ISSN 0030-5987. Orthopaedics, traumatology and prosthetics. 2023. № 2

УДК 616.718.4-001.5-089.844:615.461]:616-092.9

DOI: http://dx.doi.org/10.15674/0030-59872023233-42

Changes in markers of bone tissue remodeling and the inflammatory process in the blood serum of white rats in case of defect filling of the femur with implants based on polylactide and tricalciumphosphate with mesenchymal stem cells

N. M. Gontar

Educational and Scientific Institute of Postgraduate Education of Kharkiv National Medical University. Ukraine

Objective. Based on the analysis of markers of inflammation and metabolism of bone tissue in the blood serum of laboratory rats, to evaluate the course of bone remodeling after filling the defect in the distal metaphysis of the femur with 3D-printed implants based on polylactide and tricalcium phosphate (3D-I) alone or in combination with mesenchymal stromal cells (MSCs). Methods. 53 white rats were used, which were divided into groups: intact (5 animals) — the operation was not performed; Control (15) — 3D-I; Experiment I (15) — 3D-I + cultured alloMSCs; Experiment II (15) — 3D-I + introduction of alloMSCs into the area of surgical intervention 7 days after implantation. The following were studied: the content of glycoproteins (GP), interleukin-6 (IL-6), osteocalcin, chondroitin sulfates (CS), total protein, calcium, alkaline (AlP) and acid phosphatase (AP) activity, and their ratio, mineralization indices were calculated. Results. Compared with intact animals, higher indicators were determined in the rats of the Control group: the content of GP by 39.73; 32.88; 23.29 %; CS — 250.00; 222.09 and 196.51 %, AlP activity — 81.67, 51.03, 39.36 %, on the 15th, 30th, and 90th days of the experiment; IL-6 — 44.89; 60.06 % on the 15th and 30th days. In the rats of the Experiment I group: the content of GP — by 82.19; 65.75, 57.53 %, IL-6 — 72.14; 96.59; 79.88 %, CS — 306.98; 276.74; 253.49 %; AlP activity — 63.73; 129.70; 51.28 %, on the 15th, 30th and 90th days of the experiment. In the Experiment II group: on the 15th, 30th and 90th days, the content of GP was higher by 27.40; 26.03; 129.18 %; CS — by 175.58; 137.21 and 115.12 %; AlP activity — 192.99; 178.02, 76.31 %; on the 15th and 30th days: IL-6 — by 37.46; 20.74 %. Conclusions. In the case of filling the defect with 3D-printed implants, biochemical signs of moderate inflammation were determined; 3D-printed implants together with MSCs — pronounced inflammation, slowing of bone formation, formation of connective tissue; 3D-printed implants with postoperative injection of MSCs — moderate inflammation and optimal conditions for healing the defect with bone tissue.

Мета. На підставі аналізу маркерів запалення та метаболізму кісткової тканини в сироватці крові лабораторних щурів оцінити перебіг ремоделювання кістки після заповнення дефекту в дистальному метафізі стегнової кістки 3D-друкованими імплантатами на основі полілактиду та трикальційфосфату (3D-I) самостійно або в комбінації з мезенхімальними стромальними клітинами (МСК). Методи. Використано 53 білих щурів, яких розподілили на групи: інтактна (5 тварин) — операцію не виконували; Контроль (15) — 3D-I; Дослід I (15) — 3D-I + культивовані алоМСК; Дослід II (15) — 3D-I + введення алоМСК у ділянку хірургічного втручання через 7 діб після імплантації. Досліджено: вміст глікопротеїнів (ГП), інтерлейкіну-6 (ІЛ-6), остеокальцину, хондроїтинсульфатів (ХС), загального білка, кальцію, активності лужної (ЛФ) та кислої фосфатаз і розраховано їхнє співвідношення, індекси мінералізації. Результати. Порівняно з інтактними тваринами визначено більші показники у щурів групи Контроль: вмісту ГП на 39,73; 32,88; 23,29 %; ХС — 250,00; 222,09 і 196,51 %, активності ЛФ — 81,67, 51,03, 39,36 %, на 15-ту, 30-ту та 90-ту доби досліду; ІЛ-6 — 44,89; 60,06 % на 15 та 30-ту доби. У щурів групи Дослід I: вміст ГП — на 82,19; 65,75, 57,53 %, ІЛ-6 — 72,14; 96,59; 79,88 %, ХС — 306,98; 276,74; 253,49 %; активність ЛФ — 63,73; 129,70; 51,28 %, на 15-ту, 30-ту та 90-ту доби досліду. У групі Дослід II: на 15-ту, 30-ту та 90-ту доби був більшим вміст ГП на 27,40; 26,03; 129,18 %; ХС — на 175,58; 137,21 та 115,12 %; активність ЛФ — 192,99; 178,02, 76,31 %; на 15-ту та 30-ту доби: ІЛ-6 — на 37,46; 20,74 %. Висновки. У разі заповнення дефекту 3D-друкованими імплантатами визначено біохімічні ознаки помірного запалення; 3D-друкованими імплантатами разом із МСК — вираженого запалення, уповільнення кісткоутворення, формування сполучної тканини; 3D-друкованими імплантатами з післяопераційним введенням МСК — помірного запалення і оптимальних умов для загоєння дефекту кістковою тканиною. Ключові слова. Дефект, моделювання, регенерація, ксеноімплантат, полілактид, трикальційфосфат, мезенхімальна стовбурова клітина, біохімія, сполучна тканина.

Key words. Defect, modeling, regeneration, xenoimplant, polylactide, tricalcium phosphate, mesenchymal stem cell, biochemistry, connective tissue

Introduction

Bone tissue has a natural ability to heal, which is usually enough to repair fractures, but sometimes it is not enough [1]. The incidence of non-unions for fractures of the humerus diaphysis is known to be 32 % in patients aged 55 and over [2]. In a study involving 147 patients with non-operated fractures of the diaphysis of the humerus, their union was recorded in 126 individuals [3].

The imbalance of the opposing actions of osteoblasts and osteoclasts is one of the reasons for impaired fracture consolidation [4]. At the same time, functionally aged stem cells have a reduced potential for bone and cartilage formation, but produce more clones that express high levels of pro-inflammatory and pro-resorptive cytokines [5].

Bone grafts are the second most common tissue transplanted in the United States. They are necessary in the field of emergency and reconstructive orthopedic surgery. The soreness of the donor site and the limited amount of material make an autograft not an ideal option. Among bone graft substitutes, synthetic materials based on phosphate and calcium sulfate are attractive due to their affinity for bone tissue, osteoconductive and osteoinductive qualities [6, 7].

A promising approach to the treatment of bone fractures is cell therapy based on mesenchymal stem cells (MSCs) [8]. The actions of MSCs may include direct differentiation into bone cells, involvement and recruitment of other cells, or creation of a regenerative environment due to the production of growth factors [1].

The healing process of bone defects filled with grafts can be accelerated by other factors, in particular, cytokine background [6].

Purpose: based on the analysis of markers of inflammation and metabolism of bone tissue in the blood serum of laboratory rats, to evaluate the course of bone remodeling after filling a defect in the distal metaphysis of the femur with printed implants based on polylactide and tricalcium phosphate under conditions of immediate or delayed supplementation with mesenchymal stem cells.

Material and methods

Experimental studies were carried out in compliance with the requirements of humane treatment of experimental animals established in the Law of Ukraine «On the Protection of Animals from Cruelty Treatment» (No. 3447-IV dated 21.02.2006) and the European Convention «On the Protection of Vertebrate Animals Used for Research and Other Scientific Goals» (Strasbourg, 18.03.1986) [9–11]. The plan of experimental research was discussed and approved at the meeting of 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. 217 dated 14.06.2021).

Animals

The study was carried out on 53 white 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 Medical Sciences of Ukraine (at the beginning of the experiment, the age of the animals was 5–6 months, body weight 200–260 g).

The rats were randomly divided into groups depending on the implant used:

– intact (5 animals) — the operation was not performed;

– Control (15) — 3D-printed implant;

– Trial I (15) — 3D-printed implant in combination with cultured allogeneic MSCs;

– Trial II (15) — 3D-printed implant with injection of 0.1–0.2 ml of cultured allogeneic MSCs into the surgical site 7 days after implantation.

3 rats were used to obtain allogeneic MSCs according to the described method [12].

Surgical interventions

Surgical interventions were performed under aseptic and antiseptic conditions under general anesthesia (ketamine, 50 mg/kg of body weight, intramuscularly). Using the anterolateral approach, a transcortical defect of the femur of a critical size (diameter 2.6 mm, depth 3 mm) was made with a dental bur. A defect of critical size is considered to be a minimal hole that does not heal on its own during the life of the animal or during the experiment [13]. The minimum size of a critical defect for the zone of the distal metaphysis of the femur of laboratory rats is considered to be damage with a diameter and depth of at least 2.5 mm [14]. Defects were filled with 3D-printed implants. Before implantation in the bone of the rats of the Experiment 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. Rats of Experiment II group were injected with 0.1–0.2 ml of cultured allogeneic MSCs into the surgical site 7 days after implantation.

After surgery, the wound was sutured in layers and treated with Betadin® antiseptic. 5 animals each were removed from the experiment on the 15th, 30th, and 90th days after surgery by decapitation under open inhalation anesthesia with diethyl ether (the method is due to the need for blood sampling for research).

Implants

The implants were made of a 1.75 mm thick polylactide (PLA) tricalcium phosphate (TCP) composite filament made by mixing 60 % PLA granules and 40 % mineral compound (20 % PLA + 80 % TCP), heating and extrusion on a 3D- Easy3DPrint printers with an extruder (printing technology using composite filament welding). During the creation of the composite thread, PLA granules (L-polylactide, manufactured in China) and compound granules (TCP medical, diameter 10 μm, manufactured in China) were used. The structure of the implants: internal — a frame made of interweaving composite threads forming vertical and horizontal channels (pore size 300 μm, porosity 45 %); the outer — cylinders with a diameter of 2.5 mm and a length of 30 mm, which were mechanically divided into 3 mm-long fragments.

Biochemical research

After obtaining blood, the serum was separated by centrifugation at 1500 rpm for 30 min followed by determination of the content of glycoproteins according to the modified method of O. P. Shtenberg and Y. N. Dotsenko [15], chondroitin sulfates (CS) according to the Nemeth–Csoka method in the modification of L. I. Slutsky [16], calcium by the potentiometric method using the AEK-01 electrolyte analyzer; activity of alkaline and acid phosphatases by reaction with diethanolamine by kinetic methods according to the instructions «Alkaline phosphatase — kin Sp.L» and «Acid phosphatase — kin Sp.L» using the electrophotocolorimeter «KFK-3» and biochemical analyzer «GBG STAT FAX 1904 Plus».

The calculation of the integral indicators of the ratio of alkaline phosphatase activity to acid phosphatase activity, as well as the degree of mineralization (the ratio of calcium content (for this, the measurement units of mmol/l in g/l) to the protein content in blood serum) was performed.

The content of the non-collagenous protein osteocalcin (OC) and interleukin-6 (IL-6) was determined in the blood serum of rats by the method of solid-phase immunoenzymatic analysis in accordance with the instructions of the manufacturers of the OC kits (osteocalcin kit (N-MID® Osteocalcin ELISA kit, Immunodiagnostic Systems Limited, Great Britain), IL-6 (Interleukin-6-IFA-BEST kit, JSC VECTOR-BEST, A-8768).

To measure the results of enzyme-linked immunosorbent assays, an enzyme-linked immunosorbent assay LisaScan (Erba® Diagnostics Mannheim GmbH, Germany) was used.

Statistical methods

Data analysis was performed using the IBM SPSS Statistics 20 and Microsoft Office Excel 2007 software. Measurement results are presented as mean ± standard deviation in the case of a normal distribution. The normality of the distribution was checked using the Kolmogorov–Smirnov analysis method. Comparison of the results of different groups with a normal distribution was performed using the Student-Fisher method. The difference was considered statistically significant in $p< 0.05$ [17].

Results and their discussion

Control group (3D-printed implant)

15^{th} day of observation

In the blood serum of animals, an increase in the content of total protein by 14.89 %, glycoproteins by 39.73 %, and IL-6 by 44.89 % was observed (Table 1), which is a sign of the development of the inflammatory process with the production of an excess of acute phase proteins. At the same time, a 250.00 % increase in the content of CS was noted, which indicates the activation of the formation of connective tissue in the body, probably due to its formation in the defect, which is undesirable. At the same time, a higher activity of alkaline phosphatase was recorded, which exceeded the indicator in intact animals by 81.67 %, which in conditions of almost unchanged activity of acid phosphatase led to an increase in their ratio by 66.10 % (Table 2). This characterizes the probable activation of the mineralization of the newly formed bone tissue with the predominance of bone formation processes over the lysis of the affected tissue.

30^{th} day

Compared to the parameters of the intact group, a 15.20 % higher level of total protein was found in the blood serum, as well as activation of inflammatory processes with the corresponding manifestation of inflammatory markers — a 32.88 % higher content of glycoproteins and a 60.06 % higher IL-6 (Table 1). In this group of animals, as in the previous period of observation, an increase in the content of CS in the blood serum by 222.09 % was found, which reflects the formation of a connective tissue defect in the area. At the same time, the activity of alkaline phosphatase in blood serum increased by 51.03 %, acid phosphatase by 24.25 %, as well as the value of their ratio, which reflects the initial stages of bone tissue remodeling, by 21.74 % (Table 2).

Compared with the values of the studied markers of the Control group, on the 14^{th} day after the operation, an increase in the level of osteocalcin in the blood serum by 21.28 %, which stimulates the healing of the bone defect, was determined. On the contrary, based on the indicators of activity

in blood serum of alkaline (15.63 % less) and acid (14.01 % more) phosphatases together with a 26.21 % decrease in their ratio (Table 2), it can be assumed that regenerative and remodeling processes in bone tissue subside along with simultaneous acceleration of its lysis.

90th day

Normalization of the total protein and IL-6 content in the blood serum of the Control group rats was revealed.

Compared with the group of intact rats, an increase in the content of glycoproteins by 23.29 %, and CS by 196.51 % was observed, which indicates an intensive metabolism of connective tissue (Table 1).

Alkaline phosphatase activity in the blood serum remained at a high level, which exceeded the level of intact animals by 39.36 %, while the values of acid phosphatase practically did not differ in all groups. This led to an increase in the ratio of alkaline and acid phosphatase activity by 26.09 % relative to

Table 1

Changes in markers of inflammation and general somatic parameters in the blood serum of laboratory rats with a transcortical defect of the femur of a critical size with different types of defect filling in time course (M ± m)

Group	Indicator				
	total protein, g/l	total calcium, mmol/l	degree of mineralization, $\cdot 10^{-3}$	glycoproteins, units	interleukin-6, pg/ml
Intact, n = 5	68.5 ± 2.3	2.33 ± 0.04	1.36 ± 0.03	0.73 ± 0.02	0.323 ± 0.027
Control:					
15 days, n = 5	78.7 ± 3.3 +14.89 %[1)6)]	2.03 ± 0.02 –12.88 %[1)5)]	1.40 ± 0.07 +0.80 %[1)5)]	1.02 ± 0.03 +39.73 %[1)8)]	0.468 ± 0.009 +44.89 %[1)8)]
30 days, n = 5	78.9 ± 2.5 +15.20 %[1)6)] +0.30 %[4)5)]	2.19 ± 0.03 –6.00 %[1)5)] +7.90 %[4)5)]	1.39 ± 0.03 +2.2 %[1)5)] +2.21 %[4)5)]	0.97 ± 0.04 +32.88 %[1)8)] –4.90 %[4)5)]	0.517 ± 0.039 +60.06 %[1)8)] +10.47 %[4)5)]
90 days, n = 5	76.3 ± 1.5 +11.20 %[1)5)] –3.30 %[4)5)]	2.41 ± 0.03 +3.43 %[1)5)] +10.00 %[4)5)]	1.27 ± 0.04 –6.60 %[1)5)] –8.63 %[4)5)]	0.90 ± 0.02 +23.29 %[1)7)] –7.22 %[4)5)]	0.298 ± 0.027 –7.74 %[1)5)] –42.36 %[4)8)]
Trial I:					
15 days, n = 5	97.5 ± 1.9 +42.34 %[1)8)] +23.89 %[2)7)]	2.34 ± 0.03 +0.42 %[1)5)] +15.27 %[2)6)]	1.39 ± 0.03 +2.20 %[1)5)] –0.71 %[2)5)]	1.33 ± 0.04 +82.19 %[1)8)] +30.39 %[2)8)]	0.556 ± 0.030 +72.14 %[1)8)] +18.80 %[2)6)]
30 days, n = 5	95.1 ± 1.4 +38.83 %[1)8)] +20.53 %[2)6)] –2.50 %[4)5)]	2.30 ± 0.04 –1.29 %[1)5)] +5.02 %[2)5)] –1.71 %[4)5)]	1.42 ± 0.02 +4.41 %[1)5)] +2.16 %[2)5)] +2.20 %[4)5)]	1.21 ± 0.03 +65.75 %[1)8)] +24.74 %[2)6)] –9.02 %[4)5)]	0.635 ± 0.032 +96.59 %[1)8)] +22.82 %[2)7)] +14.21 %[4)6)]
90 days, n = 5	88.4 ± 3.5 +29.06 %[1)7)] +15.86 %[2)6)] –7.05 %[4)5)]	2.45 ± 0.02 +5.15 %[1)5)] +1.65 %[2)5)] +6.50 %[4)5)]	1.11 ± 0.04 –18.40 %[1)8)] –12.60 %[2)5)] –13.40 %[4)6)]	1.15 ± 0.04 +57.53 %[1)8)] +27.78 %[2)6)] –4.96 %[4)5)]	0.581 ± 0.024 +79.88 %[1)8)] +94.97 %[2)8)] –8.50 %[4)5)]
Trial II:					
15 days, n = 5	62.1 ± 2.5 –9.34 %[1)5)] +14.99 %[2)6)] –36.30 %[3)8)]	2.22 ± 0.05 –4.72 %[1)5)] +9.36 %[2)5)] –5.13 %[3)5)]	1.43 ± 0.04 +5.15 %[1)5)] +2.10 %[2)5)] +2.87 %[3)5)]	0.93 ± 0.02 +27.40 %[1)7)] –8.84 %[2)5)] –30.07 %[3)8)]	0.444 ± 0.031 +37.46 %[1)8)] –5.13 %[2)5)] –20.14 %[3)7)]
30 days, n = 5	66.3 ± 1.5 –3.21 %[1)5)] –15.97 %[2)6)] –30.28 %[3)8)] +6.80 %[4)5)]	2.27 ± 0.04 –2.57 %[1)5)] +3.65 %[2)5)] –1.30 %[3)5)] +2.30 %[4)5)]	1.41 ± 0.04 +3.68 %[1)5)] +1.44 %[2)5)] –0.70 %[3)5)] –1.40 %[4)5)]	0.92 ± 0.02 +26.03 %[1)7)] –4.12 %[2)5)] –23.97 %[3)6)] –1.08 %[4)5)]	0.390 ± 0.030 +20.74 %[1)7)] –24.56 %[2)7)] –38.58 %[3)8)] –12.16 %[4)5)]
90 days, n = 5	73.3 ± 2.8 +7.00 %[1)5)] –3.90 %[2)5)] –17.08 %[3)6)] +10.60 %[4)5)]	2.53 ± 0.04 +8.58 %[1)5)] +4.98 %[2)5)] +3.26 %[3)5)] +11.45 %[4)5)]	1.38 ± 0.04 +1.47 %[1)5)] +8.66 %[2)5)] +2.20 %[3)5)] –2.13 %[4)5)]	0.87 ± 0.02 +19.18 %[1)6)] 0.00 %[2)5)] –8.42 %[3)5)] –5.44 %[4)5)]	0.364 ± 0.029 +12.69 %[1)5)] +22.15 %[2)7)] –37.35 %[3)8)] –6.67 %[4)5)]

Notes: Comparison of parameters: [1)] — intact group of rats, [2)] — rats of the Control group for the same period of observation, [3)] — Trial I group for the same period of observation, [4)] — within one group for the previous term of the trial, [5)] — $p > 0,05$; [6)] — $p < 0,05$; [7)] — $p < 0,01$; [8)] — $p < 0,001$

the value in intact rats, which indicates an acceleration of bone tissue remodeling in the early stages of mineralization.

A decrease of 18.40% in the value of the mineralization indicator was observed, which indicates a delay in the last phase of mineralization of bone tissue (Table 2).

Compared with the indicators of the same group at the previous term of the study, a sharp decrease in IL-6 content in blood serum was determined (by 42.36 %, Table 1), which is a reflection of a decrease in the activity of inflammatory processes.

Trial I group (3D-printed implant in combination with cultured allogeneic MSCs)

15th day

Compared to the intact group, the content of IL-6 glycoproteins was found to be 72.14 % higher — by 82.19 %. At the same time, probably due to proteins

Table 2

Changes in markers of bone and connective tissue metabolism in the blood serum of laboratory rats with a transcortical femoral defect of critical size with different types of defect filling in time course (M ± m)

Group	Indicator				
	CS	osteocalcin, ng/ml acid	alkaline phosphatase, unit/l	phosphatase, units/l	Relationship between the activity of alkaline and acid phosphatases
Intact, n = 5	0.086 ± 0.005	228.3 ± 11.4	199.7 ± 8.7	33.4 ± 1.2	5.98 ± 0.09
Control:					
15 days, n = 5	0.301 ± 0.026 +250.00 %[1)8)]	196.9 ± 14.9 –13.75 %[1)5)]	362.8 ± 24.0 +81.67 %[1)8)]	36.4 ± 1.3 +8.98 %[1)5)]	9.93 ± 0.30 +66.10 %[1)8)]
30 days, n = 5	0.277 ± 0.015 +222.09 %[1)8)] –7.97 %[4)5)]	238.8 ± 14.3 +4.60 %[1)5)] +21.28 %[4)6)]	301.6 ± 19.5 +51.03 %[1)8)] –15.63 %[4)6)]	41.5 ± 1.3 +24.25 %[1)6)] +14.01 %[4)6)]	7.28 ± 0.48 +21.74 %[1)6)] –26.69 %[4)6)]
90 days, n = 5	0.255 ± 0.016 +196.51 %[1)8)] –7.94 %[4)5)]	239.1 ± 10.5 4.73 %[1)5)] +0.13 %[4)5)]	278.3 ± 23.8 +39.36 %[1)8)] –7.73 %[4)5)]	36.7 ± 1.5 +9.88 %[1)5)] –11.57 %[4)5)]	7.54 ± 0.39 +26.09 %[1)6)] +3.57 %[4)5)]
Trial I:					
15 days, n = 5	0.350 ± 0.013 +306.98 %[1)8)] +16.28 %[2)6)]	205.0 ± 12.7 –10.21 %[1)5)] +4.11 %[1)5)]	307.0 ± 16.7 +53.73 %[1)8)] –15.38 %[2)6)]	48.7 ± 1.4 +45.81 %[1)8)] +33.79 %[2)8)]	6.29 ± 0.23 +5.18 %[1)5)] –36.66 %[2)8)]
30 days, n = 5	0.324 ± 0.019 +276.74 %[1)8)] +17.00 %[2)6)] –7.43 %[4)5)]	243.4 ± 13.6 +6.61 %[1)5)] +1.80 %[2)5)] +18.73 %[4)6)]	458.7 ± 21.4 +129.70 %[1)8)] +52.09 %[2)8)] +49.41 %[4)8)]	50.4 ± 1.8 +50.90 %[1)8)] +60.00 %[2)8)] +3.49 %[4)5)]	9.10 ± 0.18 +52.20 %[1)8)] +15.20 %[2)6)] +44.67 %[4)8)]
90 days, n = 5	0.304 ± 0.021 +253.49 %[1)8)] +19.22 %[2)6)] –6.17 %[4)5)]	204.0 ± 10.9 –10.64 %[1)5)] –14.68 %[2)6)] –16.19 %[4)6)]	302.1 ± 18,3 +51.28 %[1)8)] +8.55 %[2)5)] –34.14 %[4)8)]	45.1 ± 1.9 +35.03 %[1)8)] +68.91 %[2)8)] –10.52 %[4)5)]	6.67 ± 0.15 +11.50 %[1)5)] –35.74 %[2)6)] –26.70 %[4)6)]
Trial II:					
15 days, n = 5	0.237 ± 0.019 +175.58 %[1)8)] –21.26 %[2)7)] –32.30 %[3)7)]	372.7 ± 14.5 +61.25 %[1)8)] +89.28 %[2)8)] +81.80 %[3)8)]	585.1 ± 18.6 +192.99 %[1)8)] +88.83 %[2)8)] +90.59 %[3)8)]	31.5 ± 1.7 –5.69 %[1)5)] –13.46 %[2)5)] –18.60 %[3)6)]	18.38 ± 0.46 +207.40 %[1)8)] +85.10 %[2)8)] +132.36 %[3)8)]
30 days, n = 5	0.204 ± 0.026 +137.21 %[1)8)] –26.35 %[2)7)] –37.00 %[3)8)] –13.92 %[4)5)]	478.3 ± 14.9 +109.51 %[1)8)] +100.29 %[2)8)] +96.50 %[3)8)] +28.33 %[4)6)]	555.2 ± 18.6 +178.02 %[1)8)] +84.08 %[2)8)] +21.04 %[3)7)] –5.11 %[4)5)]	28.2 ± 1.7 –15.57 %[1)6)] –32.00 %[2)8)] –44.05 %[3)8)] –10.48 %[4)5)]	19.80 ± 0.55 +231.10 %[1)8)] +150.60 %[2)8)] +117.58 %[3)8)] +7.73 %[4)5)]
90 days, n = 5	0.185 ± 0.020 +115.12 %[1)8)] –47.10 %[2)8)] –39.15 %[3)8)] –9.31 %[4)5)]	409.3 ± 22.9 +79.28 %[1)8)] +71.18 %[2)8)] +100.64 %[3)8)] –14.43 %[4)6)]	352.1 ± 26.1 +76.31 %[1)8)] +26.52 %[2)7)] +16.55 %[3)6)] –36.58 %[4)8)]	22.4 ± 3.3 –32.34 %[1)8)] –16.10 %[2)6)] –50.33 %[3)8)] –20.57 %[4)6)]	15.77 ± 0.36 +163.70 %[1)8)] +51.90 %[2)8)] +136.43 %[3)8)] –20.40 %[4)6)]

Notes: Comparison of parameters: [1)] — intact group of rats, [2)] — rats of the Control group for the same period of observation, [3)] — Trial I group for the same period of observation, [4)] — within one group for the previous term of the trial, [5)] — $p > 0,05$; [6)] — $p < 0,05$; [7)] — $p < 0,01$; [8)] — $p < 0,001$

of the acute phase, the content of total protein in blood serum increased by 42.34 % (Table 1). A very high qualified rate of metabolism of connective tissue indicates an increase in the concentration of CS in blood serum by 306.98 %. Markers of bone tissue formation and lysis — the activity of alkaline and acid phosphatases in blood serum increased quite synchronously relative to the level of indicators in intact animals (by 53.73 and 45.81 %, respectively), and their ratio did not differ significantly (Table 2).

Compared to the indicators of the animals of the Control group, the content of glycoproteins in blood serum was significantly higher by 30.39 %, IL-6 by 18.80 %, and total protein by 23.89 % (Table 1), which indicates a more active inflammation process in rats of the Trial I group, which can be explained by the reaction to the introduction of MSCs immediately after the formation of the defect.

In addition, the animals of the Trial I group were found to have an excess of the level of the control group by 16.28 % in terms of the content of CH in blood serum (Table 2), which indicates a higher rate of connective tissue development. Alkaline phosphatase activity was 15.38 % lower, and acid phosphatase activity was 33.79 % higher than in the Control group. As a result, the value of the ratio of the activity of alkaline and acid phosphatases in blood serum was lower by 36.66 % (Table 2). That is, under conditions of additional administration of MSCs simultaneously with an implant based on PLA and TCP, the formation of bone tissue is inhibited and its lysis is accelerated, possibly due to excessive activation of inflammatory processes (Table 1). Remodeling of the newly formed bone tissue was suppressed already at the beginning of mineralization processes.

30th day

In the Trial I rats, the development of inflammatory processes continued, which was reflected by an increase in the manifestation of specific inflammatory markers in the blood serum (the content of IL-6 by 96.59 %, glycoproteins by 65.75 %) and indirect markers (the content of total protein by 38.83 %) (Table 1).

Presumably, active development of connective tissue continued in the defect zone with an increased serum level of CH by 276.74 % (Table 2).

In addition, an increase in the activity of alkaline phosphatase by 129.70 % and acid phosphatase by 50.90 % in blood serum was established. The value of the ratio of the activity of alkaline and acid phosphatases was also increased by 52.20 % compared to the indicator in the animals of the Control group for the same period of the trial (Table 2).

Within the Trial I group, compared to the indicators obtained on the 15th day of the experiment, an increase in IL-6 content in blood serum by 14.21 % was recorded (Table 1), which indicates further intensification of inflammatory processes. As for markers of bone tissue formation, some improvement of the situation can be noted. In particular, the content of osteocalcin in blood serum was higher by 18.73 %, the activity of alkaline phosphatase by 49.41 %, the ratio of activity of alkaline and acid phosphatase by 44.67 % (Table 2). That is, the process of remodeling and mineralization of bone tissue came out of the pause that was characteristic of the 15th day of the experiment.

90th day

An active course of the inflammatory process was determined, which was manifested in a significant increase in the content of glycoproteins in blood serum by 57.53 %, IL-6 by 79.88 %, and total protein by 29.06 % (Table 1). Indicators of markers of connective tissue formation were quite pronounced: the content of CH in blood serum was higher than that of intact animals by 253.49 % (Table 2). At the same time, both anabolic processes (alkaline phosphatase activity exceeded that of intact animals by 51.28 %) and catabolic processes (acid phosphatase activity was higher than in the group of intact animals by 35.03 %) were activated in the bone tissue (Table. 2).

Compared with the results obtained in the rats of the Control group, the content of glycoproteins in the serum was determined to be significantly higher by 27.78 %, IL-6 by 94.97 %, and total protein by 15.86 % (Table 1), which indicates sharp activation of inflammation also on the 90th day of the trial. The content of CH in the blood serum of Trial I group was higher by 19.22 % than in control animals, and osteocalcin was lower by 14.68 % (Table 2), which indicates the formation of a connective tissue defect in the area. Alkaline phosphatase activity in blood serum was comparable to that of the Control group, and acid phosphatase activity was increased by 68.91 %. Accordingly, the ratio of the activity of alkaline and acid phosphatases decreased by 21.83 %, indicating an activation of the destruction of bone tissue near the defect zone (Table 2).

Compared to the previous period of the study, the value of mineralization indicator was determined to be 13.40 % lower (Table 1). At the same time, the level of osteocalcin in blood serum was lower by 16.19 %, the activity of alkaline phosphatase by 34.14 %, the ratio of activity of alkaline and acid phosphatase by 26.70 % (Table 2).

Group Trial II (3D-printed implant with injection of cultured allogeneic MSCs on the 7th day after surgery)

15th day

The content of glycoproteins in the blood serum was increased by 27.40 % and IL-6 by 37.46 %, which is significantly less than in the Experiment I group by 30.07 % and 20.14 %, respectively. The content of total protein and CH in the blood serum of animals of the considered group, although it was higher than that of intact rats, by 14.99 and 175.58 %, respectively, but these values were lower than that of animals of Trial I group (by 36.30 % and 32.30 %, respectively) (Table 1). This indicates a lower level of inflammation and limited activation of connective tissue metabolism than in the comparison groups.

Regarding markers of bone tissue metabolism in laboratory rats of the Trial II group, the following was found in blood serum: osteocalcin was 61.25 % more than in intact animals, 89.28 % more than in the Control group, and 81.80 % more than in the Trial I group; higher activity of alkaline phosphatase by 192.99, 88.83 and 90.59 %, respectively; activity of acid phosphatase comparable to the intact group, but lower than that of the Control group by 18.60 % (Table 2). The ratio of alkaline and acid phosphatase activity in blood serum was higher than that of intact animals by 207.40 %, the Control group — by 85.10 %, and the Trial I group — by 132.36 % (Table 2). That is, activation of bone tissue remodeling with a predominance of bone formation over destruction was recorded in the Trial II group.

30th day

During this period of the experiment, further inhibition of the inflammatory process was determined, which was reflected by a decrease in the concentration of inflammatory markers in the blood serum of experimental animals: the content of glycoproteins and IL-6, although it was higher by 26.03 and 20.74 %, respectively, than in intact animals, but it turned out lower by 23.97 and 38.56 %, respectively, than the indicators of the Trial I group. The content of total protein was comparable to the value in intact animals, but by 15.97 and 30.28 % less than at the same observation period in the Control and Trial I, respectively (Table 1). That is, in the animals of the Trial II group, the inflammatory process was significantly less pronounced than in the Control and Trial I groups.

According to the content of calcium and the degree of mineralization, the parameters of the laboratory rats of the Trial II group did not differ significantly from the comparison groups. They were also found to have relatively low levels of CS in blood serum: 137.21 % higher than in intact animals, but 26.35 and 37.00 % lower than in the Control and Trial I groups, respectively (Table 2), which reflects a lower content of connective tissue in the area of the defect.

An increase in the level of osteocalcin in blood serum compared to the 15th day of observation by 28.33%, compared to the indicators of the rats of the Trial I and Control groups on the 30th day — by 96.50 % and 109.51 % (Table 2) can be interpreted as the formation of a larger amount of bone tissue in the area of the defect in rats of the Trial II group. This statement is also supported by the higher activity of alkaline phosphatase in their blood serum by 178.0; 84.08 and 21.04 %, as well as a lower activity of acid phosphatase (a marker of bone tissue lysis) by 15.57; 32.00 and 44.05 % compared to the intact, Control and Trial I groups, respectively (Table 2). The ratio of serum alkaline and acid phosphatase activity in rats of Trial II group was higher by 231.10; 150.60; 117.58 % than in the intact, Control and Trial I groups, respectively (Table 2). This indicates that the most favorable conditions for the formation of bone tissue and its remodeling were created in the Trial II group.

90th day

At this stage of the trial, a moderate manifestation of markers of the inflammatory process was determined, namely: a higher content of glycoproteins in blood serum by 19.18 % compared to intact rats; IL-6 was higher by 22.15 % compared to the Control group, but lower by 37.35 % compared to the Trial I group; of total protein was lower by 17.08 % compared to the Trial I group (Table 1). This indicates a lower activity of the inflammatory process in the animals of the Trial II group compared to the Trial I group. No statistically significant differences with the comparison groups were found for the calcium content in the blood serum and the degree of mineralization in the rats of the Trial II group.

The concentration of CH in blood serum was recorded to be 115.12 % higher than that of intact animals, but lower than the values found in the Control and Trial I groups on the 90th day, by 47.10 and 39.15 %, respectively (Table 2).

As in the previous term of the study, the content of osteocalcin in blood serum was at a fairly high level: higher by 79.28; 71.18 and 100.64 % for the indicators of the intact, Control and Trial I groups, respectively. It was established that during this period there was a decrease of 36.58 % in the activity of alkaline phosphatase in the blood serum of rats of the Trial II group compared to the 30th day, but the indicator remained

higher by 76.31; 26.52 and 16.55 % for the value on the 90th day in the intact, Control and Trial I groups, respectively. The activity of acid phosphatase compared to the same groups was lower by 32.34; 16.10 and 50.33 %, respectively. The calculated indicator of the ratio of alkaline and acid phosphatase activity was 163.70 higher than in the intact, Control and Trial I groups; 51.90 and 136.43 %, respectively. Compared to the indicator of the Trial II group for the previous period of the study, the specified parameter was significantly lower by 20.40 % (Table 2).

Discussion

The presented research involved a comparative study of the effect (by metabolic markers) on the bone tissue of a new biomaterial — 3D-printed implants based on PLA and TCP, used alone or in combination with MSCs, which were either saturated with the implant before installation or injected into defect site on the 7th day after surgery. A hole defect in the distal metaphysis of the rat femur of critical size (diameter 2.6 mm, depth 3 mm) was chosen as the model.

Biochemical analysis of blood serum showed that in the conditions of filling the defect with 3D-printed implants, the self-regenerative potential of bone tissue remained limited and against the background of moderate manifestation of markers of the inflammatory process, there was a moderate increase in markers of bone tissue formation and its mineralization. The reconstruction of the connective tissue was quite active, which was evidenced by the excess of CS in the blood serum.

As noted by T. Rolvien et al. [18], in the best case, under the conditions of using MSCs, reparative osteogenesis and reconstruction of the graft material takes place in conjunction with the bioframe. At the same time, it was experimentally shown that the saturation of MSCs of bone allografts led to a slowdown in bone formation in cases of fresh traumatic bone injuries, with the formation of significant areas of connective tissue on the 14th and 28th days after implantation, regardless of the age of the rats [12]. It can be assumed that it was the use of allogeneic MSCs at the early stage of defect healing that caused excessive activation of inflammation by the immune mechanism, resulting in the formation of connective tissue. It was determined that MSCs can stimulate the release of various active factors: growth factors, inhibitors, activators of inflammation with immunomodulating effects [19]. These findings are not contradicted by the results of our study: in a group of rats in which a 3D-printed implant saturated with cultured allogeneic MSCs was installed, the highest levels of CS in the blood serum were recorded, which is a sign of the activation of connective tissue remodeling, presumably due to its excessive formation in the area of the defect.

Of particular interest are the changes in biochemical markers of inflammation and bone tissue formation in the conditions of postoperative (on the 7th day) introduction of MSCs into the defect zone, where a 3D-printed implant based on PLA and TCP is installed. Under these conditions, the situation regarding the development of inflammation was found to be significantly calmer than in the Control (3D-printed implant) and Trial I (3D-printed implant, saturated with MSCs) groups, which was recorded by the manifestations of markers of the inflammatory process — the content of glycoproteins, IL-6, total protein, which could change mainly due to the biosynthesis of acute phase inflammatory proteins in the body. This feature was observed at all stages of the experiment. At the same time, a higher rate of bone formation and remodeling was recorded, according to indicators of markers of bone tissue formation (osteocalcin content, alkaline phosphatase activity, ratio of alkaline and acid phosphatase activity), probably in the defect area and around it, since other bone tissue remained intact. The mechanism of acceleration of bone tissue remodeling in conditions of delayed use of MSCs may be their promotion of neovascularization, in particular due to paracrine action [20]. Also, in rats that were injected with MSCs into the defect area on the 7th day after the installation of the 3D-printed implant, a moderate activation of connective tissue metabolism was recorded with a slight increase in the content of CS in the blood serum, which was lower than in the other groups from the beginning of the experiment.

Conclusions

Based on the analysis of the results of a biochemical study of blood serum indicators of laboratory rats under the conditions of filling the defect with 3D-printed implants based on PLA and TCP, signs of moderate inflammation with an increase in the content of glycoproteins, IL-6, and total protein were revealed. Slowing down of bone formation was determined by a slight increase in alkaline phosphatase activity, a high level of acid phosphatase activity, and excessive formation of connective tissue by a significant level of chondroitin sulfates.

In the case of using 3D-printed implants saturated with cultured allogeneic MSCs to fill a bone defect, biochemical signs of pronounced inflammation were recorded with a significant increase in the content

of glycoproteins, IL-6 and total protein in the blood serum of rats. This creates unfavorable conditions and slows down the healing of the defect by bone tissue, which is evidenced by a decrease in the content of osteocalcin, the activity of alkaline phosphatase, and the ratio of the activity of alkaline and acid phosphatases.

Under the conditions of installation of 3D-printed implants in the metaphyseal defect of the femur of rats and the post-operative (7th day) administration of cultured allogeneic MSCs, the conditions for the healing of the defect with bone tissue were created, as evidenced by a significant increase in the activity of alkaline phosphatase against the background of low variability of acid phosphatase activity, lower content of CH in blood serum, which characterizes a small amount of connective tissue in the area of the defect.

Under the conditions of installation of 3D-printed implants in the metaphyseal defect of the femur of rats and the postoperative (7th day) introduction of cultured allogeneic MSCs, the conditions for the healing of the defect with bone tissue were created, as evidenced by a significant increase in the activity of alkaline phosphatase against the background of low variability in the activity of acid phosphatase, a lower content of CS in the blood serum, which characterizes a small amount of connective tissue in the defect area.

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

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The article has been sent to the editors 25.04.2023

CHANGES IN MARKERS OF BONE TISSUE REMODELING AND THE INFLAMMATORY PROCESS IN THE BLOOD SERUM OF WHITE RATS IN CASE OF DEFECT FILLING OF THE FEMUR WITH IMPLANTS BASED ON POLYLACTIDE AND TRICALCIUMPHOSPHATE WITH MESENCHYMAL STEM CELLS

N. M. Gontar

Educational and Scientific Institute of Postgraduate Education of Kharkiv National Medical University. Ukraine

✉ Nazar Gontar, MD: gontarnazar@ukr.net