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Bone remodeling markers after experimental augmentation of trabecular bone defects with resorbable and non-resorbable osteoplastic materials in rabbits

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Objective To study the effect of bone defect augmentation on the dynamics of bone remodeling markers. **Material and methods** The effect of resorbable xenoplastic material (RXM), synthetic beta-tricalcium phosphate (b-TCP), porous titanium implant (PTI) and nanostructured carbon implant (NCI) on the markers of bone remodeling (osteocalcin, OC; bone alkaline phosphatase, BALP; C-terminal telopeptide of type I collagen, CTX-1) and inflammation marker (C-reactive protein, CRP) was investigated using bone defect model in rabbits. 24 animals were divided into 4 groups (n = 6 in each group) according to the type of osteoplastic material. Control group (n = 6) was without augmentation. An impression fracture of the proximal tibia was modeled. Blood samples were taken on days 1, 3, 7, 14, 45, 90, 180 after surgery. **Results** CTX-1was not detected in the control, b-TCP, PTI, and RXM groups after 90 days, but in the NCI group CTX-1 remained elevated until the end of the study. OC in the control, b-TCP, PTI groups reached a maximum at 14-45 days. No significant increase in OC was found in the NCI group. The BALP in the control group peaked at 90 days. In the b-TCP and PTI groups the concentration of BALP increased more rapidly. The dynamics of CRP in the RXM, b-TCP and PTI groups was similar to the dynamics in the control group, in the NCI group an increased level of CRP remained until the end of the study. **Conclusion** When a bone defect was augmented with both resorbable b-TCP and non-resorbable PTI, high osteogenesis activity and low osteoresorption activity were detected. The use of xenoplastic material did not reveal any advantages in comparison with surgery performed without augmentation. An increase in osteoresorption and a low level of osteogenesis were found by using NCI.
Keywords: bone remodeling markers, osteoplastic materials, augmentation, bone defects, experiment

INTRODUCTION

Substitution (augmentation) of bone defects refers to the priorities of current clinical medicine. Bone defects occur in the surgical treatment of intra- and periarticular fractures, partial osteochondral defects, in degenerative diseases of large joints of the limb bones and oncopathology of the musculoskeletal system [1, 2]. The need for surgical management of a bone defect necessitates a constant search for new materials and structures for osteoplasty that can be successfully integrated into the human body, have good biocompatibility and the ability to stimulate osteoconduction and osteoinduction mechanisms [3].

Currently, there is a wide range of replacement materials for osteoplasty of a bone defect [4–6]. Non-mineralized xenomaterial is obtained from purified animal bones, which are processed to a highly purified bone matrix, resulting in the preservation of collagen and mineral components [7]. Another resorbable osteoplastic material is synthetic β-tricalcium phosphate, the porous structure of which supports osteoblasts and promotes the integration of bone tissue. Small size of the particles improves the osteoconductive properties of the augment and ensures its integration during the process of bone remodeling [8]. Titanium is one of the most inert and biotolerant metals, which has a widespread use in traumatology and orthopedics [9]. Selective laser melting technology allows obtaining titanium augments with a given architectonics [10]. Non-resorbable nanostructured carbon implants also have a sufficient set of characteristics (osteoinduction, bioinertness, safety) allowing their use in traumatology and orthopedics [11].

An important element of the research on the use of various types of augments to bone tissue is to obtain information about the nature and dynamics of the repair process. Molecular markers of bone remodeling have been shown to be informative both for assessing the effectiveness of bone repair after surgical treatment of fractures and for early detection of osteogenesis disorders. It was found that the indices of bone markers differ in various types of fractures and their location, [12–14]. However, there is no data on the features of bone metabolism by filling bone defects with various resorbable and non-resorbable augments. The aim of this work was a comparative study of the effect of various resorbable and non-resorbable materials for bone tissue filling on the dynamics of resorption and bone formation markers in a rabbit trabecular bone model of an impression fracture.

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MATERIAL ΦTA METHODS

Study object The study was performed on 30 sexually mature female Chinchilla rabbits weighing 3–3.5 kg at the beginning of the experiment. The animals were kept in the vivarium of the Ural State Medical University, were healthy; veterinary certificates of quality and health status were provided. The study design was approved by the local ethics committee of the Federal State Budget Educational Institution of Higher Medical Education of the Ural State Medical University of the Russian Ministry of Health. The study was carried out in accordance with the "Guidelines for the maintenance of laboratory animals in vivariums of research institutes and educational institutions" RD-APK 3.10.07.02-09 and Directive 2010/63 / EU of the European Parliament and the Council of the European Union "On the Protection of Animals Used for Scientific Purposes" Animals were kept in identical conditions of feeding and maintenance. Euthanasia was performed by overdosing a solution of 20 % sodium thiopental.

Osteoplastic materials To fill in bone defects, the following materials were used: 1) resorbable xenoplastic material Osteomatrix, which is a chemically processed non-demineralized lyophilized bone matrix of an animal, manufactured by Connectbiopharm (Russia); 2) synthetic beta-tricalcium phosphate (β-TCP) (Science & BioMaterials, France), which is an osteoconductive resorbable augment with a porosity of 60 %; 3) porous (volume porosity of 80 %) titanium implants (cylindrical samples d = 5 mm, h = 5 mm) produced with additive technologies (3D printing), were experimental medical products made by direct laser metal sintering (DMLS) with the EOSINT-280 system at the state corporation Rosatom (Russia); 4) a nanostructured carbon implant, which is a hard composite of carbon fibers bonded by a nanostructured carbon matrix, cube-shaped (face size = 5 mm), manufactured by Nanotechmedplus (Russia).

Fracture model Interventions were performed under general anesthesia, 2 % rometar intramuscular injection of 8 mg/kg (Rometar 2 %, SPOFA, Czech Republic) and zoletil 6 mg/kg (Zoletil-100, Virbac Sante Animale). For local anesthesia, a 0.25 % novocaine solution was used, also for hydraulic tissue dissection. Intra-articular impression fracture of the proximal tibia was modelled according to the technique described previously in our works [15]. Upon modeling an impression fracture, the impact site was elevated, osteoplastic material of the "press fit" type was augmented into the defect formed, and the wound was sutured in layers. A day after the operation, the condition of the rabbits in all groups was satisfactory and corresponded to the early postoperative period. Mild edema was observed in the surgical intervention area for several days which did not require additional therapeutic measures. Postoperative wound healed by first intention, the sutures were removed on the 10th day after the operation.

Study designs All animals were divided into 5 groups, six rabbits in each. Animals of four experimental groups underwent bilateral modeling of an impression fracture of the trabecular bone with subsequent augmentation by resorbable xenoplastic material (RXM); synthetic beta-tricalcium phosphate (β-TCP); porous titanium augment (PTI); nanostructured carbon implant (NCI). As a control group (CG), we used rabbits that simulated a fracture without subsequent surgical correction. Peripheral venous blood was taken in the morning from the marginal vein of the ear before surgery, on days 1, 3, 7, 14, 45, 90, 180 after the operation.

Immunochemical study Blood samples were collected in Improvacuter vacuum tubes (China). Animal blood serum was obtained by precipitation after centrifugation of blood (3000 rpm, 20 minutes), poured into aliquots and stored at -70 °C until the study. To study the dynamics of markers of bone remodeling and inflammation in peripheral blood serum, concentration of C-terminal telopeptides (C-CT), osteocalcin (OC), bone isoenzyme of alkaline phosphatase (BALP), C-reactive protein (CRP) were determined using kits of enzyme-linked immunosorbent assay kit (Cloud-Clone Corp) Organism Species (UK) according to the protocols attached to the sets using controls. To perform the analysis, a complex was used, including a Termo Scientific Multiskan GO enzyme-linked immunosorbent analyzer (Japan); washer Termo Scientific 112 Wellwash (Japan), shaker-thermostat Elmi ST-3L (Latvia).

Statistical processing of the findings Statistical processing of the findings was carried out by methods of variation statistics using Statistica 8.0 software. To compare the groups, the Kruskal-Wallis test was used, followed by a multiple analysis. The dynamics of markers in the postoperative period was evaluated using the Friedman test (ANOVA). Value $p < 0.05$ was considered statistically significant. Data are presented as median [interquartile range].

RESULTS

Data on changes in the osteoresorption marker are presented in Table 1. Blood level of CTX on the first day after surgery showed a slight or moderate increase in most of the animals included in the study, without significant differences between the groups. On the 3rd day after the operation, there was a sharp increase in the concentration of CTX in the blood in the β-TCP and PTI groups, in contrast, in

the CG and NCI groups a low level remained, and in the RXM group there was a very wide variability of this marker. From day 7, the level of CTX tended to normalize in the β-TCP and PTI groups, in the RXM group, most animals showed an increase in this indicator; in the NCI group, the concentration of CTX was practically not detected. By day14, gradual normalization of the CTX concentration in the β-TCP and PTI groups continued; in the RXM group, a high level of resorption marker remained, and in the CG and NCI groups there was a sharp increase in the concentration of CTX in the blood. On day 45, the concentration of CTX in the β-TCP, PTI and CG groups continued to decrease, but remained high in the RXM and NCI groups. From day 90 until the end of the study, an increased level of bone resorption marker was maintained only in the NCI group.

An analysis of the dynamics of osteogenesis markers showed that the concentration of OC in the blood on day 1 after surgery was increased only in the RXM group (Table 2). On day 3, the level of OC in all experimental groups did not differ significantly from the value of this indicator in the group of control animals. From 7 days, an increase in this marker was detected in all the groups studied. By 14 days, the maximum level of OC was achieved in the β-TCP and CG groups, the lowest values of this indicator were in the NCI group. After 45 days, an elevated OC level remained in the β-TCP and PTI groups, by 90 days – only in the β-TCP group. By the end of the study period, the concentration of OC in any of the experimental groups did not exceed the level of the control group (Table. 2). It should be noted that in the NCI group, in contrast to other groups, postoperative changes in the OC concentration were not so pronounced (Friedman test, p = 0.115).

The concentration of BALP in the control group gradually increased from the first week after surgery, reaching maximum values by 90 days. In the RXM, β-TCP and PTI groups, the concentration of this marker of osteogenesis increased more rapidly than in the control group and the enzyme level was higher, while the highest values were observed in the PTI group. In the NCI group, an increase in ALP was observed only in the earliest postoperative period, and it was apparently not associated with repair osteogenesis (Table 3).

The nature of changes in the concentration of CRP in the RXM, β-TCP and PTI groups corresponds to the dynamics of the postoperative period in the control group with an early recovery and gradual normalization to 90 days after surgery. However, in the NCI group, a significant increase in CRP levels persisted until the end of the study period (Table 4).

Table 1

Blood concentration of C-terminal telopeptides of collagen type I (pg/ ml) by augmentation of the tibia of rabbits with osteoplastic materials

Term after operation (days)	Group					p1
	Control	RXM	β-TCP	NCI	PTI	
	1	2	3	4	5	
1	0 [0; 0]	5 [0; 70]	0 [0; 50]	10 [0; 70]	50 [30; 65]	0.301
3	10 [0; 40][2,5]	18 [20; 265]	144 [125; 165][1,4]	0 [0; 50][2,5]	135 [120; 140][1,4]	0.007
7	20 [0; 90]	113 [60; 190][4]	112 [65; 123][4]	0 [0; 0]	73 [50; 100][4]	0.002
14	375 [350; 400]	110 [102; 240]	60 [50; 80]1	123 [0; 285]	70 [55; 80][1]	0.002
45	0 [0; 50][4]	63 [13; 355]	0 [0; 10][4]	187 [100; 410][1,3,5]	5 [0; 15][4]	0.015
90	0 [0; 0][4]	0 [0; 0][4]	0 [0; 0][4]	120 [120; 230][1,2,3,5]	0 [0; 0][4]	< 0.001
180	0 [0; 0] [4]	0 [0; 0][4]	0 [0; 0][4]	140 [140; 210][1,2,3,5]	0 [0; 0][4]	< 0.001
p2	< 0.001	< 0.001	< 0.001	0.004	0.001	

Note: results are presented as median [interquartile range]; p1 – statistical significance of differences between study groups; [1,2,3,4,5] – differences with groups 1, 2, 3, 4, 5 are statistically significant (p < 0.05); p2 – statistical significance of postoperative changes in the indicator in the group

Table 2

Blood osteocalcin level (ng/ml) by augmentation of the tibia of rabbits with osteoplastic materials

Term after operation (days)	Group					p1
	Control	RXM	β-TCP	NCI	PTI	
	1	2	3	4	5	
1	11.5 [10.2;14.0]	24.3 [15.3; 29.2][4,5]	10.0 [10.0; 13.0]	8.0 [7.0; 11.0][2]	8.5 [7.0; 9.0][2]	0.003
3	11.5 [9.6;14.0]	8.0 [7.5; 12.5]	12.0 [8.0; 15.0]	9.5 [9.0;11.0]	9.5 [8.0; 11.0]	0.473
7	16.7 [13.3; 23.0]	11.5 [9.0; 17.0]	19.0 [15.0; 22.0]	13.0 [11.0; 15.0]	12.0 [8.0; 13.0]	0.056
14	23.2 [21.6; 24.0][4]	13.0[11.5;17.5]	35.0 [16.0; 53.0][4]	8.0 [8.0; 10.0][1,3,5]	18.0 [10.0; 29.0][4]	0.010
45	10.0 [8.4; 12.0][3,5]	11.5 [10.1; 14.5][3,5]	26.5 [15.0;36.0][1,2]	13.0[11.0; 15.0][5]	25.0 [19.0;28.0][1,2]	< 0.001
90	8.0 [7.4; 8.0][5]	9.5 [8.0; 15.6]	13.0 [12.0;18.5][1,5]	10.0 [7.0; 14.0]	8.0[5.0; 10.0][5]	0.027
180	9.2 [7.0; 11.1]	8.5 [5.5; 11.0]	10.5 [8.0; 13.0]	8.0 [8.0; 13.0]	7.0 [5.0; 10.0]	0.462
p2	<0.001	0.001	<0.001	0.115	0.001	

Note: results are presented as median [interquartile range]; p1 – statistical significance of differences between study groups; [1,2,3,4,5] – differences with groups 1, 2, 3, 4, 5 are statistically significant (p < 0.05); p2 – statistical significance of postoperative changes in the indicator in the group

Table 3

Blood concentration of bone alkaline phosphatase (ng/ml) by augmentation of the tibia of rabbits with osteoplastic materials

Term after operation (days)	Group					p1
	Control	RXM	β-TCP	NCI	PTI	
	1	2	3	4	5	
1	8.2 [6.5; 9.4][3,4]	11.8 [8.2; 15.1]	27.5 [18.0; 35.0][1]	24.0 [19.0;30.0][1]	6.9 [1.8; 13.0]	0.006
3	6.9 [6.6; 7.6]	11.6[11.6;11.9]	15.6 [7.3; 29.0]	17.8 [16.3;26.3][1]	3.3 [2.1; 14.0]	0.023
7	14.1 [7.0; 24.7]	10.6 [8.2; 18.4][3]	41.0 [32.0; 45.0][1,2,4]	7.3 [5.1; 10.2][3]	15.6 [6.4; 26.0]	0.010
14	12.1 [10.6; 13.4][5]	14.8[12.0; 16.0][5]	40.0 [27.0;54.0][1]	14.5 [7.4; 40.0]	46.5 [38.0; 85][1,2]	0.004
45	15.8 [14.7; 16.8][5]	36.5 [32.0; 48.9]	26.0 [21.0;27.0][5]	5.8 [5.7; 9.9][5]	85.0 [64.0; 112.0][1,3,4]	< 0.001
90	25.9 [24.1; 27.5][3,4]	18.2 [16.0;24.5][4]	14.2 [8.7; 17.3][1]	9.1 [7.8; 9.8][1,2,5]	18.4 [17.7; 21.0][4]	< 0.001
180	14.6 [13.6; 15.5]	10.0 [8.9; 11.2]	10.0 [7.1; 14.0]	9.8 [8.9; 10.1][1]	12.8 [10.9;14.3]	0.032
p2	< 0.001	< 0.001	< 0.001	0.004	< 0.001	

Note: results are presented as median [interquartile range]; p1 – statistical significance of differences between study groups; [1,2,3,4,5] – differences with groups 1, 2, 3, 4, 5 are statistically significant ($p < 0.05$); p2 – statistical significance of postoperative changes in the indicator in the group

Table 4

Blood concentration of C-reactive protein (ng/ml) by augmentation of the tibia of rabbits with osteoplastic materials

Term after operation (days)	Group					p1
	Control	RXM	β-TCP	NCI	PTI	
	1	2	3	4	5	
1	0.77 [0.73; 0.84]	0.70 [0.40;0.98]	1.45 [1.0; 1.60]	1.49 [1.00;1.53]	1.11 [1.00;1.20]	0.056
3	0.82 [0.66; 1.08]	0.99 [0.83;1.55]	1.30 [1.20;1.80]	1.76 [1.20;1.90]	1.22 [1.08;1.60]	0.054
7	0.58 [0.56; 0.62]	0.67 [0.42;0.84]	0.63 [0.56;1.25]	1.60 [0.55; 4.50]	0.58 [0.31;0.84]	0.627
14	0.21 [0.16;0.30][4]	0.39 [0.33;0.41][4]	0.42 [0.21;0.53][4]	1.50 [0.63; 2.10][1,2,3,5]	0.35 [0.12;0.54][4]	0.003
45	0.03 [0.00;0.09][4]	0.12 [0.03;0.36][4]	0.21 [0.16;0.28][4]	1.50 [0.65; 2.80][1,2,3,5]	0.12 [0.11;0.24][4]	0.001
90	0.00 [0.00; 0.00][4]	0.00 [0.00;0.12][4]	0.0 [0.0; 0.10]4	0.99 [0.70; 1.28][1,2,3,5]	0.00 [0.00; 0.00][4]	0.005
180	0.00 [0.00; 0.00][4]	0.00 [0.00;0.04][4]	0.00 [0.0; 0.00][4]	0.46 [0.41; 0.58][1,2,3,5]	0.00 [0.00; 0.00][4]	< 0.001
p2	< 0.001	< 0.001	< 0.001	0.094	< 0.001	

Note: results are presented as median [interquartile range]; p1 – statistical significance of differences between study groups; [1,2,3,4,5] – differences with groups 1, 2, 3, 4, 5 are statistically significant ($p < 0.05$); p2 – statistical significance of postoperative changes in the indicator in the group

DISCUSSION

The processes of bone resorption and bone formation after injuries and surgical interventions are accompanied by a change in blood levels of markers that reflect the functional activity of osteoclasts and osteoblasts performing bone remodeling [16, 17]. The C-terminal telopeptides of type I collagen split from its molecule and appear in the blood at the earliest stages of bone tissue destruction. In uncomplicated surgical treatment of fractures, the concentration of CTX begins to increase in the blood from week 1 after surgery, reaches the maximum by 4 to 8 weeks and returns to its original values by 24 weeks [18]. It is noted that the level and dynamics of osteoresorption markers is dependent on the volume of the injured tissue [12, 19]. We observed activation of bone resorption during the first two weeks after surgery in rabbits operated without implantation and augmentation with resorbable and non-resorbable materials, but the dynamics of this process were different. In β-TCP and PTI implantation, intensive bone resorption in experimental animals started earlier than in control animals, which apparently reflects the normal remodeling process during augmentation with these osteoplastic materials.

In the cases of RXM, the prolongation of CTX release into the blood is apparently associated with the influence of degradation of the collagen component of the implant itself. High level of bone degradation over a long period of time during augmentation using a carbon implant is obviously due to high intensity and duration of the inflammatory reaction that was found in this group of experimental animals in the postoperative period, since it is known that inflammation stimulates the process of bone resorption [13].

Osteocalcin refers to non-collagenic proteins, is expressed in the process of bone formation and controls the mass, size, orientation of the mineral component, participates in the organization of the extracellular matrix [20]. Previously, a large variety in the dynamics of OC after bone tissue damage was shown [17], which is also true for filling bone defects with various osteoplastic materials. The most pronounced and long-lasting, exceeding control values, increase in the concentration of this osteogenesis marker was observed by using β-TCP, which can serve as evidence of the effectiveness of the regenerative process. At the same time, a low

level of OC in NCI may indicate insufficient success in restoring bone tissue. An explanation of the early postoperative increase in OC level with the use of xenomaterial may be the incorporation of this protein into the bone matrix [21], which makes it possible to release it early in the RXM group. Low values of OC concentration in the subsequent time points of the study using RXM may be explained by insufficient activity of osteogenesis.

Bone isoenzyme of alkaline phosphatase is a tetrameric glycoprotein found on the cytoplasmic membrane of osteoblasts and has the ability to produce extracellular inorganic phosphorus. The level of ALP is considered to be associated with the level of bone formation [22]. A previous study on modeling a femoral diaphysis fracture in experimental animals showed an increase in the expression of informative RNA BALP in the period from 10 to 14 days. Clinical studies found a significant increase in the concentration of this marker in the blood 2–4 weeks after injury [14, 23], and the level of the marker may remain elevated even after consolidation of the fracture [24, 25]. However, violation of reparative osteogenesis after trauma leads to a less pronounced and/or later increase in the concentration of ALP in the blood compared with normal bone formation [26]. In our study, augmentation with osteoplastic materials led to an earlier and more pronounced increase in blood glucose concentration compared with surgery without augmentation, which may indicate a more effective regenerative process. An inflammatory reaction has a significant negative effect on bone remodeling [21]. In particular, high postoperative intensity of the inflammatory response inhibits the expression of ALP [27]. Apparently, it is precisely the high activity of the inflammatory process that explains the increased level of resorption markers and the reduced level of osteogenesis markers during augmentation of bone defects with nanostructured carbon implants.

CONCLUSION

Using a model of an impression fracture of the proximal tibia in rabbits, it was found that if a bone defect is filled in with resorbable β-TCP or nonresorbable PTI, a combination of a high level of osteogenesis markers and a low concentration of osteoclastic resorption marker indicates an active course of bone tissue reparative regeneration and effective integration of osteoplastic material in the area of an impression bone defect. Considering the obtained data, a porous titanium implant produced with additive 3D printing technology seems to be a promising augment for filling impression defects in intraarticular fractures. The use of xenoplastic material, augmented into the bone interface, did not show any advantages in comparison with surgery performed without augmentation. The nanostructured carbon implant did not possess osteogenesis-inducing properties, but its augmentation led to an increase in the processes of osteoclastic resorption. It can be assumed that the use of NCI for intraarticular fractures may result in a defect in the integration of augment and bone and worsen treatment results.

Conflict of interests *The authors declare no apparent and potential conflicts of interest related to this study.*

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Compliance with ethical principles *All manipulations with animals were carried out in accordance with the "Guidelines for the maintenance of laboratory animals in vivariums of research institutes and educational institutions" RD-APK 3.10.07.02-09 and Directive 2010/63 / EU of the European Parliament and the Council of the European Union "On the Protection of Animals used for scientific purposes". The study design was approved by the ethics committee of the Federal State Budget Educational Institution of Higher Medical Education of the Ural State Medical University of the Ministry of Health of Russia.*

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