# Regeneration of Skull Bones in Adult Rabbits after Implantation of Commercial Osteoinductive Materials and Transplantation of a Tissue-Engineering Construct

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We performed a comparative study of reparative osteogenesis in rabbits with experimental critical defects of the parietal bones after implantation of commercial osteoinductive materials "Biomatrix", "Osteomatrix", "BioOss" in combination with platelet-rich plasma and transplantation of a tissue-engineering construct on the basis of autogenic multipotent stromal cells from the adipose tissue predifferentiated in osteogenic direction. It was found that experimental reparative osteogenesis is insufficiently stimulated by implantation materials and full-thickness trepanation holes were not completely closed. After transplantation of the studied tissue-engineering construct, the defect was filled with full-length bone regenerate (in the center of the regenerate and from the maternal bone) in contrast to control and reference groups, where the bone tissue was formed only on the side of the maternal bone. On day 120 after transplantation of the tissue-engineering construct, the percent of newly-formed bone tissue in the regenerate was 24% (the total percent of bone tissue in the regenerate was 39%), which attested to active incomplete regenerative process in contrast to control and reference groups. Thus, the study demonstrated effective regeneration of the critical defects of the parietal bones in rabbits 120 days after transplantation of the tissue-engineering construct in contrast to commercial osteoplastic materials for directed bone regeneration.

**Key Words:** *tissue engineering; multipotent stromal cells from the adipose tissue; rabbits; defect of the parietal bones*

Regeneration of calvarial and facial skull bones is still an actual problem, because of not only shortness of commercial osteoplastic materials or methods of autoplastics, but also the absence of optimal effective, safe, and pathogenetic approaches to regeneration of skull bones. The use of osteoplastic materials for directed bone regeneration is based on the assumption that they induce the formation of the new bone via stimulation of reparative osteogenesis from the host bed and induction of osteogenesis upon contact with the granulation tissue (osteoinduction) and osteoconduction via controlled terms of resorption or the absence of resorption [5].

The following groups of osteoplastic materials are used for local replacement of bone defects in oral and maxillofacial surgery: on the basis of natural or synthetic minerals, on the basis of animal biopolymers (native collagen, stromal cell-free matrix, demineralized

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or partially demineralized bone matrix), biodegradable synthetic polymers on the basis of organic acids, and their combinations (composites). Mineral materials are primarily presented by nonresorbable hydroxyapatite (Interpore 200, BioOss), resorbable hydroxyapatite (*e.g.* Osteoben), and β-tricalciumphosphate. Resent studies demonstrated predominance of fibrosis over osteogenesis after implantation of osteoinductive materials in experiments [1,7] and their low efficiency in clinical use.

Tissue engineering is a promising method of stimulation of reparative osteogenesis considerably improving its efficiency. Tissue-engineering constructs (TEC) for reparative osteogenesis are created on the basis of multipotent stromal cells from the bone marrow [8], adipose tissue [3,9], and periosteum. These constructs are effective and safe in experimental studies and in clinical studies [6].

Here we used commercial osteoinductive materials declared by the manufacturer as materials for targeted bone regeneration (BioOss, Osteomatriks, Konnectbiofarm) and additionally exhibiting properties of resorbed membranes (Biomatriks, Konnectbiofarm). The reference material was TEC for reparative osteogenesis on the basis of multipotent stromal cells (MSC) of stromal and vascular fraction of the adipose tissue (SVFAT) committed to osteogenic differentiation using RMosteo-AT (ReMeTeks) and Osteomatriks (Konnectbiofarm) protocol.

## MATERIALS AND METHODS

**Platelet-rich plasma** Platelet-rich plasma was isolated from donor platelet concentrate (N. V. Sklifosovskii Institute of Emergency Care) unsuitable for transfusion because of changed biochemical parameters of donor blood. Plasma aliquots (5 ml) were centrifuged at 1500 rpm and the supernatant was collected (3 ml). Polymerization was performed with bovine thrombin in the presence of $CaCl_2$.

**SVFAT MSC culture.** Primary MSC cultures were isolated from adipose tissue samples obtained from the neck region. The material was delivered to the laboratory within 1 h after surgery in a special transporting container. The adipose tissue was repeatedly washed with Hanks solution (PanEko) and incubated with sterile type I collagenase solution (250 U/ml, PanEko) at 37°C for 2-3 h. The isolated cells were pelleted by centrifugation (1100 rpm for 10 min) and transferred into NUNC culture flasks with complete growth medium (DMEM/F12, 1:1, PanEko) supplemented with 10% FCS (HyClone-Perbio), L-glutamine (4 mM, PanEko), and amikacin (500 mg/liter, Sintez AKO). The flasks were placed in a $CO_2$-incubator 37°C, 5% $CO_2$), the medium was replaced after 24 h, nonadherent cells were removed during medium replacement.

After 1-1.5 weeks, the MSC culture attained subconfluence and the cells were subcultured 5-7 times. Passage 4-5 cells were used in the study.

**Osteogenic differentiation.** For targeted differentiation the cells were seeded to 90-mm Petri dishes or 6-well plates and after attaining confluence the growth medium was replaced with differentiation medium: growth medium supplemented with1, 25 dihydroxycholecalciferol (vitamin D3, $10^{-8}$ M), L-ascorbic acid (50 mg/liter), β-glycerophosphate ($10^{-2}$ M, Sigma). For detection of mineralization foci, the cells were twice washed with BPS (PanEko), fixed with cold 70% ethanol, and stained with alizarin red S (40 mM; pH

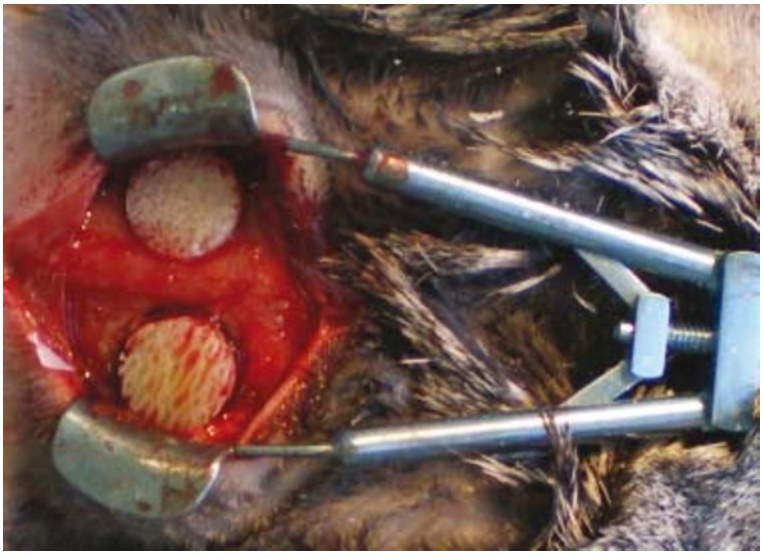

**Fig. 1.** Transplantation of TEC into parietal bone defects.

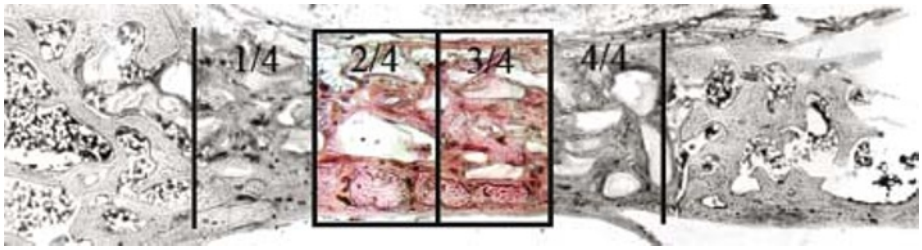


**Fig. 2.** Arbitrary zonal gradation of the regenerate. 1/4 and 4/4 are the zones with high fluctuation of tissue structures, 2/4 and 3/4 are the zones with low fluctuations suitable for statistical analysis.

4.1; Mosreaktiv) for 5 min. Unbound dye was washed out with PBS.

**Tissue-engineering construct and implantation material.** The blocks were washed with Hanks solution (PanEko) containing cephazolin (1 g/liter, Sintez), platelet-rich plasma (PRP) was carefully layered onto the blocks, and thrombin solution was added drop-by-drop (P.Z. Cormay, 50 U/ml in 10% $CaCl_2$, Dal'khimfarm) until polymerization.

**Experimental protocol** The study was approved by Ethical Committee of Institute of Human Morphology, Russian Academy of Medical Sciences. Mature male and female rabbits (age >1.5 years) were used. The animals were randomized into groups using a random number generator (Random). A total of 17 rabbits were divided into 6 groups.

In rabbits weighing 4 kg, soft tissues were dissected under combined narcosis (Zoletil 100/Rometar) and the periosteum was exposed. After separation of the periosteum, 2 or 4 full-thickness bone defects with a diameter 1 cm reaching the dura mater were made with a trephine. The test material with PRP as an additional inductor of regeneration was placed into the defect. Control group 1 ($n$=3) received only PRP and in control group 2 only bone defects were made ($n$=3). In the experimental groups, implantation/transplantation of osteoinductive materials in the form of cylinders (1 cm in diameter and 0.4 cm in height) with PRP polymerized on them was performed (Fig. 1): Osteomatriks in group 3 ($n$=3), Biomatriks in group 4 ($n$=3), BioOss in group 5 ($n$=3), and SVFAT-osteo in group 6 ($n$=2). The periosteum above the defects was sutured. The animals were sacrificed on day 120 of the experiment.

**Histological methods.** The histological samples were processed routinely using electrolytic decalcification. The sections were stained with hematoxylin and eosin after Bock. Morphometry was performed using the method of separate calculation of relative content of the tissue structures and implanted material in the regenerate using a ImageJ 1.41 freeware (National Institutes of Health).

The center of the regenerate was located by microphotograph and the regenerate was then divided into 4 equal parts (Fig. 2). In order to not include additional fluctuation at the boundary between the regenerate and maternal bone, the areas of different tissues on central quarters of the defect section were calculated on digital microphotographs at ×12. The data were processed using OpenOffice.org Calc and OpenOffice.org Math software.

During morphometric analysis of regeneration of the skull defect, the percentage of the lamellar and woven (reticulofibrous) bone tissues, osteoid, adipose tissue, and loose and dense connective tissues was calculated. The relative content of tissue components in the regenerate was calculated as the ratio of component area to the total area of the field of view and expressed in percents. The group arithmetic mean (M) and standard error of the mean for the parts (SEM) were calculated. The correspondence to normal distribution in the group was verified using Shapiro–Wilk test. For samples with normal distribution, the intergroup differences were analyzed by one-way dispersion analysis followed by Newman–Keuls and $t$ test (for paired comparison of the data in the experimental and control groups). For samples not conforming the normal law, Kruskal–Wallis rank dispersion analysis followed by Dunn test and Mann–Whitney test (for paired comparison of the experimental and control groups) was used. Significance level was 5% for all comparisons.

## RESULTS

The study revealed considerable differences between the groups (Table 1).

Solitary foci of bone tissue formation around BioOss were observed in the central zones of the regenerate by the 4th month (0.08±0.01%, Fig. 3). The young bone tissue was primarily found near the host bone close to the dura mater (10±4%). Hydroxyapatite granules were resorbed by giant cells (macrophages) without excessive infiltration with these cells. The implanted material was "embedded" into the fibrous tissue. The regenerate had a developed vascular network. The adipose tissue penetrated into the regenerate

**TABLE 1.** Relative Content of Tissue Components in Central Quarters of the Regenerate (*M±SEM*)

| Tissue structure | BiOss | Biomatriks | Osteomatriks | TEC |
|---|---|---|---|---|
| Lamellar bone | 0.08±0.01* | 2.75±0.06* | 0 | 15.19±0.09* |
| Woven bone | 1.26±0.03*+ | 9.02±0.10*+ | 0 | 23.40±0.11*+ |
| Osteoid | 0 | 0.28±0.02* | 0 | 0.78±0.02* |
| Adipose tissue | 5.11±0.06* | 28.23±0.16*+ | 1.32±0.03** | 21.65±0.10*+ |
| Connective tissue | 65.13±0.13* | 59.72±0.17* | 74.92±0.10* | 30.60±0.12* |
| Implantation material | 28.42±0.12* | 0 | 23.76±0.09* | 8.38±0.07* |

**Note.** $p<0.05$ compared to: *values in the same group, +values in other groups.

from the subcutaneous fat (Fig. 3, *d*). Its content was 5.11±0.06%.

Biomatriks was not found in the regenerate. The regenerate primarily looked like fields of dense connective tissue. The foci of young bone tissue formation were found near the host bone, it actively spread along the dura mater. The newly formed bone constituted 11.17±0.16% (Fig. 4). The connective tissue was infiltrated with eosinophils and lymphocytes. The regenerate had a developed vascular network. The adipose tissue constituted 28.23±0.16% (Fig. 3, *b*).

Osteomatriks did not induce osteogenesis in the center of the regenerate (Fig. 4). The formation of the new bone was seen on the side of the host bone. Macrophages and osteoclasts resorbed the material in zones of contact of the material with newly-formed bone. Foci of lymphocytic infiltration were seen somewhere. The adipose tissue constituted 1.32±0.03%. The bulk of the regenerate was presented by dense connective tissue (74.92±0.10%; Fig. 3, *c*).

The formation of the bone tissue in the regenerate after transplantation of the tissue-engineering construct was observed in all zones of the regenerate, primarily around Osteomatriks trabeculae and evenly throughout the thickness of the regenerate (Fig. 5). The bone tissue had the following composition: lamellar bone constituted 15.19±0.09% (Fig. 4) and woven tissue and osteoid constituted 23.40±0.11 and 0.78±0.02%, respectively. The newly-formed bone tissue on the side of the host bone merged with the bone tissue in the transplant, but in none cases connections with the external cortical plate were observed. Osteoclasts participated in resorption of Osteomatriks, it constituted 23.76±0.09%. The areas of newly-formed bone tissue contained diffuse areas of the loose connective tissue with adipose tissue inclusions, which were also seen in the spongy layer and along the margins of the defect. The regenerate contained numerous venous, arterial, and capillary vessels (Fig. 3, *d*).

In control groups with unfilled defect (group 1) and with defect filled with PRP (group 2), the regenerate was primarily presented by dense connective tissue (it constituted 85±3%, Fig. 3, *a*).

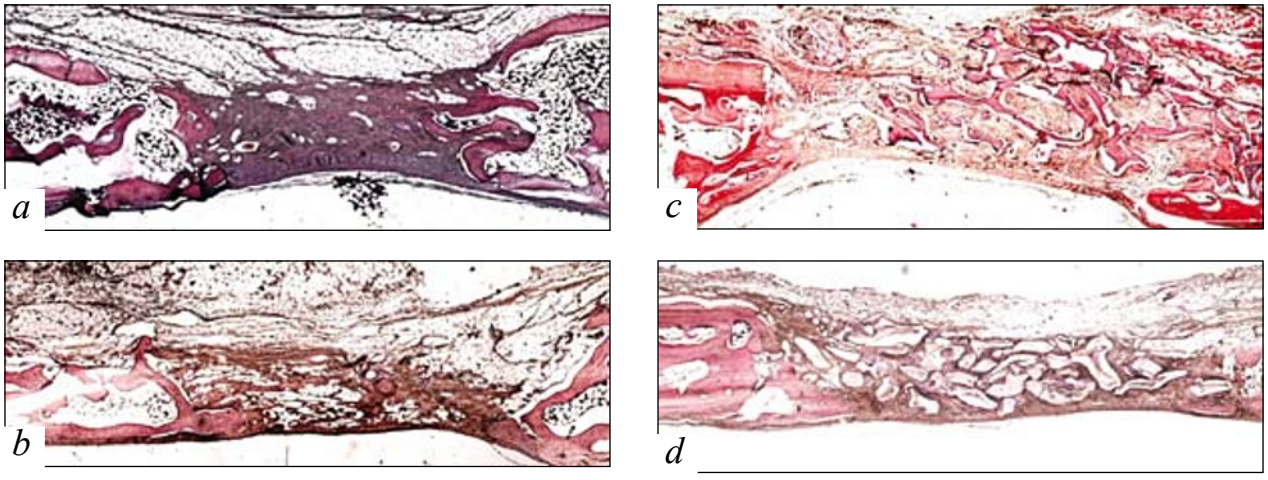


**Fig. 3.** Panoramic histological image of bone regenerate 120 days after implantation/transplantation of PRP (*a*), Biomatriks+PRP (*b*), Osteomatriks+PRP (*c*), and BioOss"+PRP (*d*). Hematoxylin and eosin staining, ×2.5.

Morphometry of the newly-formed bone tissue in central zones (2/4 and 3/4; Fig. 2) of the regenerate revealed considerable differences in the percentage of woven and lamellar bones between the groups with implantation of BioOss and Biomatriks and transplantation of TEC.

The volume content of the bone tissue in the groups with TEC transplantation was always lower than in other groups. The relative content of woven bone tissue and loose and dense connective tissues in the central quarters of the regenerate also significantly differed in all groups.

However, morphometry of the whole regenerate revealed no significant differences due to pronounced fluctuations of the relative content of the bone tissue along the edges of the regenerate, which was determined by stochastic regenerative potencies of the host bone and the type of damage.

It should be noted that analysis of the central part of the defect provides better information of the contribution of osteoinductive materials into regeneration of the bone tissue in the parietal skull bone defect.

Thus, the test commercial osteoplastic materials did not induce regeneration of the skull bone on the model of parietal bone defect in adult rabbits. The major contribution into the regeneration process was made by the host bone. The regeneration process quenches soon after beginning and does not result in complete regeneration. The rapid and predominant development of the connective tissue suppresses spreading of the regenerate. The periosteum and dura matter do not take a part in the regeneration at a distance from the host bone. Biomatriks moderately stimulates the reparative osteogenesis and is rapidly resorbed by osteoclasts and macrophages (the osteoinductive effect is absent). The osteoinductive factors released during this processes (non-collagen extracellular matrix proteins) probably stimulate reparative osteogenesis from the side of the host bone. However, the material does not maintain the volume at late terms and does not prevent the formation and growth of dense connective tissue and adipose tissue. Osteomatriks only slightly stimulates reparative osteogenesis via osteoinduction of the adjacent connective tissue; it exhibits pronounced osteoconductive properties, is resorbed by osteoclasts, and maintains the volume for at least 120 days. BioOss is a weak osteoinductive material; it maintains the volume due to the absence of resorption and in fact is embedded into the fibrous tissue. Reparative osteogenesis in the bone defect after transplantation of the tissue-engineering construct on the basis of SVFAT MSC had some peculiarities. First, the regenerate contained loose connective tissue and only small amount of dense connective tissue. Second, osteogenesis was observed both along the dura mater and in the depth of the regenerate. The presence of the adipose and loose connective tissues did not hamper the development of the bone tissue, but contributed to the trophic function of the transplant due to the presence of numerous blood vessels of different diameters. Third, *de novo* bone formation is underlain by the mechanism of intramembranous

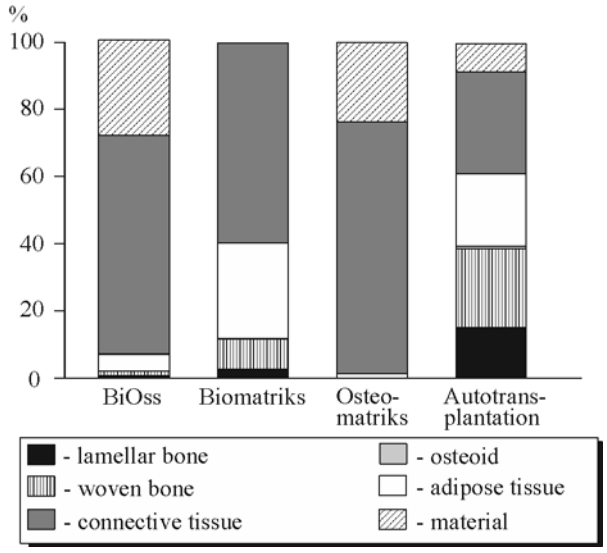


**Fig. 4.** Structural component of parietal bone regenerate.

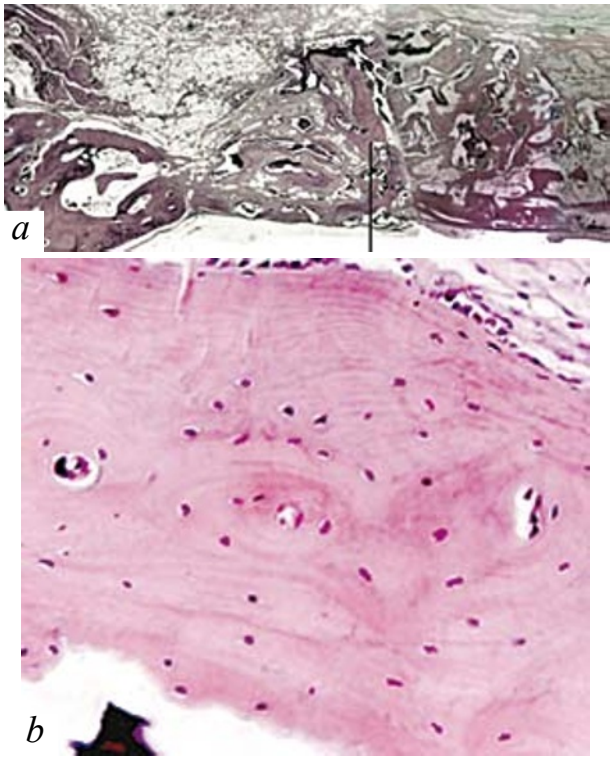


**Fig. 5.** Histological picture of the bone regenerate 120 days after transplantation (*a*) and formation of mature lamellar bone in the center of the defect (*b*). Hematoxylin and eosin staining, ×40.

ossification without signs of the cartilaginous tissue. Fourth, it can be hypothesized that the formation of young bone tissue all over the material surface can be directly related to activity of the transplanted cell culture and its osteogenic properties proved in *in vitro* experiments.

In out study, we did not monitor the dynamics of bone tissue maturation and did not analyze the mechanisms of reparative osteogenesis after transplantation of TEC. However, we outlined the ways of solving the key problems of regeneration of calvarial bones.

Our findings agree with previous reports on early and effective regeneration of the skull bones in rabbits [4,6].

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