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# The effect of mineralized bone matrix on reparative osteogenesis

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## ABSTRACT

**Background:** The development and experimental-and-clinical evaluation of osteoplastic implantation materials one of the most urgent problems of modern medicine. The purpose of the present work consists in studying the characteristics of reparative osteogenesis for filling bone defect under implantation of the allogenic mineralized bone matrix (MBM). **Materials and Methods:** The special features of reparative osteogenesis for implantation of granulated MBM obtained without thermal and demineralizing treatment into a tibial defect have been studied experimentally in 50 Wistar rats using scanning electron microscopy, X-ray electron-probe microanalysis, and histological techniques. **Results:** MBM been established to possess marked osteoinductive and osteoconductive properties, the prolonged activation of reparative osteogenesis is observed during implantation, as well as deep bone tissue sprouting into the implant, acceleration of organotypic remodeling of regenerated bone, intense angiogenesis and early restoration of the bone involved. **Conclusion:** The use of the implant from the MBM appears theoretically sound and promising, particularly in the surgical treatment of bone defects, osteomyelitic seizures, bone cysts, and foci of osteonecrosis.

KEY WORDS: Bone defect, implantation, mineralized bone matrix, reparative osteogenesis

## INTRODUCTION

The development and experimental-and-clinical evaluation of osteoplastic implantation materials possessing bioactivity, biocompatibility, osteoinductivity, and osteoconductivity remains one of the most urgent problems of modern medicine [1-3]. Different materials are used for bone replacement, both biological and synthetic [4,5]. The following materials are used the most often: Demineralized bone matrix, bioceramics, collagen matrices and cryogels, bioglass and biosital, bone mineral analogs - hydroxylapatite, tricalcium phosphate, and polysaccharides of natural origin as well [6,7]. However, while studying long-term results, it has been established that these implants do not osteointegrate, but they are surrounded by a fibrous capsule regardless of whether dense or porous material used [8]. Their drawbacks also include low osteoinductive and osteoplastic effectiveness, the absence of osteoconductive activity, biocompatibility limitation. The purpose of the present work consists in studying the characteristics of reparative osteogenesis for filling bone defect under implantation of the allogenic mineralized bone matrix (MBM).

## MATERIALS AND METHODS

### The Experimental Procedure

The experiments were performed using 50 Wistar pubertal rats, males, and females, with the body weight of 340-390 g. The keeping, experiments, and euthanasia of animals were made according to the guidelines of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [9], and approved by Ethics Committee of Russian Ilizarov Scientific Center Restorative Traumatology and Orthopaedics. Two groups of animals were formed, 25 rats each, - a control group and experimental one. The animals of both groups were kept under the same vivarium conditions with the standard diet. In the animals of control and experimental groups the modeling of non-through fenestrated defects of 2.5-3 mm diameter at the boundary of tibial shaft proximal third and metaphysis was made in the operating room with penetration into the medullary cavity by unilateral perforation of cortical bone with a dental drill using general anesthesia (8 mg of Rometar and 4 mg of Zoletil per 1000 g of body weight IM). Sterile biomaterial, i.e., granulated MBM

of 2-3 mg mass, was placed into the zone of the formed defect immediately after surgery in the animals of the experimental group. The biomaterial was obtained from allogenic tubular bones organic components of which were removed using 6% solution of sodium hypochlorite (NaOCl) with further grinding to a powder [10]. The microrelief and structure of MBM granules were examined with JSM-840 scanning electron microscope (Jeol, Japan), the content of osteotropic macro-elements (calcium, phosphorus, sulfur, sodium, and magnesium) was determined with INCA-200 energy X-ray electron probe microanalyzer (Oxford Instruments, England).

### Research Methods

After 2, 5, 7, 14, and 21 days the animals were withdrawn from the experiment (five animals were used for each time point). Tibias were fixed in 2% solution of paraformaldehyde and glutaraldehyde on phosphate buffer at 7.4 pH, embedded in paraffin (after decalcification) and in araldite (without decalcification). Paraffin sections were stained with hematoxylin-eosin, and with picrofuchsin by Van Gieson. Araldit blocks were examined using X-ray electron probe microanalyzer and JSM-840 scanning electron microscope (Jeol, Japan). The activity of osteogenesis process was determined by the content of bone tissue and unmineralized components in the regenerated bone. The index of regenerated bone compactness was calculated by the ratio of these values, it characterized the osteogenesis intensity and maturity degree of newly formed bone tissue in which the content of osteoid and mineralized matrix was determined as well. The results of quantitative investigations were processed using the methods of variation statistics. The significant differences of the parameters compared were calculated using Student's *t*-test. The differences were considered significant at the level of $P < 0.05$.

## RESULTS

Implanted MBM granules are of 50-200 $\mu$m in diameter and have a well-ordered highly porous structure, their surface pattern is characterized by pronounced roughness and fractality with granule size in nanorange [Figure 1]. They are nanostructured, have multiple macro- and micropores of an irregular shape the size of which is 10-20 $\mu$m, and some of them are <100 nm in size. The pores correspond to the places of localizing bone lacunae and bone tubules from which cells and other organic components were removed when obtaining the implant. Microarchitectonics of MBM granules is close to the natural architectonics of bone matrix [Figure 1b].

The content of the main osteotropic macroelements comprising MBM granules is shown in Table 1.

As histological investigations have shown, alternative-and-destructive changes are revealed in the zone of injury in both groups of animals 2-5 days after surgery, as well as the acute inflammatory reaction occurs. Foci of organized hematoma are found with fibrin clots, little-differentiated cellular elements, neutrophilic granulocytes, macrophages,

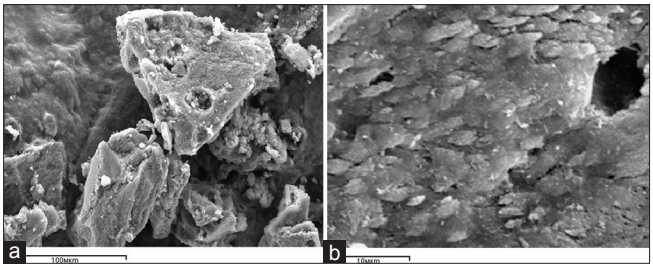


**Figure 1:** The implanted granules of the mineralized bone matrix (MBM): (a) General view of the implanted MBM granules; (b) macro- and micropores, and nano-structuring of MBM granule. Scanning electron microscopy, magnification: (a) ×320, (b) ×2200

**Table 1: Relative content of different chemical elements in the implanted granules of mineralized bone matrix (M±m, %)**

| Chemical elements | Proportion of the total mass, % |
|---|---|
| Sodium | 0.440±0.02 |
| Magnesium | 0.350±0.01 |
| Phosphorus | 13.61±0.60 |
| Sulfur | 0.281±0.01 |
| Calcium | 26.82±1.30 |

mast cells, extravasal erythrocytes, and lymphocytes. Leukocytic necrotic masses containing lysed cells and fibrin strata are identified. Cellular elements of inflammation and bone chips produced in the process of surgery form the centrally located focus of inflammation on the site of which restorative processes are developed by 7 days, as well as reparative bone formation is observed by intramembranous osteogenesis type, and a regenerated bone is developed which gradually fills the perforation cavity [Figure 2].

Two zones, peripheral and central, are clearly identified in the regenerated bone of the animals of the control group by 7 days after surgery. New formation of coarse-fibered bone tissue is observed in the peripheral zone, only isolated thin trabeculae and small bone-osteoid islands are formed by this period in control group [Figure 2a], wherein reparative osteogenesis develops in the direction from the periphery to the center (centripetally). Little-differentiated connective tissue with edema signs containing few blood vessels, exudate, and cellular detritus is observed in the central zone of the regenerated bone of control group. An extensive small-looped network of thickened trabeculae is formed in the peripheral zone of the regenerated bone of animals from experimental group, wherein the trabeculae are covered with rows of numerous osteoblasts, which form spongy bone substance closely knitted with compact substance surface [Figure 2b]; numerous bone trabeculae, osteoblastic and fibroblastic elements, as well as a significant amount of enlarged and filled with blood vessels are identified in the central zone of the regenerated bone.

The cells of inflammatory reaction are not identified in the animals of the experimental group; reparative osteogenesis in the regenerated bone develops all over the defect diameter in both directions - centrifugally and centripetally. The zones of active neoangiogenesis and appositional osteogenesis are

identified around MBM granules [Figure 3a]. There are no hemorrhagies and destruction foci in these areas, the active proliferation of fibroblasts and preosteoblasts is observed, a layer of osteogenic cells, osteoid, and bone matrix is formed on the surface of implanted MBM granules.

A phase of organogenesis and remodeling is observed in the regenerated bone of the animals of experimental group by 14 and 21 days after surgery, as evidenced by reorganization of primary trabeculae to organotypical osteon structures, and by a large number of commissural lines. Massive stratifications of newly formed bone tissue are identified along the defect edge; wherein the tissue approaches the intact cortex by calcification degree [Figure 2c and d]. The osteocyte lacunae of the characteristic structure are often found in the regenerated bone, and one of their walls is represented by the surface of implanted MBM granules [Figure 3b]. Empty lacunae of osteocytes are not numerous. Immured in bone tissue remains of MBM granules formed during bio-destruction can be seen among lamellar bone trabeculae; and the functionally active osteoclasts contacting the granule surface and having multiple nuclei and invaginations of brush-like rim are found within these remains. This indicates that the rate of biodegradation of the implant corresponds to the speed of new bone tissue formation. By 21 days after surgery, most of the pores of implanted MBM granules have been filled with osteogenic cells, which form bone tissue within the implant, thereby providing to acquire its osteogenic properties [Figure 3c]. The formation of tissue-specific regenerated bone in the defect area occurs slower, and in later periods in the animals of control group, only gradual reorganization of coarse-fiber bone tissue trabeculae to more mineralized and mature ones is observed by 21 days after surgery.

As quantitative studies have demonstrated [Table 2], the volume of bone tissue (both osteoid and mineralized matrix) in the regenerated bone of the animals of experimental group increases almost 2-fold compared with control values by 7 days after surgery ($P < 0.01$).

The index of compactness increases from 0.09 ± 0.001 in control group to 0.21 ± 0.002 - in experimental one ($P < 0.001$) thereby evidencing the increase of maturity degree of newly formed bone tissue in experimental group of animals. The volume of bone tissue in the regenerated bone increases significantly by 14 and 21 days after surgery in both groups, but most significantly - in the experimental group of animals ($P < 0.01$). The index of compactness in this period is the following: by 14 days - 1.35 ± 0.06 for control, and 2.31 ± 0.1 - for experiment ($P < 0.01$); by 21 days - 5.80 ± 0.20 for control, and 12.7 ± 0.5 - for experiment ($P < 0.001$).

## DISCUSSION

The studies performed have demonstrated that MBM granules obtained without exposure to high temperatures and demineralizing agents have a three-dimensional structure of interrelated pores and retain natural micro architectonics of bone tissue. The chemical composition of granules corresponds to the mineral composition of the cortical layer of the tibial shaft intact adult rats [11]. The value of Ca/P coefficient in the implanted MBM granules is 1.97 ± 0.11, and according to the literature [5,12,13], it indicates that MBM, as well as mineralized matrix of intact bones, is not an analog of stoichiometric hydroxylapatite, $Ca_{10}[PO_4]_6[OH]_2$, but it represents dahllite - carboxyl hydroxylapatite, $Ca_5[PO_4] \times [CO_3]_3OH$, with the labile structure of amorphous apatite surface layer. Formation of this surface carbonate-apatite layer in organism biological environment on MBM granule pores which has more pronounced intensity of ion exchange comparing with natural bone provides affinity to

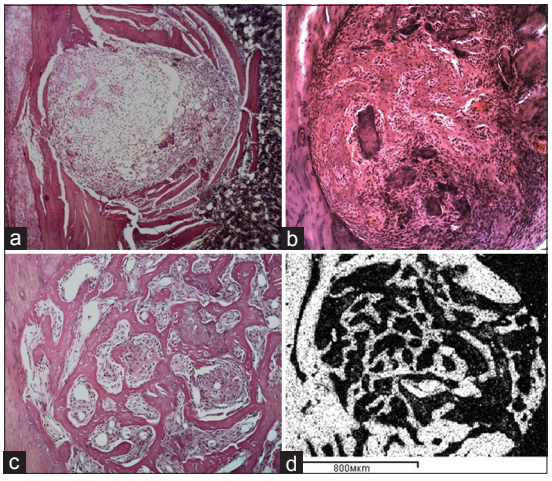


**Figure 2:** Reparative bone formation in the cavity perforating tibial 7 (a,b) and 14 (c,d) the day after the operation: (a) Regenerate control animals, trabeculae are formed only on the periphery of the defect; (b) regenerate animal experimental group, bone trabeculae fill the entire cavity of the perforation; (c,d) regenerates the animals of the experimental group at 14 days after surgery; (a,b,c) paraffin sections, hematoxylin - eosin. The lens 10, an eyepiece 10; (d) Card X-ray electron probe microanalysis, image characteristic X-ray of calcium

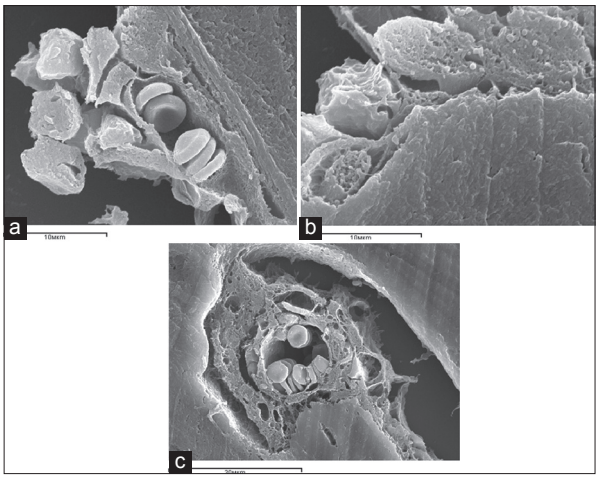


**Figure 3:** Osteogenic cells and blood vessels on the surface of the implanted granules of mineralized bone matrix (MBM) through 7 (a), 14 (b), and 21 (c) day after surgery: (a) Capillary kidney and perivascular osteogenic cells, forming layers of osteoid; (b) Mature osteoblasts on the surface of the granules MBM; (c) Capillary terminals and perivascular osteogenic cells, grown into the porous structure of the implanted granules MBM. Scanning electron microscopy

Table 2: Proportion of various components comprising the regenerated bone being formed in tibial perforation cavity of rats (M±m, %)

| The components studied | The period after surgery, days | | | | | |
|---|---|---|---|---|---|---|
| | 7 | | 14 | | 21 | |
| | Control | Experiment | Control | Experiment | Control | Experiment |
| Unmineralized components | 91.42±4.31 | 82.11±4.10* | 42.53±2.13 | 30.21±1.40* | 14.70±0.71 | 7.32±0.31* |
| Osteoid | 2.44±0.10 | 5.63±0.22* | 11.42±0.51 | 18.11±0.90* | 17.12±0.84 | 19.33±0.92* |
| Mineralized bone matrix | 6.21±0.22 | 12.33±0.52* | 46.11±2.20 | 51.84±2.32* | 68.31±3.30 | 73.44±3.53* |

*Significant changes comparing with control values

the bone tissue of regenerated bone, as well as its ingrowth, close contact with the surface of implanted granules, and formation of osteointegrative connection. It is known that growth factors and bone morphogenetic proteins being effective osteoinductors, which release during natural bone remodeling, traumatic injury, and pathological process are localized in bone tissue mineralized matrix [14-16]. Consequently, noted by us gradual prolonged osteoclastic resorption of implanted MBM granules provides them osteoinductor properties and the most favorable conditions for adhesion, proliferation, differentiation, and functioning of osteogenic cells facilitated by roughness and nanostructuring of the implant surface.

## CONCLUSION

Thus, prolonged activation of reparative osteogenesis, deep bone tissue sprouting into the implant, acceleration of the process of the regenerated bone remodeling, and reducing the periods of damaged bone healing can be observed in case of MBM granule implantation into the zone of bone defect. Relative atraumaticity of surgical intervention, simplicity of the technology of implantation material harvesting and conservation, as well as osteoconductive, osteoinductive, and osteogenic properties, characteristic rate of biodegradation, which corresponds to reparative osteogenesis rate, absence of biological rejection reaction place the studied biomaterial among the most optimal osteoplastic materials, especially under the conditions of reducing the individual osteogenetic potential in mature and elderly patients. The application of MBM implant as a reparative osteogenesis stimulator seems theoretically grounded and promising, in particular for surgical treatment of bone defects formed as a result of osteomyelitic sequesters, bone cysts, osteonecrosis foci, and oncologic diseases.

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