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Modern Approaches to Studies of New Osteogenic Biomaterials on the Model of Regeneration of Critical-Size Cranial Defects in Rats

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Osteoinductive characteristics of new osteoplastic materials based on demineralized bone matrix of xenogenic origin with high and controlled degree of purification were studied on the model of regeneration of critical-size cranial defects in rats using modern approaches, including histological analysis, evaluation of morphological parameters of the bone tissue obtained by micro-computed tomography, and estimation of bone tissue growth rate using *in vivo* fluorochrome label. Demineralized bone matrix and, to a much greater extent, its activated form containing modified recombinant growth factor rhBMP-2 with high content of the dimeric form exhibited osteoinductive activity.

Key Words: *demineralized bone matrix; BMP-2; osteoinductive activity; bone tissue regeneration; defect of critical size*

Modern requirements to safety and efficiency of osteoplastic materials based on demineralized bone matrix (DBM) with bioactive components necessitate the development of new protocols for creation of highly purified DBM [3,9] and standardization by the osteoinductor content. Quantitative evaluation of osteogenic activity of these materials implies analysis of numerous parameters on standard experimental models *in vivo*. One of the most promising is the model of regeneration of critical-size cranial defects (CSD) in rats [8].

We evaluate the osteoinductive characteristics of a new material based on highly purified DBM and its activated form containing modified growth factor rhBMP-2, on the model of cranial CSD in rats using osteotropic fluorescent labels *in vivo* and high-resolution micro-computed tomography.

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MATERIALS AND METHODS

Preparation of membranes from DBM. In order to prepare membranes, cattle femoral diaphyses were cut (with a belt saw) into 1-mm layers. Purification was carried out as described previously [3].

Deproteinization and immobilization of DBM. We previously developed a method for preparing DBM with osteoinductive characteristics with controlled pH and residual levels of calcium and lipids [1,3]. In order to minimize possible side effects of DBM application, we optimized the method by standardizing the content of potentially allergenic noncollagen proteins [7]. For elimination of noncollagen proteins, the membranes were processed for 11 days in 8 M urea in 50 mM sodium phosphate buffer (pH 5.8) in 1:25 proportion at constant stirring; this resulted in 12-fold decrease of the content of noncollagen proteins: from 1.2 to 0.1 mg/g matrix.

Immobilization of rhBMP-2 on DBM membranes included their equilibration with buffer (0.5 M NaCl, 50 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.5), lyophiliza-

tion, 3-h incubation with rhBMP-2 in the same buffer, washing in buffer, and drying at 20°C. In membranes used in the experiments, the rhBMP-2:DBM ration was 0.5:100 mg. Membranes with different levels of the factor could be prepared by this method, which was essential for optimization of the osteogenic activity of the materials, as very low and very high doses of BMP-2 impair osteogenesis [5].

Preparation of rhBMP-2. Modified rhBMP-2 containing the domain, promoting the formation of active dimeric protein form during synthesis, was produced in *E. coli* and isolated according to a previously developed protocol [4] with subsequent chromatography on heparin-sepharose. The resultant protein contained 95% active dimeric form.

***In vivo* experiments.** The study was carried out on 10-week-old male Wistar rats in accordance with the Directive 2010/63/EU and Supplement A to European Convention ETS No. 123. The rats were distributed into 3 groups, 6 per group. The animals were intraperitoneally narcotized with zoletil 100 (15 mg/kg) and rometar (6 mg/kg). After full-thickness incision was made in the scalp in the sagittal plane, a standardized 8-mm defect was created with the use of Surgic AP machine (NSK Nakanishi Inc.) in the parietal bones under conditions of constant cooling with saline. In group 1, CSD remained empty (control), in group 2 it was filled with the membrane, and in group 3 with the membrane with rhBMP-2. In order to evaluate the bone tissue growth rate, the animals were subcutaneously injected with a single dose of tetracycline hydrochloride (25 mg/kg) after implantation and with Alizarin Red S (35 mg/kg) twice (on days 40 and 50 after the operation). Euthanasia was carried out on day 60 after implantation by CO_2 inhalation, and necropsy of calvaria sites with CSD was carried out.

Micro-computed tomography. Scanning was carried out *in vivo* on a SkyScan 1176 tomograph (Bruker) with 0.5 mm aluminum filter at 60 kV, 575 mA current, and 35 μ resolution, with subsequent analysis of bone volume (BV), tissue volume (TV), bone surface area (BS), total tissue surface (TS), bone tissue percentage estimated as bone volume/tissue volume (BV/TV), bone surface compactness estimated as bone surface/tissue volume (BS/TV), and trabecular pattern factor (Tb.Pf).

Histology. Specimens for microscopy were fixed in 10% neutral buffered formalin (7-10 days) and decalcified in Richman—Gelfand—Hill fluid (3 days, 4°C). Paraffin sections were stained with hematoxylin and eosin. Specimens for fluorescent microscopy were fixed in 70% ethanol, after which thin sections were sliced. Tissue status in the regeneration focus was scored [2]. Bone tissue growth rate was estimated as the ratio of the distance between fluorochrome binding fronts to the time between fluorochrome injections.

The data were processed by the nonparametric Kruskal—Wallis test using Statistica 12.0 software. The values were considered significant at $p<0.05$.

RESULTS

Pathomorphological studies showed that CSD was not completely filled with the bone tissue in any of the groups (Fig. 1, *a*).

In group 1, defects had hard edge roughness, the central part was filled by soft connective tissue film. In group 2, CSD contained non-resorbed DBM with a single moderately manifest ossification focus of irregular shape. In group 3, CSD was filled with connective tissue containing fragments of implanted membrane with several ossification foci of various sizes. These observations were documented by X-ray examination (Fig. 1, *b*) and tomography (Fig. 1, *c*).

Histological studies (Fig. 1, *d*) showed that CSD in group 1 were filled with loose fibrous connective tissue with foci of cartilage tissue and sites of reticulofibrous bone tissue adjacent to the maternal bone. In group 2, a significant amount of fragmented DBM surrounded by connective tissue fibers was found. In group 3, just few fragments of DBM with multifocal depositions of woven bone tissue of various sizes were found in the marginal and central parts of CSD, and there were numerous osteoblasts. The rate of new bone tissue growth (Fig. 1, *e*) and summary efficiency of regeneration were the maximum in group 3 (Table 1).

Statistical processing of tomography data demonstrated significant differences from the control for all parameters except bone surface compactness (BS/TV). In groups 2 and 3, significant ($p<0.05$) differences in comparison with group 1 were observed for tissue surface area (TS) and volume (TV). The rest morphological parameters differed significantly between groups 1 and 3 (Fig. 2). These results correlated with the results of studies of BMP-2 osteoinduction on the model of cranial CSD regeneration in rats [6].

TABLE 1. Parameters of Osteogenesis in CSD Region (*M*±*m*)

Parameter	Group 1	Group 2	Group 3
Area of mineralized bone matrix, % of visual field	33±5	51±3	72±7
Growth rate, μ/day	0.5±0.2	0.8±0.4	1.2±0.1
Summary efficiency of regeneration, points	2	4	7

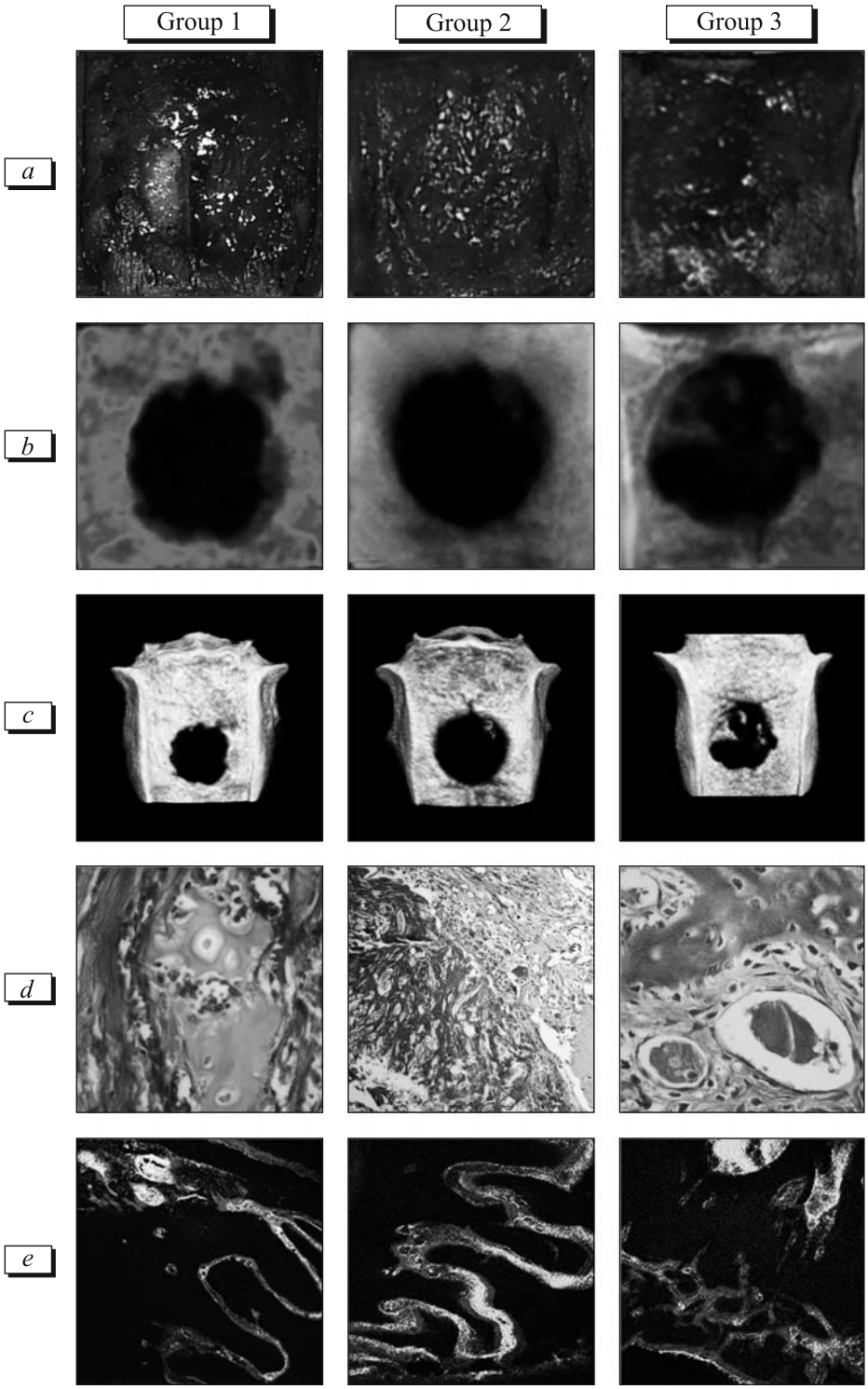

Fig. 1. Macrophotograms (*a*), roentgenograms (*b*), tomograms (*c*), microphotograms (*d*, ×100), and fluorescent microphotograms (*e*, ×200) of CSD region in rats.

Analysis of more parameters of osteogenic activity of new materials with the use of a standardized highly reproducible model of CSD incapable of complete osteoreparation without application of osteogenic materials, is expected to enable more detailed studies including studies of dose-dependent effects of two and more growth factors added to the implanted material.

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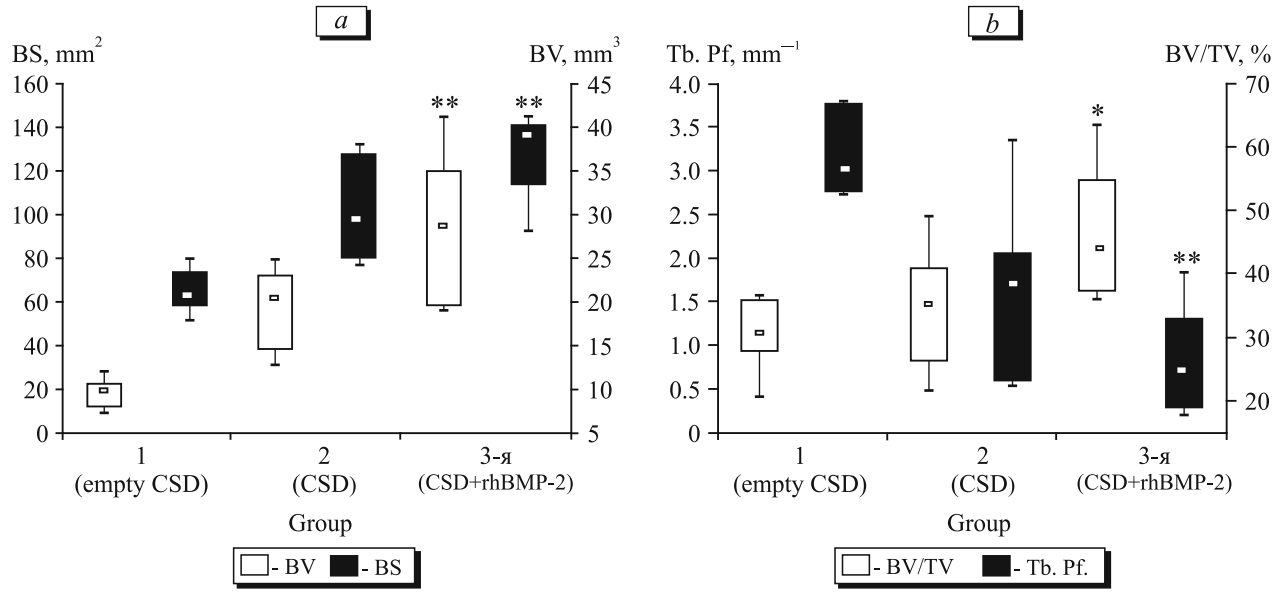

Fig. 2. Bone surface area (BS) and volume (BV) (*a*), percentage of bone tissue (BV/TV) and index of fragmentation (Tb.Pf) (*b*) in CSD. **p*<0.05, ***p*<0.01 in comparison with group 1.

REFERENCES

1. Bartov MS, Karyagina AS, Gromov AV, Mishina DM, Trunova GV, Sidorova EI, Andreeva EV, Donchenko SV, Mukhametov FF, Mukhametov UF, Mirgazizov MZ, Mirgazizov AM, Khafizov RG, Lunin VG, Filippova NE, Gintsburg AL. New generation osteoplastic materials GAMALANT, containing growth and regeneration protein factors. Kafedra Travmatologii Ortopedii. 2012;(2):21-25. Russian.
2. Grigor'yan AS, Toporkova AK.Problems of Implant Integration into the Bone Tissue (Theoretical Aspects). Moscow, 2007. Russian.
3. Gromov AV, Nikitin KE, Karpova TA, Bartov MS, Mishina DM, Subbotina ME, Shevliagina NV, Sergienkov MA, Soboleva LA, Kotnova AP, Sharapova NE, Semikhin AS, Didenko LV, Kariagina AS, Lunin VG. Development of a method for obtaining of dimineralized bone matrix with high residual content of native bone tissue growth factors. Biotechnology in Russia. 2012;(5):66-75.
4. Sharapova NE, Kotnova AP, Galushkina ZM, Lavrova NV, Poletaeva NN, Tukhvatulin AE, Semikhin AS, Gromov AV, Soboleva LA, Ershova AS, Sergienko OV, Lunin VG, Karyagina AS, Zaitsev VV. Production of the recombinant human bone morphogenetic protein-2 in Escherichia coli and testing of its biological activity in vitro and in vivo. Molecular Biology. 2010;44(6):923-930.
5. Boerckel JD, Kolambkar YM, Dupont KM, Uhrig BA, Phelps EA, Stevens HY, García AJ, Guldberg RE. Effects of protein dose and delivery system on BMP-mediated bone regeneration. Biomaterials. 2011;32(22):5241-5251.
6. Cowan CM, Aghaloo T, Chou YF, Walder B, Zhang X, Soo C, Ting K, Wu B. MicroCT evaluation of three-dimensional mineralization in response to BMP-2 doses in vitro and in critical sized rat calvarial defects. Tissue Eng. 2007;13(3):501-512.
7. Drosos GI, Kazakos KI, Kouzoumpasis P, Verettas DA. Safety and efficacy of commercially available demineralised bone matrix preparations: a critical review of clinical studies. Injury. 2007;38(Suppl. 4):S13-S21.
8. Hollinger JO, Kleinschmidt JC. The critical size defect as an experimental model to test bone repair materials. J. Craniofac. Surg. 1990;1(1):60-68.
9. Russell JL, Block JE. Clinical utility of demineralized bone matrix for osseous defects, arthrodesis, and reconstruction: impact of processing techniques and study methodology. Orthopedics. 1999;22(5):524-531.