Osteoinductive potential of highly porous polylactide granules and Bio-Oss impregnated with low doses of BMP-2

A V Vasilyev1,2, V S Kuznetsova1, T B Bukharova3, T E Grigoriev3, Yu D Zagoskin3, E V Galitsina2, N L Fatkhudinova1, I I Babichenko1, S N Chvalun3, D V Goldstein2 and A A Kulakov1

1Central Research Institute of Dental and Maxillofacial Surgery, Timur Frunze st., 16, Moscow, 119021, Russia
2Research Centre for Medical Genetics, Moskvorechye st., 1, Moscow, 115478, Russia
3NRC “Kurchatov Institute”, Academician Kurchatov pl., 1, Moscow, 123098, Russia

E-mail: vav-stom@yandex.ru

Abstract. Existing osteoplastic materials based on osteoconductive matrices lose their relevance. In this connection, to find the most effective and biocompatible carriers for osteoinductors delivery is an important task. Among the existing growth factors BMP-2 is the most effective and researched. Highly porous polylactide (PLA) granules and granular deproteinized bone material Bio-Oss were studied as carriers. Highly porous PLA granules were more effective than Bio-Oss. When using the minimum effective dose of BMP-2 for rats of 10 μg/ml impregnated in highly porous PLA granules, osteogenesis was induced in the calvarial critical-size defect and 18 ± 8% of the defect was filled with a new bone. There were no inflammatory reactions in the PLA granules implantation area. During subcutaneous implantation in rats it was shown that giant multinuclear cells took part in the resorption of the material. Their number was statistically significantly higher in the PLA granule implantation area than Bio-Oss and amounted to 71 ± 23 versus 30 ± 8 cells per 1 mm2. The obtained data showed that highly porous PLA granules were a promising basis for osteoinductive osteoplastic materials.

1. Introduction

Existing osteoplastic materials based on osteoconductive matrices lose their relevance because of the introduction of materials activated by growth factors [1]. The bone morphogenetic protein-2 (BMP-2) is the most studied among such factors. Its importance in skeleton formation is confirmed by the fact that knockout of the BMP-2 gene leads to the death of embryos [2]. Having high efficacy, the protein is characterized by a short half-degradation period, which is 6.7–16 minutes [3]. In this regard, there are two methods to maintain the bioefficacy of BMP-2. The first is to increase the concentration of BMP-2 by orders of magnitude. Thus, in the existing drug Infuse Bone Graft (Medtronic, USA), the concentration of BMP-2 is 1.5 mg/ml [4]. The second method is aimed at protein protecting from the body enzymes and ensures its prolonged action due to the put in the bioresorbable and semi-permeable scaffolds. Earlier we carried out a comparison of the BMP-2 release kinetics from various biopolymer matrices. In vitro studies have shown that highly porous (98%) polylactide granules obtained by freeze-drying from emulsions allowed the release of BMP-2 over a period up to 6 days [5] and had a...
high biocompatibility [6]. There is evidence that the effective concentration of BMP-2 for different mammals is different and for rats it is 10 μg/ml [7]. The use of a scaffold capable of realizing a minimum effective BMP-2 dose is economically feasible due to the high cost of recombinant proteins. An in vivo study is required to evaluate the practical effectiveness of using polylactide granules as a scaffold for BMP-2.

2. Materials and Methods

2.1. Highly Porous Polylactide (PLA) Granules
Porous granules from PLA were prepared by spraying and freeze-drying polymer emulsions of "Nature Works PLLA (4032D)", dissolved in 1,4-dioxane. PLA granules had a 98% porosity and a 1-2 mm diameter. Granules were sterilized by the radiation method. The absorbed dose of ionizing radiation was 15 kGy.

2.2. Bio-Oss (control group)
Small granules (S) of deproteinized bone matrix Bio-Oss (Geistlich, Switzerland) were used as a control.

2.3. BMP-2 impregnation
Stock solutions were prepared as follows: 10 μg of recombinant human BMP-2 (rhBMP-2, AkronBiotech, USA, SKU: AK8356, obtained in E. Coli) was dissolved in 100 μl of buffer containing 100 μg of BSA and 20 μl of 10mM acetic acid. The rhBMP-2 solution moistened the granules of the materials and evacuated five times. Subsequent freezing and lyophilization led to protein adsorption on the surface and in the pores of polylactide granules.

2.4. Material implantation
The experiment involved male Wistar rats weighing 250-350 g total number of 20 animals (5 rats in each group). Groups were formed depending on the type of material and method of implantation. The experiment was in accordance with the recommendations of the local bioethical committee, and its setting was guided by ISO 10993 “Biological evaluation of medical devices - Part 2: Animal welfare requirements” and “European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes ETC 123, 1985 [7, 8]. For anesthesia of laboratory animal intraperitoneal injections of Zoletil (Virbac, France) and Xylazine (Interchemie Werken "de Adelaar“ BV, Netherlands) at a dose of 30 mg / kg and 5 mg / kg was used. Subcutaneous implantation. Rats were shaved off their hair at the withers, antiseptic treatment was performed, a longitudinal skin incision was performed under sterile conditions. Subcutaneous pockets were formed by a blunt way and the materials were placed there (figure 1). After the wound was sutured with interrupted sutures by "Vicryl 5/0“ material (Ethicon, USA). Implantation in the area of a critical defect. Rats were shaved off the hair on their heads, a transverse and vertical laterally-displaced incision of the scalp was made, forming a triangular flap, and the parietal bones were subsequently exposed by a blunt and sharp way. A round opening was formed in the middle of the sagittal suture on the parietal bones using a C-reamer trepan 5.5 mm in diameter and 1.5 mm high from the "Neobiotech SLA kit" (Korea), avoiding perforation of the sagittal venous sinus (figure 1). After that, the studied materials were introduced into the formed bone defect. The wound was sutured in layers.

Animals were withdrawn from the experiment by introducing an overdose of Zoletil on day 28. During planned euthanasia of experimental animals, tissues of the area of implantation of the studied materials were taken and transferred for histological examination.

2.5. Histological examination and morphometry.
The material was fixed with a 10% neutral formalin solution, dehydrated for at least a day in a gradient of alcohols and xylene and enclosed in paraffin. Next, sections were made with a thickness of
5-7 μm. Slides were stained with hematoxylin and eosin and according to Masson and aniline blue (Biovitrum, Russia). The slides were inspected using an Axioimager M.1 light microscope (Carl Zeiss, Germany). Morphometry was performed in 5 fields of view on each of 5 serial sections. For subcutaneous implantation, the central region and peripheral zones were studied on the right, left, top, and bottom, and the number of giant multinucleated cells per 1 mm² was estimated. For intraosseous implantation, the areas adjacent to the maternal bone, the central part of the defect and the area between the center and the periphery were examined and the relative volume of the newly formed bone tissue was evaluated. When conducting morphometry were guided by generally accepted recommendations [9-11].

2.6. Statistical processing
Graphing and statistical processing of the results were performed in GraphPad Prism 7.0 (USA). Differences between groupes were detected using Student's t-test. Differences were considered significant if the probability level was below 5% (p <0.05).

Figure 1. Left: surface of highly porous polylactide granules and granules of deproteinized bone matrix Bio-Oss (SEM). In the center are the stages of intradermal implantation in the withers of rats. Right: the formation of a critical defect in the parietal bones of rats followed by implantation of the material.

3. Results and discussion

3.1. Subcutaneous implantation
As a result of subcutaneous implantation there was no bone formation in both deproteinized bone matrix Bio-Oss and highly porous PLA granules groups (figure 2, figure 3). Bio-Oss and PLA granules were surrounded by a high number of multinucleated giant cells or foreign body cells. There were 2 times more cells around PLA than Bio-Oss granules and their number was 71±23 and 30±8 on 1 mm², respectively (figure 4). In some cases structures resembling bone tissue were observed inside PLA granules (figure 3). However, the staining of these formations showed that mineralization did not occur and bone tissue did not form. The space between PLA granules was smaller than between Bio-Oss granules and they were arranged closely. These spaces were filled with collagenous fibers of connective tissue stained blue and a full blood vessels network (figure 2, 3). The absence of acute
inflammation cells indicated a high biocompatibility of the studied materials. Obtained data indicated that the PLA granules retained their shape and volume for up to 28 weeks and acted as a scaffold for bone regeneration (figure 3). They also have a shorter resorption period compared to Bio-Oss which undergoes degradation in a period of 20 weeks or more [12]. Low degradation rate of demineralized bone matrix prevents the formation of bone tissue.

Figure 2. Bio-Oss granules with BMP-2 after subcutaneous implantation. Left – low power magnification. In center – magnification in the peripheral zone. Right – magnification in the center. 28 day. Microscopic examination.

Figure 3. PLA-granules with BMP-2 after subcutaneous implantation. Left – low power magnification. In center – magnification in the peripheral zone. Right – magnification in the center. 28 day. Microscopic examination.

Figure 4. Histomorphometric Results. a – the number of multinucleated giant cells at the site of regeneration after subcutaneous materials implantation. b – the area of newly formed bone tissue in relation with area of calvarial critical-size defect.

3.2. Implantation into calvarial critical-size defects
In the region of a critical defect in rats highly porous PLA granules impregnated with 10 μg / ml BMP-2 concentration induced neo-osteogenesis. Masson’s stained dark blue areas of the newly formed bone tissue surrounding the highly porous PLA granules were visible (figure 5). The new bone was
penetrated by blood vessels. In certain fields of view layering of bone tissue around the vessels formed the primary osteons (figure 5).

Intraosseous implantation of Bio-Oss with BMP-2 at a concentration of 10 μg/ml did not lead to the bone formation. There was no newly formed bone in the centre but it was found on the periphery due to the osteoinductive effect of host bone. As a result, the volume of bone tissue formed in highly porous PLA granules group was higher than in the Bio-Oss group and amounted to 18 ± 8 and 2.6 ± 1.1, respectively (figure 4).

The result could be due to the fact that highly porous polylactide granules have a large surface area and long-term protein release. Earlier we have shown, BMP-2 released from highly porous PLA granules occurred equally and peaked by 6 days [5]. It means that protein continues to be released after the postoperative inflammation phase to the beginning of the phase of active cell differentiation while the peak of BMP-2 release from Bio-Oss granules occurs on the first day [13]. We suppose that this ability allows highly porous PLA granules with small doses of BMP-2 to provide the induction of neo-osteogenesis. Also BMP-2 has a high affinity for the demineralized bone matrix which contributes to retention of the protein and, possibly, limits its action [14].

![Figure 5. PLA-granules with BMP-2 after calvarial critical-size defects implantation. 28 day. Microscopic examination.](image-url)
Figure 6. Bio-Oss granules with BMP-2 after calvarial critical-size defects implantation. 28 day. Microscopic examination.

4. Conclusion
Well-known ordinary material Bio-Oss showed its low efficacy as a carrier for an osteoinductive protein BMP-2. The highly porous PLA granules were biocompatible and effective for protein delivery. The use of the minimum effective dose of BMP-2 for rats in the composition with highly porous PLA granules induced osteogenesis. The volume of newly formed bone was $18 \pm 8\%$. The obtained data showed that highly porous PLA granules can be used as a basis for the activated osteoplastic materials.

Acknowledgements
The research was supported by the Russian Science Foundation [grant number 16-15-00298].

References
[1] Deev R V, Drobyshev A Y, Bozo I Y and Isaev A A 2015 Ordinary and activated bone grafts: applied classification and the main features BioMed Res Int 365050
[2] Zhang H and Bradley A 1996 Mice deficient for BMP-2 are nonviable and have defects in amnion/chorion and cardiac development Dev Camb Engl 122 2977–86
[3] Poynton A R and Lane J M 2002 Safety profile for the clinical use of bone morphogenetic proteins in the spine Spine 27 40–8
[4] McKay W F, Peckham S M and Badura J M 2007 A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE® Bone Graft) Int Orthop 31 729–34
[5] Vasilyev A V, Bukharova T B, Kuznetsova V S, Zagoskin Yu D, Minaeva S A, Grigoriev T E, Antonov E N, Osidak E O, Galitsyna E V, Babichenko I I, Domogatsky S P, Popov V K, Chvalun S N, Goldstein D V and Kulakov AA 2019 Comparison of impregnated bone morphogenetic protein-2 release kinetics from biopolymer scaffolds Inorg. Mater. Appl. Res 10(5) 1093-100
[6] Grigoriev T E, Bukharova T B, Vasilyev A V, Leonov G E, Zagoskin Y D, Kuznetsova V S, Gomzyak V I, Salikhova D I, Galitsyna E V, Makhnach O V, Takoev K V, Chvalun S N, Goldstein D V and Kulakov AA 2018 Effect of Molecular Characteristics and Morphology on Mechanical Performance and Biocompatibility of PLA-Based Spongious Scaffolds BioNanoScience 8(4) 977-83
[7] Kamal A, Siahaan O and Fiolin J 2019 Various dosages of BMP-2 for management of massive bone defect in Sprague Dawley Rat Arch Bone Jt Surg 7(6) 498-505
[8] ISO 10993-2 2006 «Biological evaluation of medical devices — Part 2: Animal welfare requirements».
[9] European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1985) Council of Europe (Convention ETS 123)
[10] Dempster D W, Compston J E, Drezner M K, Glorieux F H, Kanis J A, Malluche H, Meunier P
J, Ott S M, Recker R R and Parfitt AM 2013 Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR histomorphometry nomenclature committee J Bone Miner Res 28(1) 2–17

[11] Compston J 1997 Bone histomorphometry In: Methods Bone Biol., eds. Arnett T R and Henderson B (Boston, MA:US/Springer US) 177–97

[12] Wallace S S, Froum S J and Tarnow D P 1996 Histologic evaluation of a sinus elevation procedure: a clinical report. Int J Periodontics Restorative Dent 16(1) 46-51

[13] Huh J B, Yang J J, Choi K H, Bae J H, Lee J Y, Kim S E and Shin S W 2015 Effect of rhBMP-2 immobilized anorganic bovine bone matrix on bone regeneration Int J Mol Sci 16(7) 16034–52

[14] Hänseler P, Ehrbar M, Kruse A, Fischer E, Schibli R, Ghayor C and Weber FE 2015 Delivery of BMP-2 by two clinically available apatite materials: in vitro and in vivo comparison J Biomed Mater Res A 103 628–38