Remineralization of Demineralized Bone Matrixes with Preserved Fibrillary Structure as a Promising Approach to Obtain Highly Effective Osteoplastic Materials

Vladislav Minaichev[1], *Polina* Smirnova[2], *Anatoliy* Senotov[1], *Anastasia* Teterina[2] and *Irina* Fadeeva[1,3]*

[1]Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Russian Academy of Sciences, Pushchino, Russia
[2]A.A. Baikov Institute of Metallurgy and Material Science Russian Academy of Sciences, Moscow, Russia
[3]Pushchino State Institute of Natural Science, Pushchino, Russia

Abstract. The development of highly effective osteoplastic materials capable of providing bone tissue regeneration still remains an urgent and unresolved problem. In the presented work, an approach is proposed for the creation of biomimetic materials by the deposition of amorphous calcium phosphates on the surface of a xenogenic bone demineralized matrix under physiological conditions. Adsorption spectroscopy and scanning electron microscopy showed the efficiency of deposition of amorphous calcium phosphates on the trabeculae surface. The additional inclusion of the calcium-binding protein albumin was found to increase the efficiency of CPC adsorption on the trabeculae surface during DBM remineralization in vitro. In the model of heterotopic implantation for 7 weeks the osteoinductive properties of the obtained material were demonstrated, expressed in intrabecular mineralization of bone trabeculae, neovascularization and pronounced synthetic activity of osteoblasts (synthesis and structurization of neocollagen directly on the implanted material). The data obtained in the course of this work will be used to create new highly effective osteoplastic materials.

1 Introduction

Currently, in orthopedics, dentistry and other fields of medicine related to the treatment of traumatic bone injuries or diseases, there is a serious lack of effective osteopathic materials designed to compensate the lack of bone tissue. The main goal of developing the most popular osteostimulating materials is to ensure full replacement of the implant by the recipient's own bone tissue [1]. Autografts are considered the gold standard among all osteoplastic materials, but there are two natural problems with their use: shortage of material and additional traumatic loads on the patient's body [2]. Modern methods of bone

---

* Corresponding author: fadeeva.iteb@gmail.com

tissue engineering (BTE) use allografts and xenografts as an alternative, but their use is limited by the complexity of cleaning tissues from immunogenic determinants and the possibility of transmitting various pathogens. In addition, even with a sufficient degree of matrix purification with its high structural preservation, the question of osteoinductive activity of such matrices remains open, since the introduction of additional, and sometimes very expensive, osteoinductive agents into the material is required to increase the osteogenic properties of such materials.

Simultaneously with the above it is known that calcium-phosphate compounds, as synthetic analogues of the mineral part of bone tissue, can exhibit osteoinductive properties, i.e. stimulate differentiation of mesenchymal stromal cells into bone tissue cells [3]. Among all calcium phosphate compounds, synthetic hydroxyapatite (HAp) is the most widely used as the closest analogue of bone tissue HAp. Moreover, it is known that synthetic HAp has an unstable biological response from osteoinduction to rejection, which is associated with different physicochemical properties mediated by the methods of its production. Thus, high-temperature treatment leads to the creation of a highly crystalline inert HAp that is not capable of integrating in the body, and, moreover, over time, when the implant is worn, particles of such material trigger an uncontrolled inflammatory process. [4].

Due to all of the above disadvantages of modern osteoplastic implants, the search for approaches to the creation of new highly effective osteoplastic materials continues. One of the most promising approaches is the development of biomimetic materials, which consists in creating materials whose composition and structure are similar to the organic and mineral part of the bone tissue [5]. Such materials, due to composition imitation and reproduction of microarchitectonics, should be recognized by the body as native bone tissue and ensure the processes of biointegration and subsequent remodeling directly on the implanted material. Still, the questions of the optimal composition of these materials and the appropriate conditions for their production remain open. According to our opinion, biomimetic materials should not only imitate the structure of bone tissue, but also the process of its biomineralization, for which the conditions (temperature, pH, etc.) should be similar to those in the natural calcification of native bone tissue. Collagen mineralization is known to be a cell-mediated process during which bone tissue hydroxyapatite is formed from an amorphous precursor through a series of intermediate states involving non-collagen proteins and lipids [6]. In the literature there are single examples of the use of amorphous calcium phosphates as biomaterials, and we believe that it is the combination of amorphous calcium phosphates as natural precursors of HAp and demineralized bone-collagen matrix (DBM) with preserved fibrillary structure that should be recognized by the body as immature bone tissue and remodeled to a mature state. In this regard, an approach for remineralization of the xenogenic bone matrix by amorphous calcium phosphates ACP, under in vitro conditions as close as possible to physiological ones, has been proposed. Also of particular interest is the study of the role of noncollagen proteins (containing natural phospholipids) as important participants in remineralization, performing calcium-binding and regulatory roles. Thus, the aim of this work was to create a biomimetic material based on xenogeneic DBM, ACP and albumin and to study its biological activity under in vivo conditions.

2 Materials and methods

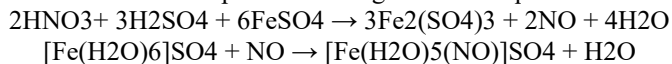### 2.1 Preparation of Demineralized bone matrix

DBM was obtained according to the author's technique (patent for invention of the Russian Federation №2686309) [7], which is a multistage treatment of xenogeneic spongy bone

tissue of cattle, including complete decellularization, delipidization and demineralization of bone tissue with maximum preservation of structure and microarchitectonics of the fibrillar extracellular matrix. Directly before remineralization, the blocks were sterilized by incubation in sterile phosphate-buffered saline (PBS) with the addition of the following antibiotics and antimycotics: gentamicin sulfate (0.02 mg/mL) (Dalchimpharm, Russia), diflucan (0.04 mg/mL) (Pfizer, France) and ciprofloxacin in (0.008 mg/mL) (Kurgan Sintez, Russia) at a ratio of the volume of samples to the volume of the resulting solution of 1:20. The blocks placed in PBS with antibiotics were incubated for 48 hours with constant stirring and a temperature of 37°C in a shaker-incubator (Biosan, Latvia). After that, for 24 hours, the blocks were washed three times in sterile PBS, pH 7.4.

2.2 Obtaining amorphous calcium phosphates and hydroxyapatite

Amorphous calcium phosphates (ACP) were prepared by quantitative mixing of saturated solutions of $Ca(NO_3)_2 \times 4H_2O$ (analytical grade, Himmed, Russia) and $NH_4H_2PO_4$ (analytical grade, Himmed, Russia).

The sediment was washed from nitrates by centrifugation of the reaction mixture at 6000 rpm for 15 minutes (UNIVERSAL 320 R, Hettich, Germany) followed by the determination of NO_3^- ions in the supernatant using a standard qualitative reaction:

$$2HNO_3 + 3H_2SO_4 + 6FeSO_4 \rightarrow 3Fe_2(SO_4)_3 + 2NO + 4H_2O$$
$$[Fe(H_2O)_6]SO_4 + NO \rightarrow [Fe(H_2O)_5(NO)]SO_4 + H_2O$$

The supernatant was replaced with an equivalent volume of PBS, the sediment was evacuated, and nitrate determination was performed again according to the above scheme. The process of washing the precipitate of nitrates was carried out until negative qualitative reaction.

2.3 Remineralization of bone-collagen matrixes

Remineralization of demineralized bone matrices (DBM) was carried out by incubating the samples in a suspension of amorphous calcium phosphate under physiological conditions (pH 7.4, 37°C). For this purpose, 125 mm3 bone matrixes of similar porosity and size were placed under sterile conditions in ACP suspension or ACP solution with the addition of 4% bovine albumin (fraction V) (MP biomedicals, USA). The bone fragments in the prepared incubation mixture were vacuumed for 30 minutes at 20 mbar using a Millivac Maxi membrane pump (Millipore, USA). Then, the vacuumed bone matrixes with deposited ACP were placed in an incubator shaker at 37°C at 60 RPM for 24 h until a uniform coating formed on the trabecular surface of the materials. The obtained material was then lyophilized (FreeZone 2.5 Liter Benchtop Freeze Dry System, Labconco, Canada) and stored until implantation at room temperature in sterile kraft bags (Meridian, Russia).

2.4 Quantification of mineralization

The calcium content in the control and modified matrixes before and after implantation was determined by absorption spectroscopy. The material samples were dried for 12 hours at 90°C in a hot air sterilizer (Binder, Germany), after which the dry weight of the samples was measured. Then each sample was dissolved in 1 ml of 1M HCl for 24 h at 20-25°C. The amount of mineralized calcium was measured using a standard calcium determination kit Calcium AS Arsenazo III (DiaSys, Germany). Optical density was measured using an Infinite F200 tablet reader (Tecan, Austria). Calcium mineralization values in the samples (µg calcium per mg of sample dry weight) were calculated according to the manufacturer's instructions.

2.5 Scanning electron microscopy

Samples of control and remineralized bone units were also analyzed by scanning electron microscopy (SEM) on a VEGA3 device (Tescan, Czech Republic) with preliminary deposition of gold particles on the surface of the materials using a Q150R ES vacuum atomizer (Quorum Technologies, England). Images of the material surface were obtained at a pressure of $7.3*10-2$ Pa in the column and $1.5*10-1$ Pa in the chamber.

2.6 Heterotopic material implantation

To examine the osteogenic potential of the obtained matrixes in vivo, a model of heterotopic subcutaneous implantation of biomaterials was used. Male Wistar rats weighing 180-200 g were used as experimental animals. The animals were kept under standard vivarium conditions. All manipulations with animals were performed in accordance with the Regulation on Research on Experimental Animals (Order № 755 of the Russian Ministry of Health of August 12, 1997). The protocol was approved by the Biosafety and Ethics Commission of the Institute of Theoretical and Experimental Biophysics RAS (05.03.2022, protocol N26/2022). Materials were implanted for 7 weeks.

2.7 Material fixation and preparation of cryosections

Immediately after explantation, the samples were fixed in a solution of neutral buffered formalin for 24 hours at +4°C. After fixation, the samples were washed of excess fixative by triple rinsing in distilled water.

Cryosectioning of modified matrixes before and after implantation was performed in a cryotome chamber (Shandon CRYOTOME 620E, Thermo Fisher Sci., USA) at the working chamber temperature from -21ºC to -23ºC (depending on external conditions). The thickness of the obtained histological sections was 9 μm.

2.8 Histochemical analysis

Cryosections of experimental samples were subjected to the following types of standard histological staining:

1. To determine the localization of mineralized calcium in tissue samples, cryosections of experimental samples were stained according to the Dahl method modified by McGee–Russell [8].

2. In order to determine the degree of maturity and preservation of collagen, cryosections were stained with trichrome staining according to the Lilly method [9].

The cryosections of preparations stained by the above methods were analyzed and their microphotographs were taken using an inverted microscope station Eclipse Ti (Nikon, Japan) with a DS-Fi1c camera (Nikon, Japan) and specialized software NIS Elements Advanced Research AR4.13.05 (Build933) (Nikon, Japan).

2.9 Statistical analysis

All experiments were performed in at least three replications ($n \geq 3$). The results are presented as mean ± m, where m is standard deviation. Significance of difference between samples of experimental data, as well as the mean values were evaluated using the Whitney-Mann test for independent samples.

3 Results and discussion

3.1 Study of DBM remineralization efficiency prior to implantation by scanning electron microscopy

In order to assess the degree of preservation of the DBM matrix, the remineralization process, as well as the morphology and particle size of the adsorbed ACP layer depending on remineralization conditions, the matrixes were examined before implantation using scanning electron microscopy (SEM). The SEM data of the remineralized matrices were compared with the data obtained for DBM not modified with CPC. Investigations of the surface morphology of the control sample DBM, which has not been remineralized, revealed a pronounced preservation of the fibrillar structure of the matrix after a multistage process of matrix purification (Fig. 1, a). In samples of the DBM group modified by ACP without the addition of BSA, trabeculae were covered with rounded particles of relatively the same shape and size. (Fig. 1, b), which on the surface of the trabeculae could form a dense layer protruding beyond the trabecular boundaries. The addition of BSA to ACP led to the formation of a continuous, uniform layer of ACP+BSA throughout the trabeculae (Fig. 2, c).
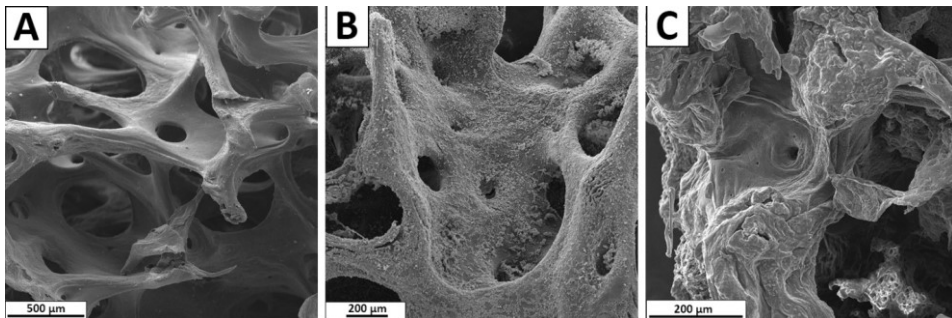

Fig. 1. Surface scanning electron microscopy (a) DBM (b) DBM+ACP and (c) DBM+ACP+BSA

As can be seen from the presented micrographs, a pronounced and uniform coating of DCM trabeculae with calcium phosphate aggregates in the absence of albumin in the incubation mixture indicates the preservation of the intrinsic sorption function of intact DBM collagen. Along with this, a significant increase in the density and coverage area of DBM trabeculae in the presence of albumin indicates that BSA molecules in this case act as centers of crystal formation due to the interaction of free carboxyl groups of albumin with Ca^{2+} and PO_4^{3-} ions, which has been previously demonstrated for titanium implants [10,11].

3.2 Study of DBM remineralization efficiency by absorption spectroscopy

Quantification of mineralization had two objectives: (1) before implantation, it provided an assessment of the effectiveness of the developed approaches to DBM remineralization under different conditions; (2) after implantation, a comparative analysis of the degree of mineralization of materials, together with the character of biomineralization determined by histological analysis, allowed to assess the potential osteoinductive properties of materials. Ca^{2+} ions were not detected in DBM samples before implantation (Fig.2).
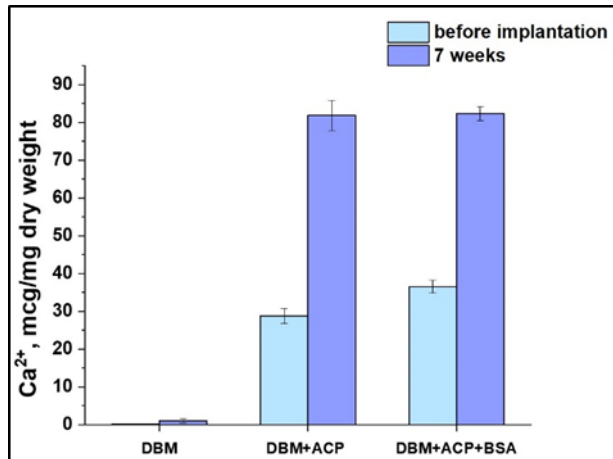
Fig. 2. Ca^{2+} concentrations in DBM, DBM+ACP and DBM+ACP+BSA samples before and after 7 weeks of implantation

In DBM+ACP samples before implantation, the Ca2+ content was 28.7±2 µg per mg of dry tissue. With the extra addition of albumin, the calcium concentration slightly increased to 36.6±3.4 µg/mg. There was no Ca2+ detected in the samples of the nonremineralized DBM group after 7 weeks of implantation, which indicates the absence of mineralization of the matrix trabeculae. In the DBM+ACP and DBM+ACP groups, the concentration of Ca2+ ions increased by 2 times to 80 µg/mg. No differences in calcium concentration were observed between the DBM+ACP and DBM+ACP+BSA groups by sorption spectroscopy. Significant increase of Ca2+ concentration in DBM+ACP and DBM+ACP+BSA samples after implantation indicated active prolongation of DBM bimineralization process in the recipient body and, consequently, increase of osteoinductive properties of the obtained materials.

3.3 Histochemical analysis of remineralized matrix samples before and after implantation

To determine the localization of mineralized calcium in the samples, cryosections of experimental materials before and after implantation were stained according to the Dahl method in the McGee–Russell modification. Analysis of the micrographs of the samples before implantation showed the preservation of the integrity of the bone matrix trabeculae and the absence of calcium deposits in the control sample. Remineralized matrixes revealed deposition of ACP in the intertrabecular space, with the extra addition of BSA there was a significant increase in the mass and area of AMP deposition in the intertrabecular space, which can be associated with increased solution viscosity and formation of calcium-protein particles, intensively binding to the intact collagen of the intact DBM.

The study of control DBM samples after 7 weeks of implantation showed a complete absence of osteoinductive effect against the expressed signs of biocompatibility of these materials. Loose connective tissue growing out of the recipient contact tissues evenly filled the intertrabecular areas without fibrous encapsulation. There were signs of partial neovascularization in the contact zones, uniform infilling of the DBM materials with recipient cells without any signs of their neosynthetic or mineralizing activity.

In contrast to the control materials, the samples of DBM+ACP and DBM+ACP+BSA groups after implantation showed both deep intratrabecular mineralization and pronounced neocollagenesis and structurization of the newly formed matrix with maturation of

neocollagen to the bone matrix stage. Regardless of the addition of BSA after implantation, there were no signs of pathological utilization calcification, preservation of the integrity of the bone matrix and the uniform nature of its mineralization, as well as a high level of biocompatibility, manifested not only in the absence of signs of leukocyte invasion, encapsulation or fibrogenesis of contact tissues, but also the appearance of pronounced signs the formation of a structured formed neocollagen matrix, moderate neovascularization with the formation of definitive vessels and the formation of mature trabeculo-like "bridges" between the trabeculae of xenogenic DBM, which together testify to the active and full grafting and osteoinductive effect of the obtained materials in the recipient's body at the studied implantation period.

It should be noted that the transition of ACP after implantation from extrafibrillar to intrafibrillar position was characteristic for both DBM+ACP and DBM+ACP+BSA samples, but this transition occurred most deeply and evenly when albumin was added, which corresponds well with the data of histochemical analysis of samples and SEM before implantation.

Approaches using collagen and calcium phosphate compounds are found in the literature, showing interest in such biomimetic approaches [12,13]. However, the presented studies did not use integral extracellular bone matrix, the ultrastructure of which is aimed at free precipitation of CPC and their further transformation in the specific histotype of bone tissue HAp, but simply separated type I collagen. It is known that the use of damaged isolated collagen leads to the initiation of a specific immunological reaction with further utilization resorption of implants without displaying any osteogenic properties by such materials [14]. The important role in the biological properties of the material is not only its composition, but also the microarchitectonics and topology of the fibrillar matrix, which determines not only the deposition of calcium phosphates in H-zones of collagen fibrils, but also the processes of migration, adhesion, proliferation and differentiation of recipient cells on the material [15]. However, based on our data it is clear that the demineralized bone matrix itself, even with a high degree of preservation, does not exhibit osteoinductive properties in the heterotopic implantation model, which seems to be related to the limitations of the heterotopic implantation model, where an appropriate osteogenic microenvironment is not formed. At the same time, the proposed approaches to DBM remineralization using ACP and ACP+BSA complex provide a pronounced ectopic osteoinductive effect of the obtained materials even in the heterotopic implantation model, indicating a pronounced synergistic osteogenic effect of the combination of ACP and intact DBM.

4 Conclusions

Thus, the proposed approach of maximum intact DBM remineralization using ACP in vitro under physiological conditions can induce ectopic neoosteogenesis and intratrabecular mineralization of DBM under in vivo conditions. Moreover, the addition of simple calcium-binding protein albumin can significantly increase the efficiency of CPC adsorption on the surface of DBM trabeculae, which is extremely important for the stability of the materials during their subsequent lyophilization and transportation.

Based on the foregoing, it can be concluded that the use of the approach of remineralization with amorphous calcium phosphates in the presence of calcium-binding proteins and phospholipids is promising, it makes it possible to significantly increase the osteoinductive properties of DBM and, on their basis, create new types of truly effective osteoplastic materials for regeneration and augmentation of bone tissue.

Acknowledgements

References

1. G. L. Koons, M. Diba, A. G. Mikos, Nature Reviews Materials 2020, **5**, 584 (2020).

2. A. H. Schmidt, Injury, **52**, S18 (2021).

3. Y. Zhang, T. Shu, S. Wang, Z. Liu, Y. Cheng, A. Li, D. Pei, Front Bioeng Biotechnol., **10**, 767 (2022).

4. F. Velard, J. Braux, J. Amedee, P. Laquerriere, Acta Biomater, **9**, 4956 (2013).

5. E. Brett, J. Flacco, C. Blackshear, M. T. Longaker, D. C. Wan, Biores Open Access, **6**, 1 (2017).

6. M.-H. Hong, J. H. Lee, H. S. Jung, H. Shin, H. Shin, Biomaterials Research, **26**, 1 (2022).

7. Federal Institute of Industrial Property, https://new.fips.ru/.

8. S. M. Mcgee-Russell, Journal of Histochemistry and Cytochemistry, **6**, 22 (1958).

9. R. D. Lillie, Stain Technol., **15**, 17 (1940).

10. Y. Wu, B. C. Yang, J. Lu, Z. W. Gu, X. D. Zhang, Key Eng Mater, 330-332, 581 (2007).

11. V. Stenport, P. Kjellin, M. Andersson, F. Currie, Y. T. Sul, A. Wennerberg, A. Arvidsson, J Mater Sci Mater Med., **19**, 3497 (2008).

12. W. R. Walsh, R. A. Oliver, C. Christou, V. Lovric, E. R. Walsh, G. R. Prado, T. Haider, PLoS One, **12**, 1 (2017).

13. C. Zhang, Y. Jiang, F. Pupilli, A. Ruffini, M. Dapporto, M. Tavoni, A. Tampieri, S. Sprio, Biomimetics, **7**, 112 (2022).

14. M. Varkey, A. Atala, *Comprehensive Biomaterials II* (2017).

15. S.-B. Han, J.-K. Kim, G. Lee, D.-H. Kim, S.-B. Han, J.-K. Kim, G. D. Lee, H. Kim, Adv Biosyst., **4**, 1 (2020).