ORIGINAL ARTICLE

Regeneration of the Experimental Defect of the Long Bone of the Skeleton after Implantation β-Tricalcium Phosphate

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Abstract:

Background: One of the main characteristics of calcium phosphate osteoplastic materials is the dynamics of rate of their resorption and replacement by the bone tissue of the regenerate. However, these data in the compact bone tissue for some drugs on the basis of β-tricalcium phosphate and electron microscopic characteristics of the structures of the regenerate are not represented in the scientific literature. *Aim and Objectives:* This study was designed to evaluate the healing of the defect of compact bone tissue after implantation of osteoplastic material «Calc-i-oss®» (β-tricalcium phosphate, granules in the size of 1-1.6 mm) with the definition of the dynamics of its resorption and morphological characteristics of the bone tissue of regenerate. *Material and Methods:* In the middle third of the femoral shaft of 24 white Wistar rats eight months of age with the weight of 250±10 g we reproduced perforated defect to the medullary canal, which was filled with β-tricalcium phosphate. After surgical intervention fragments of bones were examined on the 60th and 120th day by light microscopy with morphometry and scanning electron microscopy. *Results:* The study has established the absence of an inflammatory reaction in the area of the defect, symptoms of necrobiosis and necrosis of osteocytes in adjacent to the site of implantation maternal bone, as well as the location on the surface and inside of the particles β-tricalcium phosphate of osteogenic cells and the formation of bone tissue of regenerate. Osteoplastic material throughout the observation period was subjected to resorption and substitution by bone tissue of the regenerate, the ratio of which on the 60th day of the experiment was 25.72±2.06% to 74.28±2.06% and on the 120th day 18.31±1.54% to 81.69±1.54%. *Conclusions:* It can be concluded that β-tricalcium phosphate in the area of the defect of diaphysis of the femur shows high biocompatibility, osteoconductive properties, the ability to resorption and good integration with bone tissue of the regenerate.

Keywords: Rats; Bone, β-Tricalcium Phosphate; Reparative Osteogenesis

Introduction:

One of the most important problems that orthopedic physicians face in their practice is bone defects regeneration. The high frequency of occurrence of bone defects dictate the need to find tools that would ensure their full recovery. Transplantation of bone tissue, which is used to combat this pathology has a long history and has achieved a considerable success. However, despite this, the used auto- and xenografts still have serious drawbacks [1]. As a result, in recent years there has been a growing interest in calcium phosphate osteoplastic materials to replace bone tissue in the clinical practice [2]. The similarity of their chemical structure with that of bone tissue and inertness to biological tissues makes possible to widely use them to replace the lost bone tissue. In addition, synthetic nature of calcium phosphate material guarantees safety in practice preventing the risk of infection, and clinical trials consistently demonstrate exceptional biocompatibility the materials [3].

The drugs based on β-tricalcium phosphate by the time of its existence demonstrated high biocompatibility and excellent performance in the treatment of bone defects [4]. However, studies, on the basis of which the above facts were received, were held at the cancellous bone and on skull bone [5-7], and information on the impact of β-tricalcium phosphate on the dynamics of the healing of the defect of compact bone tissue in the scientific literature has not been found. In addition, the majority of works devoted to the study of β-tricalcium phosphate, are concerned with morphological studies, which lack electron-microscopic characteristics of the tissue-specific structures of regenerate [8-10]. Therefore the aim of our study was to investigate the healing process of the compact bone tissue defect after implantation of β-tricalcium phosphate using histological, morphometric and electron microscopic techniques.

Material and Methods:

The experiment was performed on 24 white Wistar rats eight months of age with the weight of 250±10g. All procedures were agreed with the Commission on Biomedical Ethics of Sumy State University (Minutes No. 4/14 of 06.11.2015). The study protocol was done according to the provisions "European Community Directive of 24 November 1986 on the maintenance and use of laboratory animals for research purposes". Before surgery, animals were initially injected with 2.5mg/kg of acepromazine intramuscular and in 5 minutes 75mg/kg of ketamine intramuscular (Calypsol, Gedeon Richter, Budapest-Hungary). After the introduction of the animals in anesthesia under aseptic conditions in the middle third of the femoral shaft using a portable drill with a spherical cutter at low speed with cooling we reproduced the defect to the medullary canal with the diameter of 2.5mm, without rigid fixation we filled it with the osteoplastic material «Calc-i-oss®» («Degradable Solutions Dental», Switzerland). The latter is a synthetic granular material, which is made of pure β-tricalcium phosphate (β-phase purity of > 99%, Ca / P - 1.5) with a total porosity of 50% and the size of micro pores is from 1 to 6mm (Fig. 1).

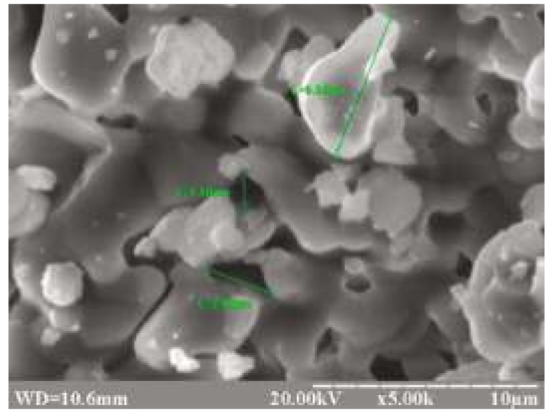

Fig. 1: Microstructure of the Osteoplastic Material Calc-I-Oss®. Mineral Crystals Measuring 6.59 Microns and Micropores between them Ranging in Size from 1, 5 to 2, 89 Microns. Electronic Scanning Image X 5000

After entering into the bone defect of osteoplastic material the wound was tightly stitched with silk thread through all layers of soft cover, the seam was treated with 3% alcohol solution of iodine. Then, during the next 3 days after operation for prevention of septic complications the after-operation seam was treated with an alcohol solution of iodine and for analgesia ketorolac was injected intramuscularly at a dose of 0.6mg 2 times a day.

Next on the 60th and 120th day after surgery animals were taken out of the experiment by decapitation under deep ether anesthesia, followed by a study of injured bones using light microscopy with morphometry and scanning electron microscopy.

For light microscopy, we extracted the fragments of femoral bones from the site of implantation of osteoplastic material and fixed them in 10 % solution of neutral formalin. After washing with water, the bone samples were subjected to decalcification in 5 % aqueous solution of Trilon B (Edetic acid), dehydrated in alcohols of increasing concentration and poured into paraffin. Histological sections were made at Sannomiya microtome "Reichert", stained with hematoxylineosin [11], analyzed at the light microscope «OLIMPUS» and photographed by digital camera.

Morphometric analysis consisted in identifying in the site of the defect of the area of bone tissue and remnants of osteoplastic material which was performed using the program for image processing "Video-Test" and "Video-Size".

For scanning electron microscopy we extracted the fragments of the femur from implanted osteoplastic material and placed samples in glutaraldehyde holder. In one day, the samples were washed in phosphate buffer, fixed in 1% OsO_4 solution and dehydrated in ethanol of increasing concentrations. Further the bone fragments were glued on metal tables with electricity conductive adhesive, sprayed with carbon dust in standard vacuum installation of VUP-5 type and examined with an electron microscope "SEM 106-I".

Using light and scanning electron microscopy we established morphological characteristics of bone tissue, the nature of its interaction with the bone material «Calc-i-oss®». In addition, by using these methods, we investigated the state of the structure of adjacent to the site of implantation maternal bone in order to establish or refute postoperative complications due to the presence or absence of signs of necrobiosis and necrosis of osteocytes [12]. The resulting digital values were treated statistically by calculating the arithmetic mean (M) and its standard error (m). The significance of differences between the indicators of the 60th and the 120th days was evaluated using Student t-test with the use of statistical computer program MS Excel XP. The differences were considered significant at $p<0.05$.

Results:

On the 60th day of the experiment the defect area was filled with lamellar bone tissue of regenerate with the integrated into its structure small remnants of osteoplastic material. At the same bone tissue in the peripheral part of the defect had continuous nature without remnants of β-tricalcium phosphate, and in the central intermittent nature, between separate parts of which are the remnants of osteoplastic material and in the deeper cuts even the elements of the bone marrow (Fig. 2).In the lacunae of bone tissue of regenerate there were well traced osteocytes and osteoblasts with appendages, and on the outer surface and in the middle of osteoplastic material - osteogenic cells and foci of osteogenesis. Near the blood vessels of channels of osteons there were observed association of the secondary osteoblasts and their precursors that formed the well-mineralized bone tissue (Fig. 3). The area of osteoplastic material for 2 months of the experiment decreased to 25.72±2.06% and was replacing by bone tissue, the relative area of which amounted to 74.28±2.06%. In the area of the defect the signs of inflammation did not show up, and adjacent to the site of the defect maternal bone was characterized by the presence in its structure of bone lacunae with typical osteocytes.

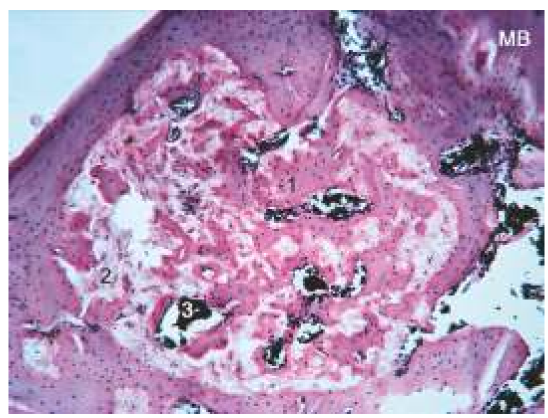

Fig. 2: The Area of the Defect of Rat Femur on the 60th Day after the Implantation of β-tricalcium phosphate. Intermittent Nature of the Lamellar Bone Tissue of the Regenerate (1) between the Individual Parts of which are the Remains of Osteoplastic Material (2) and the Elements of Bone Marrow (3) Adjacent to the Site of Implantation Maternal Bone (MB). Haematoxylin and Eosin Staining X 100

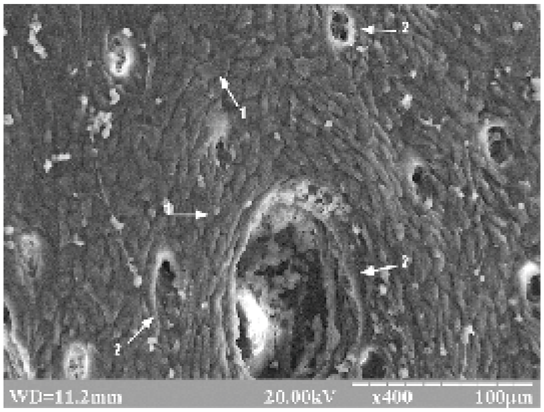

Fig. 3: The Area of the Defect of Rat Femur On the 60th Day after the Implantation of β-tricalcium phosphate. Association of Osteoblasts (1) Near Blood Vessels (2) in the Lamellar Bone Tissue of the Regenerate. Electronic Scanning Image X 400

On the 120th day of the experiment the area osteoplastic material decreased by 28.81% ($p<0.05$) in comparison with the previous observation period and was 18.31±1.54% of the total area of the defect. Simultaneously with the reduction of the number of osteoplastic material there occurred proportional increase of the bone tissue, the area of which was 81.69±1.54% ($p<0.05$). In the middle of the remaining particles of osteoplastic material there were detected osteogenic cells and foci of osteogenesis. The newly formed bone tissue of regenerate had the lamellar structure with formed osteons and differed from the maternal bone only by the presence of the integrated in their structures remnants of osteoplastic material and elements of the bone marrow (Fig. 4). In addition, in the composition of the bone tissue of regenerate there were osteocytes with long appendages, osteoblasts and moderate amount of osteoclasts, which provided its resorption (Fig. 5). It should also be noted that on the 120th day of the experiment, as in the previous period of observation, in the area of the defect of the there were no signs of inflammation, and the adjacent to the site of the defect maternal bone was characterized by the presence of typical osteocytes in its structure.

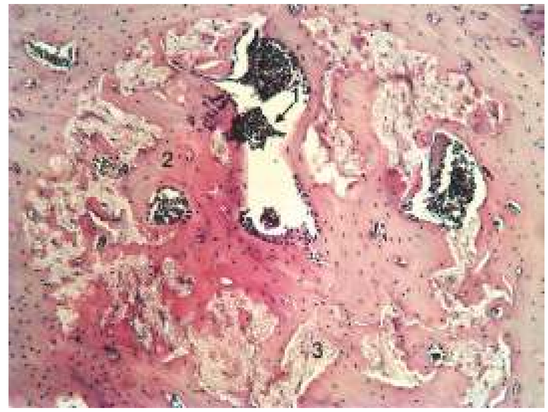

Fig.4: The Area of the Defect of Rat Femur on the 120th Day after the Implantation of β-tricalcium phosphate. Bone Marrow and Lamellar Bone Tissue of the Regenerate with Integrated into its Structure Remnants of Osteoplastic Material. Haematoxylin and Eosin Staining X 100

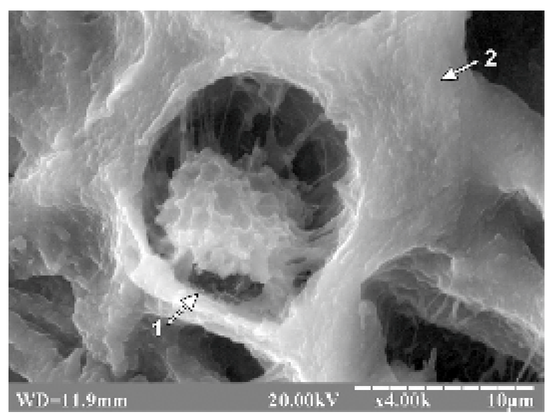

Fig.5: The Area of the Defect of Femur of a Rat on the 120th Day after the Implantation of β-tricalcium phosphate. Osteocytes (1) In the Bone Lacunae of Lamellar Osteogenic Matrix (2), Which Was Formed at the Site of Resorption of Osteoplastic Material. Electronic Scanning Image X 4000

Discussion:

The conducted microscopic examination of the diaphysis of the femur of rats after implantation into their defect of osteoplastic material β-tricalcium phosphate found that the latter has a high biocompatibility, as evidenced by the absence of an inflammatory reaction in the area of the defect and no signs of necrosis and necrobiosis of osteocytes in adjacent to the site of implantation maternal bone. With the help of the pilot study it was also determined high tropism of osteogenic cells to osteoplastic materials, as evidenced by their location on the surface and inside the particles of β-tricalcium phosphate and the formation of foci of osteogenesis. It is known that the osteoplastic material, which provides the necessary support for the cells to attach, gives them the opportunity to divide and maintain its differential phenotype. Therefore, the use of the osteoplastic material by the osteogenic cells as a platform for attaching and generating on the surface and in its cavities of new bone tissue is the proof of its osteoconductive properties [13]. In addition, microscopic examination found that the bone tissue of regenerate throughout the period of observation has a lamellar structure with a high content of the osteoblasts, osteocytes with the well-integrated into its structure remnants of osteoplastic material. The good integration of β-tricalcium phosphate with the bone tissue of regenerate is evidenced by its formation directly on the surface of the osteoplastic material and its immurement into its structures.

The results of histological studies of other authors also show the high biocompatibility of β-tricalcium phosphate, its good integration with the newly formed bone tissue and the ability to gradual resorption [14, 15]. However, these data were obtained on spongy bones and on skull bone. So, Jensen *et al* have established an almost complete disappearance of β-tricalcium phosphate from the mandibular angle defect of dwarf pigs on the 60th day, where its amount remained from 0 to 5.1% (average 2.5%), and on day 180 from 0 to 2.5% (in average 0.8%) [16]. At the same time Gotterbarm *et al* discovered the remnants of β-tricalcium phosphate (4.35%) even a year after its implantation with collagen into the defect of the epiphysis of tubular bones of dwarf pigs [17]. We also observed a gradual resorption of the osteoplastic material and its replacement by bone tissue of regenerate. However, in our study, in the defect of diaphysis of the femoral shaft the ratio of the amount of the implant and the bone tissue was 25.72 ± 2.06% to 74.28 ± 2.06% on the 60th day and 18.31±1.54% to 81.69±1.54% – on the 120th day of the experiment.

Here the resorption of the implant was not uniform throughout the area of the defect, as evidenced by the presence of remnants of osteoplastic material on the 120th day only in the central part of the defect. Moreover, in the last period of the experiment there was almost complete recovery of the defect, as indicated by its filling with the lamellar bone tissue, which was identical in structure to the maternal bone. The only thing that differs the bone tissue of regenerate from the maternal bone is the presence of integrated into its structure remnants of osteoplastic material.

Conclusion:

Thus, the β-tricalcium phosphate in the area of the defect of diaphysis of the femur showed high biocompatibility, osteoconductive properties, the ability to resorption and good integration with bone tissue of the regenerate.

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