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BIOCHEMICAL STUDIES OF RATS AFTER BONE IMPLANTATIONS OF MATERIALS ON THE BASE OF TTCP/ DCPA, TARTARIC OR ASCORBIC ACIDS

M. Gabrashanska[1], S. Tepavitcharova[2], R. Ilieva[2], M. Alexandrov[1], V. Nanev[1], N. Tsocheva-Gaytandzhieva[1], I. Vladov[1], P. Dimitrov[1]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria
E-mail: m_gabrashanska@abv.bg

ABSTRACT

The study aimed to evaluate hematological levels of markers of bone turnover in rats with calvarial defects filled with modified calcium phosphates cements. Cements were synthesized basing on TTCP/DCPA, carboxylic acids, xanthan gum and glycerin. Total and bone alkaline phosphates, osteocalcin, and their relation with oxidative stress (malondialdehyde) were studied. No differences in the markers levels were observed between the groups with implants. The level of free radicals was significantly increased in all groups. The obtained data might be useful in future experiments with new bone implants.

Key words: cements, bone markers, oxidative stress.

Introduction

For replacement of damaged bone, the most promising materials are based on calcium phosphate (CaP) that is similar in composition to the mineral component of bone tissues. CaP cements (CaPc) have many favorable properties that support their clinical use in the repair of bone defects. CaPc are cementing systems consisting of powder and liquid phases: powders are dicalcium phosphate anhydrous (DCPA) and tetracalcium phosphate (TTCP); liquid phase are carboxylic acids. They are designed for plastic filling of bone defects and joining bone fragments (Barinov & Komlev, 2011). Several attempts have been aimed to modify implant composition and morphology to optimize implant-to-bone contact and improve integration (Barinov & Komlev, 2011).

The present study aimed to evaluate hematological levels of markers of bone turnover in rats with experimental calvarial defects filled with modified calcium phosphates cements. Cements were synthesized basing on TTCP/DCPA, carboxylic acids, xanthan gum and glycerin. Total and bone alkaline phosphates (TAP and BAP), osteocalcin (OC), and their relation with oxidative stress (malondialdehyde) were studied. Bone formation markers are indicative of osteoblastic activity. TAP is an early marker for osteogenic differentiation. BAP is a key factor in determining osteoblast differentiation. OC is the most abundant non-collagenous protein in bone and it is a bone specific marker for terminal osteoblast differentiation (Allen et al., 1998; Tsocheva-Gaytandzhieva et al., 2015). Reactive oxygen species (ROS) are detrimental factor to fracture consolidation. They were measured by malondialdehyde (MDA).

Materials and methods

Modified cements preparation. DCPA ($CaHPO_4$, dicalcium phosphate) was prepa red from DCPD ($CaHPO_4.2H_2O$, dicalcium phosphate dihydrate) by thermal dehydration at 200°C. TTCP

($Ca_4(PO_4)_2O$, tetracalcium phosphate) was prepared by sintering of equimolar mixture of DCPA and $CaCO_3$ at 1500 °C for 5 hours. The as prepared solid phases were ball milled for 5 hours. Equimolar mixtures of DCPA and TTCP powders with particle size less than 28 μm and activated surfaces (by milling) were continuous mixed with liquid phases in a ratio solid to liquid 2.6 g/ml to form plastic mass. 18 mass % solutions of tartaric or ascorbic acids were used as liquid phases, modified by glycerin (5 mass %). Xanthan gum (2 mass %) was added to the initial solid mixtures to improve their mechanical characteristics (Dorozhkin, 2011).

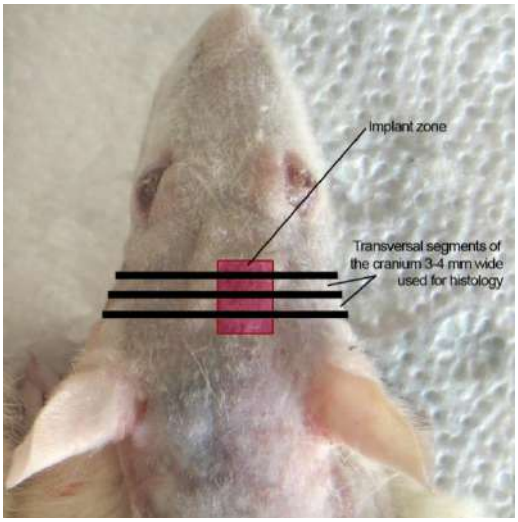

Figure 1: Schematic rat head with a calvaria defect covered with implant.

Animal model. Eight-week old male Wistar rats weighed approximately 350 g were used in the experiments. The rats were allocated to three experimental groups. Animals in the control group received a critical size skull defect (CSD) with no scaffold implantation. The rest two groups received implants as follows – material 1 (group 1) and material 2 (group 2). General anesthesia was given. To create a critical size defect (CSD) in the skull the head was shaved and cleaned with antiseptic. A lateral longitudinal incision over the head was made under aseptic conditions. The skull cortex was drilled and a calvarial bone defect 1, 8 mm wide and 6 mm long was created. The biomaterials were implanted into the defect zone and their position was checked. The wound was then closed with continuous subcutaneous stitches (Fig. 1). The animals had free access to food and water and were monitored daily in the postoperative period for any complications or abnormal behavior. The experiments were conducted in accordance with the requirements of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Biochemical studies. The blood biochemical studies were done prior-operation, at the end of the 1st week and 12th week post operation. Activity of serum enzymes TAP and BAP was measured by Human diagnostic kits using Screen master 588LiHD 111. The level of serum OC was assessed by radioimmunoassay – RIA kit Biomedical Technologies. The serum level of MDA was assessed by colorimetric assay kits (Cayman Biomol GmbH, Hamburg. MDA is the biomarker of lipid peroxidation that is the indicator of oxidative stress.

Results

Equimolar mixture of TTCP and DCPA mixed with water at a solid to liquid ratio of 4:1 was the first self-setting calcium orthophosphate cement proposed in the literature (Dorozhkin, 2011). We overcome its drawbacks as low mechanical strength; solubility in body fluids; not well-defined micro- and macroporosity by using biocompatible carboxylic acids (tartaric and ascorbic) in liquid phases and additive as biodegradable polysaccharide xanthan gum. The properties of the cements studied were described in details previously (Sezanova et al., 2014).

Two types calcium phosphate cements derived from DCPA / TTCP and differing in the liquid phase were used in this study. The liquid phases were solutions of tartaric acid or ascorbic acids, both of them biocompatible. In addition, xanthan gum and glycerin were used in order to improve the manipulation characteristics of the cement samples (Table 1). It was found the additives xanthan gum and glycerin lengthened the manipulation time due to their hydrophobicity (Sezanova et al., 2014). Further, we expect additional positive influence of xanthan gum during the cements contact with the body fluids. Xanthan gum is a natural bioresorbable polysaccharide and during its slow dissolution will ensure the necessary porosity of the cements that cannot be formed previously.

Table 1: Newly synthesized calcium phosphate (CaP) cements.

Materials	Powder phase	Liquid phase
1 material	DCPA:TTCP= 1:1	Tartaric acid 18 %
	Xanthan 2%	Glycerin 5 %
2 material	DCPA:TTCP= 1:1	Ascorbic acid 18 %
	Xanthan gum 2 %	Glycerin 5 %

The activities of TAP, BAP and OC level were similar in all groups prior the operation.

The level of serum OC was similar in all groups before the operation. Then it was reduced at the end of the 1st week post operation in all rats. At the end of the experiment OC level was increased in all groups. The levels of OC in the three groups were similar. There were no significant differences among the groups. The level of OC in the control group was nonsignificant lower compared to OC levels in the groups with implants. At the end of the experiment the level of OC was increased but it was not reached the level before the operation (Fig. 2).

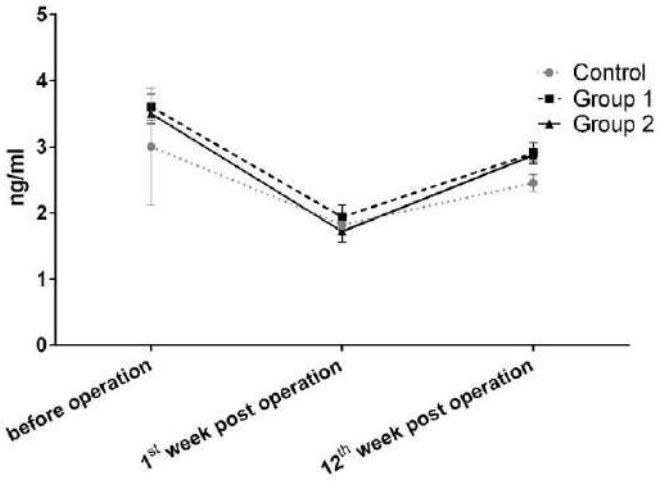

Figure 2: Levels of serum osteocalcin (OC) in rats with calvarial implants – 1 and 2 materials.

Serum activities of TAP and BAP in all rats were similar before the operation (Fig. 3 and Fig. 4). At the end of the 1st week they were decreased in all groups with nonsignificant deviations. After that the activity of TAP and BAP started to increase and at the end of the 12th week was again increased to the level before the operation. Differences in the activity of TAP were better expressed during the operative period (Fig. 3).

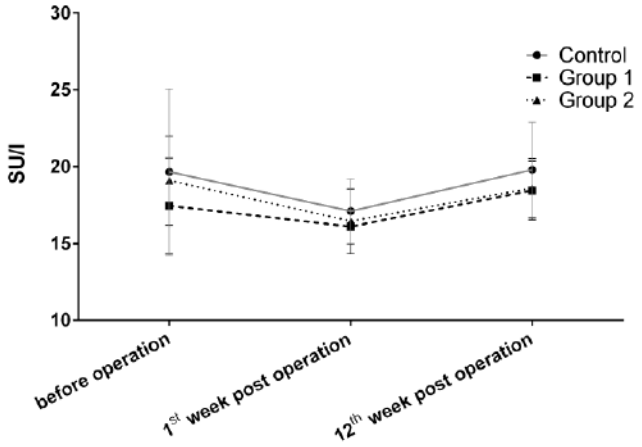

Figure 3: Serum activity of total alkaline phosphatase (TAP) in rats with calvarial implants – 1 and 2 materials.

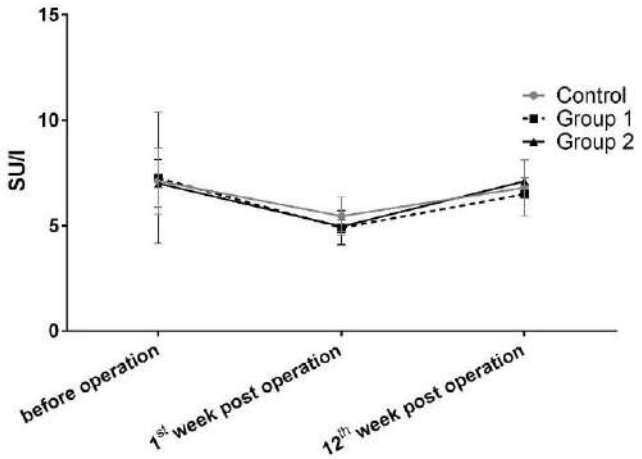

Figure 4: Serum activity of bone alkaline phosphatase (BAP) in rats with calvarial implants – 1 and 2 materials.

The present study has clearly demonstrated that the common serum markers of bone turnover in rats with calvarial implants revealed the similar dynamics during the post operation period.

Serum MDA levels were similar before the operation in all groups of rats. After the 1st week post operation (p. o.) the level of MDA was increased in all groups without any differences among the rats. After that the MDA level started to decrease. At the 12th week p. o. the levels of MDA in the both groups with implants were decreased. This process of lowering was better expressed in the 2nd group (Fig. 5).

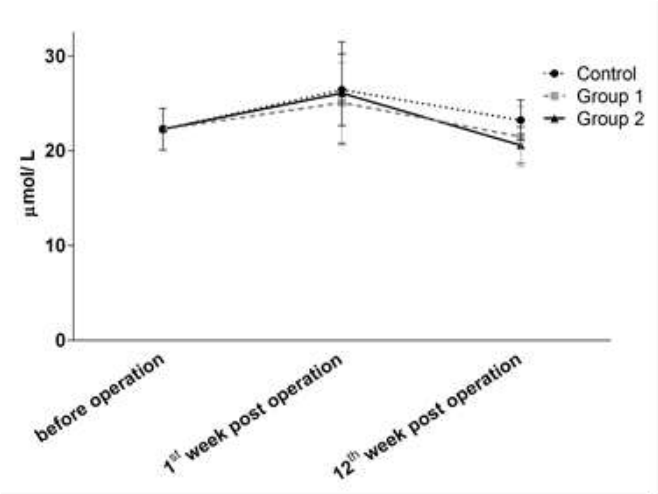

Figure 5: Serum malondialdehyde (MDA) levels in rats with calvarial implants – 1 and 2 materials.

Discussion

Changes in the blood levels of bone turnover markers (BTMs) could provide a mechanism for measuring the effects of the implantation and thus enhance the detection of the beneficial effects of such interventions. The levels of both alkaline phosphatases and osteocalcin were reduced in all groups at the end of 1th week post operation. Then BTMs had tendency to increase on the 12th week p. o. but they did not reach the levels before the operation. The reduction of TAP and BAP were less pronounced compared to the reduction of OC. At the end of the experiment increase of OC was better expressed in the groups with implants compared to the control level at the end of the experiment. Levels of TAP, BAP and OC in all treated groups were severely different from the pre-operative levels. The results are consistent with those of other authors who have registered decrease in the activity of alkaline phosphatases between the 1st and 2nd week p. o. Decreased levels of TAP, BAP and OC in the early stages of healing has been reported by Cox et al. (2010). The authors showed that the enzymes were decreased during the immediate postoperative period the 1st week, then were increased to peak at the 12th week. Others, however had shown that it is initially increased, followed by a subsequent decline at the 14th week (Barinov & Komlev, 2011).

When bone fracture occurs a remarkable yield of free radicals reactive oxygen species (ROS) is generated by the damaged tissues (Yilmar et al., 2001). However, controlled production of free radicals by normally functioning osteoclasts could accelerate destruction of calcified tissues and assist bone remodeling (Sheweita & Khoshhal, 2007). Enhancing osteoclastic production observed in bone disorders may have been responsible for increased production of ROS in the form of superoxide, which is evident by increased levels of serum MDA levels. One of the most damaging effects of ROS is lipid peroxidation, the end product of which is MDA, which also served as a measure of osteoblastic activity. The results revealed an increase MDA at the end of the 1st week p. o. and after that started its reduction. The control level of MDA at the end of the experiment was similar to that prior operation. At the end of the 12th week the MDA level in the groups with implants was lower compared to the control level. MDA level was lower in the rat in group 2 compared to that in the control group and in the first group but the statistical significance was not established. The difference among MDA level in rats with two different implants and those without

implants suggested that cellular repair system of oxidative damage was able to reduce ROS, but could not reduce MDA significantly after implantation. Lower MDA level in rats with a material 2, with a liquid phase containing ascorbic acid, could be due to of this acid. During the proliferative phase of tissue healing vitamin C is important for collage (Yilmar et al., 2001). It is appears to be essential for bone formation due to its effect on osteoblast growth and differentiation and on alkaline phosphatase expression. It was demonstrated that ROS were detrimental to fracture consolidation in rats. It seems that vitamin C plays a crucial role in homeostasis between osteoblasts and osteoclasts in terms of differentiation and activation, directly influencing the initial stages of bone repair (Yilmar et al, 2001).

Conclusions

With the development of new techniques and strategies in syntheses and characterization of CaP cements the present study clearly demonstrated the interaction among newly implants, BTMs and oxidative stress in rats with calvarial defects. Although further work is needed to fully understand the mechanism of the action of newly implants tested *in vivo*, this study has opened new avenues of research towards understanding the *in vivo* function of the additives xanthan gum, carboxylic acids and glycerin lengthened the manipulation time due to their hydrophobicity. The study revealed the potential use of these innovative CaPcs in the healing of bone lesions.

Acknowledgements

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