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Magnesium substitution in brushite cements for enhanced bone tissue regeneration

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ABSTRACT

We have synthesized calcium phosphate cements doped with different amounts of magnesium (Mg-CPC) with a twofold purpose: i) to evaluate in vitro the osteoblast cell response to this material, and ii) to compare the bone regeneration capacity of the doped material with a calcium cement prepared without magnesium (CPC). Cell proliferation and *in vivo* response increased in the Mg-CPCs in comparison with CPC. The Mg-CPCs have promoted higher new bone formation than the CPC ($p < 0.05$). The cytocompatibility and histomorfometric analysis performed in the rabbit calvaria showed that the incorporation of magnesium ions in CPC improves osteoblasts proliferation and provides higher new bone formation. The development of a bone substitute with controllable biodegradable properties and improved bone regeneration can be considered a step toward personalized therapy that can adapt to patient needs and clinical situations.

1. Introduction

Nowadays there is a high clinical demand for synthetic bone materials due to drawbacks associated with biological bone grafts [1]. Although autogenous bone is still considered to be the gold standard in bone regeneration, the harvesting of autogenous bone has disadvantages such as donor site surgery, extended surgery time with the consequent risk of complications, and limited amount of graft material. For these reasons, there has been an increased research in bone replacement biomaterials in the last decades. The calcium phosphate cement (CPC) has gained clinical acceptance for bone substitution and bone augmentation due to their similarities with the mineral bone composition [2,3], and to their biocompatibility, bioactivity and osteoconductivity [4]. Many efforts have been expended to develop CPC that mimic the bone tissue [5] and different strategies were proposed to reach that goal. Namely: development of porous cements to enhance material resorption, tissue colonization and angiogenesis [6], incorporation of some specific ions which play relevant roles in bone metabolism [7,8], and finally the addition of drugs and growth factors to the CPC matrix [9–11].

The incorporation of ions into calcium phosphate cements is of great importance because many biological tissues, such as bone or teeth are composed of an apatitic mineral phase containing tiny amounts of other elements [12] such as magnesium, strontium and silica. Magnesium (Mg) is the fourth highest abundant cation in the human body after Ca, K, and Na, and the 50% of the total Mg is found in bone tissue whereas only 1.0% is found in extracellular fluid. Mg ions are involved in various biological processes such as cellular processes of proliferation and differentiation, the cell–matrix interaction, and the normal function of parathyroid glands and metabolism of vitamin D. Furthermore, the deficiency of Mg in bone seems to be a risk factor for osteoporosis in humans [13]. The addition of Mg within CPC has attracted attention because of the potential beneficial effects of Mg on the physicochemical properties of the minerals [14,15] and also because the improvement of the bone metabolism [16]. Mg^{2+} is considered the most important ion used in calcium substitution, since its incorporation into materials leads to a change in their biological and chemical performance [17]. Recently, it was reported that the amount of Mg^{2+} into calcified tissues is associated with the apatitic phase decrease, and that strong calcification leads to changes of the bone matrix that determines the bone fragility [18]. Currently, the incorporation of Mg^{2+} is considered a promising route for increasing the bioactivity of bone-engineering scaffolds [19,20]. For the above reasons, we consider that the incorporation of Mg^{2+} ions within brushite cement presents a biological approach toward increasing the bioactivity of the brushite scaffolds.

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2. Material and methods

2.1. Cement formulations

The preparation of the new biomaterial is explained elsewhere [21] but herein a brief description of the synthesis is given. β-Tricalcium phosphate (β-TCP) was prepared by sintering calcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) (Sigma-Aldrich, Spain) and calcium carbonate ($CaCO_3$) (Sigma-Aldrich, Spain) in a molar ratio of 2:1 at 1000 °C for 12 h [Eq. (1)]. Mg-substituted β-TCP (Mg-TCP) was produced by replacing calcium phosphate dihydrate with magnesium phosphate 3-hydrate ($MgHPO_4 \cdot 3H_2O$) (Sigma-Aldrich, Spain) in the synthesis reaction of β-TCP,

$$CaCO_3 + 2(CaHPO_4 \cdot 2H_2O) \rightarrow \beta - Ca_3(PO_4)_2 + 5H_2O + CO_2 \quad (1)$$

resulting in a molar Mg/(Mg + Ca) ratio of the reactants between 0 and 40% (Table 1). The (Mg + Ca)/P ratio was maintained constant at 1.5. Thereafter, the sintered ceramics were crushed and sieved with 200 μm pore size-mesh.

The as prepared powder was mixed with monocalcium phosphate monohydrate (Sigma-Aldrich, Spain), at equimolar ratio, using a mortar with pestle and subsequently it was reacted with water at a powder to liquid ratio (P/L) of 3.0 $g \cdot ml^{-1}$, to obtain the cements. For the in vitro study, the sample dimensions were 15 mm diameter and 2 mm thick, whereas for the in vivo study, the cements were crushed and sieved to grain size between 0.5 and 0.8 mm. Finally, the samples were disinfected with ethanol 70% for 1 h.

2.2. Cell studies

2.2.1. Cell proliferation

The evaluation of the cell response to the cements was performed using the osteoblast cell line MG-63. The cells were maintained in cell culture flasks in an incubator with 5% CO_2 and 95% air atmosphere at 37 °C. The cell culture medium consists of Dulbecco's modified with Eagle (Sigma-Aldrich, UK) supplemented with 5 ml/L L-glutamine (Sigma-Aldrich, UK), 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, UK), and 100 ml/L fetal bovine serum that was changed every 2 days. The confluent cells were dissociated with trypsin (Sigma-Aldrich, UK) and subcultured to three passages which were used for tests.

Four disk-shaped samples, for each of the cements, were prepared in silicon molds of 14 mm diameter and 2–3 mm thickness and then disinfected using 70% ethanol. Sterile samples were placed in quadruplicate into 24-well plate for 3, 5, 7 and 10 days. Samples were preconditioned for 24 h by introducing 2 ml of medium per well. After preconditioning the medium was removed and osteoblasts were seeded on top of the cements disks at an initial density of 25,000 cells/ml/disk and maintained in an incubator with a humidified atmosphere of 5% CO_2 in air at 37 °C. The culture medium was exchanged every second day.

Table 1
Samples containing different amounts of Mg (from 0 to 40% of Ca substituted by Mg).

Samples	$CaHPO_4 \cdot 2H_2O$ [mol]	$MgHPO_4 \cdot 3H_2O$ [mol]	$CaCO_3$ [mol]	[Mg(Mg + Ca)] (%)
β-TCP	2	0	1	0
6.67% Mg-TCP	1.8	0.2	1	6.67%
26.67% Mg-TCP	1.2	0.8	1	26.67%
40% Mg-TCP	0.8	1.2	1	40%

The cell proliferation was evaluated at 3, 5, 7 and 10 days. At each time point the samples were rinsed twice with PBS to remove the non-attached cells. The attached cells were dissociated with 1 ml of trypsin per well in incubator with 5% CO_2 at 37 °C for 7 min. Then, the enzymatic activity was neutralized with 1 ml of the medium. Cell quantification was performed with a hemocytometer (Neubauer chamber) by capillarity, and subsequently the cells were observed with an optical microscopy.

2.2.2. Cell morphology

Morphological evaluation of the cells on the surfaces of the cement specimens was carried out as follows: the cells were cultured onto the disks as mentioned above, after of each study time (3, 5, 7 and 10 days) the medium was removed from each well containing the sample and the cell-cultured specimens were rinsed with phosphate buffered saline (PBS) twice, and then, the cells were fixed with 2 ml/well of 6.25% glutaraldehyde (Sigma-Aldrich, Spain). After 30 min, they were rinsed again and kept in PBS at 4 °C. After cell fixation, the specimens were dehydrated in ethanol solutions of varying concentration (15, 30, 50, 70, 90, and 100%) for about 30 min at each concentration. The specimens were then dried, using the critical-point dry technique, and subsequently they were coated with carbon using the evaporator Balzers MED-010 (Balzers Union, Liechtenstein), and then coated with gold using metallization K550X (Emitech, Taiwan). The surface images were recorded using scanning electron microscopy JSM-6400.

2.3. Experimental animal model

In vivo experiments were conducted in accordance with the guidelines laid down by the European Communities, Council Directive of 24 November 1986 (86/609/EEC). Before starting the in vivo animal study, the protocol was approved by the Ethics Committee for Animal Experimentation at the Universidad Rey Juan Carlos (Madrid), and adequate measurements were taken to minimize pain and discomfort in the animals.

Seven New Zealand rabbits, six-month-old, and weighing between 2.4 and 3.1 kg were used. The animals were accommodated in the official stable for animal assays of the Universidad Rey Juan Carlos, at 22–24 °C with 55%–70% humidity, light cycles of 12 h, and air renewal 15 times/h. Before surgery, they underwent a head haircut, then they were weighed and with this datum it was calculated the volume of anesthetic for each animal.

2.3.1. Operative procedure

The rabbits were anesthetized by intramuscular injection of Buprenorfina (Schering plough, Spain) 0.3 ml, Ketamina (Merial, Spain) 0.4 ml/kg and Xilacina (Bayer, Germany) 0.25 ml/kg. The animals were placed prone on the operating table and then an antiseptic solution (Iodopovidone) was applied over the surgical field. The surgery begins by making an incision in the midline of the head, with a No. 15 scalpel, and then the skin and periosteum were lifted and moved laterally using a periosteal elevator to leave the calvarial bone exposed (Fig. 1A). Bone defects were produced in the calvarial bone plate using a trephine of 10 mm in diameter and afterwards the bone block was removed with a curette (Fig. 1B). During bone drilling, the surgical field was continuously irrigated with sterile saline solution to reduce thermal damage. Once the 2 bone defects were achieved (Fig. 1C) we proceeded to fill the defects with cement granules (Fig. 1D). After this, the periosteum was repositioned in its place and sutured by simple points with resorbable sutures (3/0) and skin with was sutured with silk (3/0). Once the surgery was complete, we applied an antiseptic solution (Iodopovidone) to each animal and all of them received antibiotics (Terramycin) and Buprenorphine in the drinking water (2 doses of 3 ml per day for 2 days).

Eight weeks after surgery, the rabbits were euthanized by means of a lethal intravenous injection (30 mg/kg) of pentobarbitone sodium