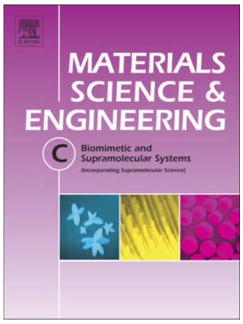
Accepted Manuscript



Magnesium substitution in brushite cements for enhanced bone tissue regeneration

Jatsue Cabrejos-Azama, Mohammad Hamdan Alkhraisat, Carmen Rueda, Jesús Torres, Luis Blanco, Enrique López-Cabarcos

PII:	S0928-4931(14)00399-3
DOI:	doi: 10.1016/j.msec.2014.06.036
Reference:	MSC 4740
To appear in:	*Materials Science & Engineering C*
Received date:	11 December 2013
Revised date:	5 June 2014
Accepted date:	30 June 2014

Please cite this article as: Jatsue Cabrejos-Azama, Mohammad Hamdan Alkhraisat, Carmen Rueda, Jesús Torres, Luis Blanco, Enrique López-Cabarcos, Magnesium substitution in brushite cements for enhanced bone tissue regeneration, *Materials Science & Engineering C* (2014), doi: 10.1016/j.msec.2014.06.036

Magnesium substitution in brushite cements for enhanced bone tissue regeneration

Jatsue Cabrejos-Azama[1,2,*], Mohammad Hamdan Alkhraisat[1], Carmen Rueda[1], Jesús Torres[3], Luis Blanco[2], Enrique López-Cabarcos[1]

[1] Departamento de Química-Física II, Facultad de Farmacia, UCM, Madrid, Spain.
[2] Departamento de Estomatología III, Facultad de Odontología, UCM, Madrid, Spain.
[3]Facultad de Ciencias de la salud, URJC, Alcorcón, Madrid, Spain

*Corresponding author: J. Cabrejos-Azama; Facultad de Farmacia, Dpto. de Química-Física II, Universidad Complutense de Madrid, Plaza Ramón y Cajal S/N, 28040-Madrid; Tel. +34 913941751; Fax: +34 913942032; e-mail: jacaza@farm.ucm.es.

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 towards personalized therapy that can adapt to patient needs and clinical situations.

Keywords: calcium phosphate cement; brushite; magnesium; osteoblast; bone regeneration.

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 [9,10,11].

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 towards increasing the bioactivity of the brushite scaffolds.

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 ($CaHPO_4.2H_2O$) (Sigma-Aldrich, Spain) and calcium carbonate ($CaCO_3$) (Sigma-Aldrich, Spain) in a molar ratio of 2:1 at 1000 ºC for 12 h [Eq. (1)]. Mg-substituted β-TCP (Mg-TCP) was produced by replacing calcium phosphate dihydrate with magnesium phosphate 3-hydrate ($MgHPO_4.3H_2O$) (Sigma-Aldrich, Spain) in the synthesis reaction of β-TCP,

$$CaCO_3 + 2(CaHPO_4.2H_2O) \rightarrow \beta\text{-}Ca_3(PO_4)_2 + 5H_2O + CO_2 \qquad (1)$$

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 $g.ml^{-1}$, 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 hour.

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 ºC. 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 discs at an initial density of 25000 cells/ml/discs and maintained in an incubator with a humidified atmosphere of 5 % CO_2 in air at 37 ºC. The culture medium was exchanged every second day.

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 minutes. 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 discs 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 ºC. 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 ºC 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 was lifted and moved laterally using a periosteal elevator to leave the calvarial bone exposed (Figure 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 (Figure 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 (Figure 1C) we proceed to fill the defects with cement granules (Figure 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 (Vétoquinol, France). The bone blocks containing the defects filled with cements were removed with a safety margin of 1 cm around the defect using a high-speed cutting disk under continuous saline irrigation. Finally, the bone samples were fixed in 10 % buffered formalin for 5 days to be processed for histological and histomorphometric analysis.

2.3.2. Histology

The bone tissue blocks were dehydrated in graded ethanol series (70–100 %), and then embedded into resin methylmethacrylate (Kulzer, Germany) and polymerized. Polymerization was carried out for 24 h under low intensity UV light, followed by 1 h under high-intensity UV light to assure complete polymerization.

The samples were cut along the longitudinal axis using a diamond circular (Remet, Italy) to obtain thin sections (500 µm) and then were polished (Remet, Italy) until a final thickness around 80 µm. The resulting sections were stained using toluidine blue (Panreac - Spain). The samples were observed using optical microscopy (Nikon, Japan) for the histological evaluation.

The defects were assessed considering the cement incorporation, the cement resorption, and the new bone formation as well as tissue behavior in the regions adjacent to the cement. Following light microscopy, the same sections were prepared for backscatter scanning electron microscopy (B-SEM). For this, specimens were glued on a copper holder and their surfaces were highly polished with diamond paste. Thereafter, the surfaces were sputtered with a carbon layer and examined using a scanning electron microscopy with the backscatter detector.

2.3.3. Histomorphometry

The histomorphometric evaluation was carried out using an optical microscope. By means of the digital camera (Nikon, Japan) the images were captured with 2x magnification and transferred to the computer. The images were analyzed using image analysis software NIS (Nikon, Japan). In each section we measured the total area, the new bone area, and the residual cement area. These data permit to calculate the following parameters: average bone volume formed (BV) and volume of the remaining graft material (RG).

$$BV\ (\%) = \frac{\text{Newly formed bone volume}}{\text{Total sample volume}} \times 100$$

$$RG\ (\%) = \frac{\text{Remaining graft volume}}{\text{Total sample volume}} \times 100$$

2.4 Statistical analysis:

Statistical analysis was evaluated using Origin 8 (OriginLab Corporation, USA). The data rows were examined with the Shapiro-Wilk test and it was proved that they are normally distributed. The data were analyzed using analysis of variance (ANOVA) (α = 5 %) to test the hypothesis that there were no differences in the parameters for all groups. Values of $p < 0.05$ were considered statistically significant. The post-hoc test, according to Tukey, allowed assessment of the significance for intra- and intergroup differences.

3. Results

3.1 *In vitro* cell study

To determine the cytocompatibility of our cements we studied the in vitro response of osteoblast cell line MG-63 on the surface of the cements. The Figure 2 shows the change in numbers of viable cells attached on cements as a function of time. After an initial period of attachment to the surface of the cements, the osteoblasts began to proliferate showing significant differences in cell numbers after 3, 5, 7 and 10 days ($p \leq 0.0001$) Table 2 on all samples. Cell proliferation was affected by the addition of magnesium in the CPC and the cell proliferation increases with increasing concentration of magnesium. The osteoblasts exhibit better proliferation on all Mg-CPC compared to control group of CPC.

Figure 3 shows the morphology of the osteoblasts cells that appear well attached onto the surface of all cements. You can view SEM images of the materials without the cells in the previous paper published [21]. The cells cultured on cements without Mg substitution (Figures 3A and 3B correspond to control sample) are well adapted to the surface of the cement after the third and fifth day of cultivation. The cells grown on 6.67 % Mg-CPC are shown in figures 3C and 3D. The cells adhered to the surface of the cement have a round shape and seem to be in the extension process in which they appear flattened, elongated and extended. After five days of culture, the cells proliferate and start to penetrate the pores as shown in figures 3F and 3H that correspond to 26.67 % Mg-CPC and 40 % Mg-CPC respectively. On the seventh day of culture, the cells are covering the entire surface. In these figures we observe some broken cells whose presence is attributed to the fixing and dehydration processes of the samples that can sometimes be very aggressive with the cells.

3.3 *In vivo* study

Once the *in vitro* studies have demonstrated the good cytocompatibility of the Mg-CPCs, the assay in animal models is the last step in evaluating the biocompatibility of such materials. It is worth to say that there were no surgical complications during the preparation of bone defects and filling with experimental biomaterials. The seven animals recovered well from surgery and no animals were lost during the study.

3.3.1. Clinical observations

The diet and activity of all rabbits were normal and it was not visually observed any sign of inflammatory tissue reactions after operation. The process of healing was uneventful in all animals as no complications were observed. At 8 weeks, we observed a normal healing of skin in the operated area in all animals. All cements were well incorporated to the adjacent bone and they did not elicit an obvious inflammatory reaction in the adjacent soft-tissue such as edema, reddening, or hypervascularity. Some residual cement granules can be appreciated after 8 weeks of surgery, however there are no signs of displacement or migration of the granules.

3.3.2. Histological and B-SEM observations

The negative control group refers to the empty cavities (without biomaterial) that was considered a critical size defect (10 mm) since it can not be filled with new bone within the investigated time period (8 weeks), even though a small amount of peripheral bone was formed, as illustrated in Figure 4A.

However, light microscopy and the B-SEM micrographs of the core biopsies of the control sample (CPC without Mg) show residual cement granules surrounded by new bone (Figure 4B and 4C), with native bone visible at both ends. The grays levels of the B-SEM view indicate different degrees of mineralization.

On the other hand, in the critical defects that were fully filled with 6.67 % Mg-CPC, 26.67 % Mg-CPC and 40 % Mg-CPC new bone formation was observed and also some residual graft granules (Figures 5). The images obtained with light microscope and B-SEM in the cements prepared with magnesium are consistent with those obtained with the control cement: residual graft granules surrounded by new bone in the core. Moreover, some graft granules shown osteoconduction while others have minimal contact with the bone.

3.3.3. Histomorphometric results:

The histomorphometric measurements of all samples were performed on the histology sections where the bone was colored blue or violet, and the residual cement appears brown or black. The results of the histomorphometric analysis gave the percentage of new bone and also the remaining graft for the biomaterials: i) CPC (without Mg), NB=11 % ± 2, RG= 40 % ± 7 ; ii) 6.67 % Mg-CPC, NB=26 % ± 5, RG=27 % ± 3; iii) 26.67 % Mg-CPC, NB=19 % ± 3, RG=52 % ± 5; and 40 % Mg-CPC, NB= 32 % ± 5, RG=20 % ± 3.

Statistical analysis of the sections, using a one way factorial ANOVA design, showed significant differences in bone formation, as well as in remaining cement, between the various cements with Mg and the control (p < 0.05) (Figure 6 and table 3 and 4).

4. Discussion

The rationale to use bone cells to analyze the cellular response of the materials is because our work aims to develop new materials with applications in bone repair and bone regeneration. We have chosen for these trials the MG-63 cell line, which consists of human osteosarcoma cells and osteoblast-like cells which are responsible for bone formation. This is a well known cell line that is commonly employed to study the interactions of cells with biomaterials [22,23].

The cellular response to a biomaterial, such as proliferation and differentiation, not only depend on the chemical composition of the material, but also on the morphology of its surface [24], which plays a crucial role in determining cell behavior and influences the amount of ions released from the biomaterial [25]. The cell proliferation studies provide information on the behavior of a material in relation to a control, in this case CPC (without Mg), indicating if the new material somehow improves or worsens the cell behavior. Our results show an increased cell proliferation in the Mg-CPCs in comparison with the control cement. These results could be attributed to the role of Mg in the deposition and bone mineralization [26], but there is no complete agreement on this point. Thus, some previous studies have demonstrated that in addition to Mg some other ions such as Ca and Si can also be released to the medium, thus also contributing to the proliferation and differentiation of osteoblasts [27,28]. Other authors refer that the release of magnesium ions does not cause local or systemic toxicity, and may have beneficial effects on the cellular response in areas where they are located [29]. Moreover, the magnesium added to calcium phosphate cements improves the adhesion of osteoblasts, it is suggested that the binding of cells to biomaterials plays an important role in the rapid restoration of the defective area [30].

The morphology of the surface also influences the behavior of the osteoblasts cultured on it [31]. The results of SEM (see Figure 3) revealed that the cells are well spread and in intimate contact with the surface of the cement during the 10 day test. Usually, once the cells have reached confluence, stops proliferating. However, the cell line used form multilayers, i.e., after reaching confluence and complete a monolayer, the cells continued to grow, forming more than one layer. Due to the growth characteristics of osteosarcoma cell lines differs significantly from that of osteoblasts. All osteosarcoma cell lines reveal a 2 to 3-fold greater mean doubling-time and a 15 to 20-fold higher saturation density than osteoblasts [32].

For the *in vivo* study, we selected rabbit as animal model because according to Frame [33], in the calvaria of this animal there is an adequate area of cortical and spongy bone that permits repairing bone cavities. Furthermore, the calvaria bone of rabbit is an excellent region for carrying out bone substitution studies that later can be used in the jaws, due to the similarity that exists between this bone and the human jaw. From an embrionary point of view they have the same intramembranous origin, morphologically they are made up of two cortical bones separated by medullar bone, and physiologically they have the same repair pattern. In addition, the rabbit is an appropriate model to study the formation and remodeling period of bone (called Sigma) since these phenomena are around three times faster in rabbits (6 weeks) than in humans (about 6 months). We chose 8 week experiments to ensure good bone neo-formation and remodeling, since in 4 weeks only is possible to evaluate the biocompatibility of the material, and 12 weeks are not necessary inasmuch as do not provide more information. In addition to the above reasons, rabbits are cheap, accessible, easy to handle and anesthetize.

It has been determined that the bone defect must not be less than the critical size defect [34], that in rabbit calvarium is controversial, as some authors consider the size of 15 mm [35] whereas for other authors the critical size is around 9 and 10 mm [36]. Our results show that after 8 weeks, the spontaneous bone repair was not sufficient to promote complete healing in the empty cavities of 10 mm. These results agree with various authors that found that in 8 weeks the spontaneous regeneration does not provide complete bone repair [37]. So, we can assume that the bone repair found in this work is related to the filling of the cavities with the calcium phosphate cements.

Through histological analysis, we observed that the new materials promote new-bone formation within the defects, when they were compared with control, being the osteoconduction the responsible for the repair in all experimental groups. Moreover, we determined the presence of histological continuity between cement and bone tissue without fibrous tissue interface. The mechanism of osseointegration of calcium phosphate cements is called osteotransduction and is a peculiarity of this group of materials. In this case, the implanted material is directly involved in the bone replacement, since formation of new bone takes place at the same time the material is degraded by osteoclasts [38].

Furthermore, together with the new bone formation, we found progressive degradation of the granules, so that, after 8 weeks, it is possible to observe rests of cement granules surrounded by bone tissue. The remodeling experienced by the newly formed bone is correlated with the cycles of resorption and bone apposition that characterize normal bone. This implies the existence of a dynamic interface, called coalescence, that relates the material implanted with the apatitic crystals deposited in the bone matrix [39].

The absence of inflammatory manifestations or postoperative infections in any rabbit is similar to the results reported by Yu et al [40] who studied a magnesium phosphate cement as a biodegradable adhesive cement for fracture healing. Overall, the studies suggest that calcium phosphate cements substituted with magnesium are safe, and may have application in fractures fixation.

Histomorphometric measurements quantify the remains of the implanted bone cements, and is an indicator of the rate of resorption of biomaterials. We have found an increased resorption of the cements with Mg, which seems to indicate that the addition of Mg accelerates the degradation of these materials in agreement with the results reported by Zeng et al [41]. Furthermore, the histomorphometric analysis is also able to quantify new bone formation. The statistical analysis of histomorphometric measurements reveals, through the Tukey test at 5 % probability, a significant difference between experimental groups.

In bone regeneration, the use of osteoconductive substitutes is contraindicated in the repair of large defects, because the bone regeneration with this type of material is restricted to the periphery of the implant. Therefore, for a complete bone repair it is important the combination of a osteoconductive matrix with osteoinductive substances, such as growth factors [42]. The clinical application of Mg-CPCs seems possible because their versatility to meet specific requirements concerning bone replacement in musculoskeletal surgery.

5. Conclusions

The development of bone substitute with controllable biodegradable properties and improved bone regeneration is a step toward personalized therapy that can adapt to patient needs and clinical situations. Herein, we suggest that the employment of magnesium ions could improve the clinical efficiency of calcium phosphate cements and modify the pace of their biodegradation.

6. Acknowledgements

The authors acknowledge financial support from the Spanish Ministry of Science and Innovation (MAT2010-15349).

7. References

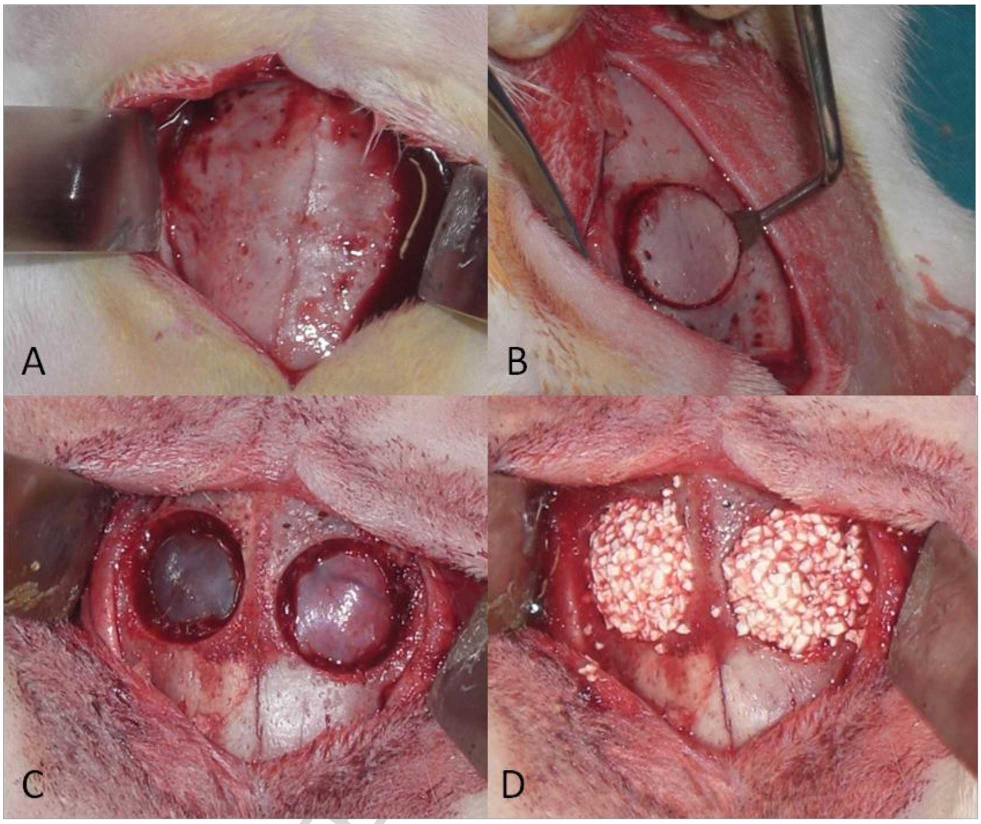


Figure 1. A) Rabbit´s calvaria exposed; B) removal bone block; C) bone defects exposed; D) bone defects filled with bone substitute.

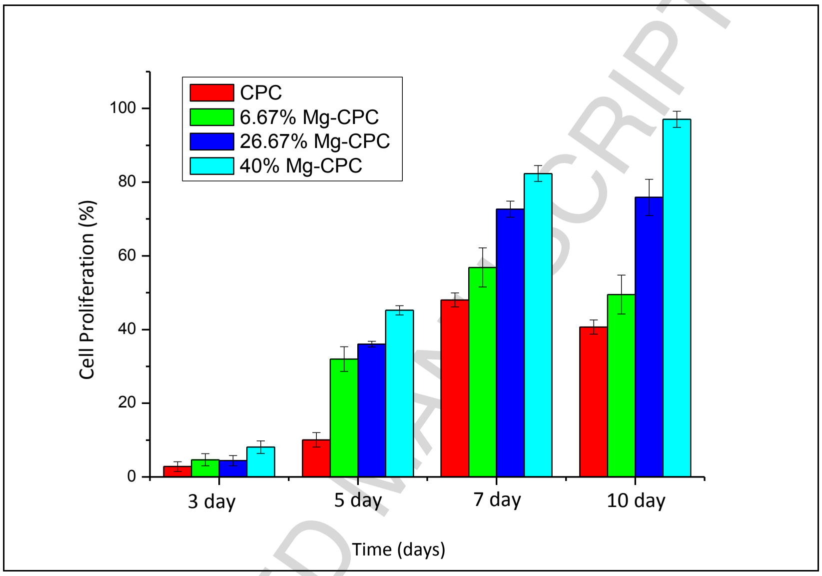


Figure 2. Cell proliferation after seeding on CPC and Mg-CPCs (Error bars represent SD).

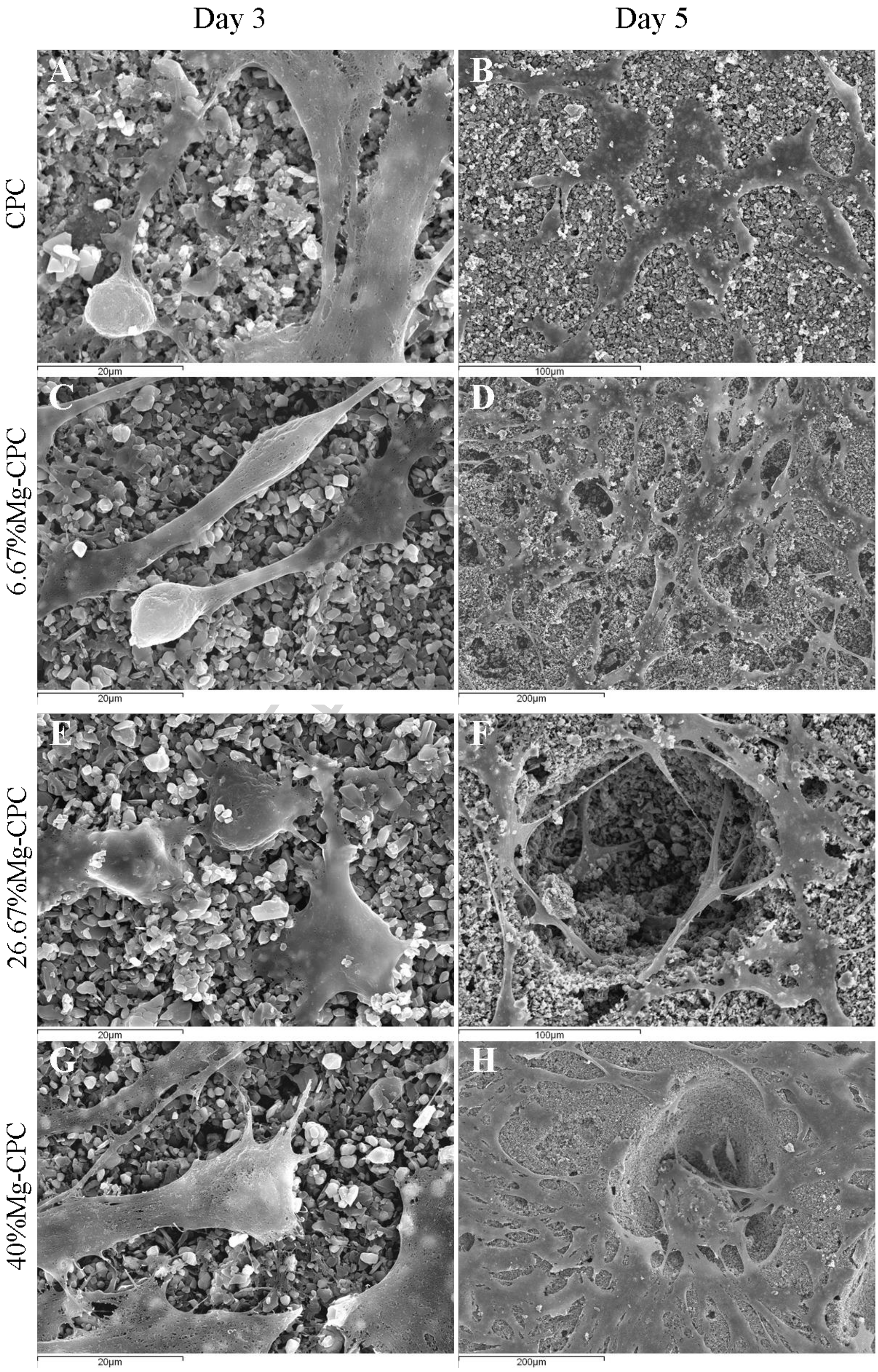

Day 3
Day 5
CPC
6.67%Mg-CPC
26.67%Mg-CPC
40%Mg-CPC
A
B
C
D
E
F
G
H
20μm
100μm
20μm
200μm
20μm
100μm
20μm
200μm

Figure 3. SEM observations of MG-63 cell morphology on different cements after three and five days of culture

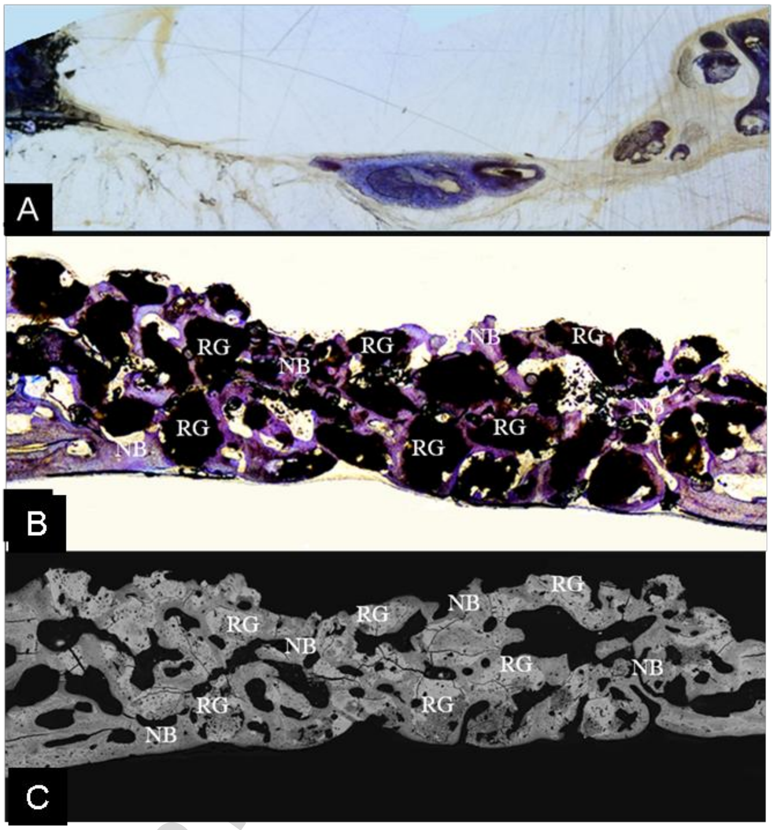


Figure 4. Bone regeneration within a critical defect without cement (A). . Section of the control sample (CPC without Mg). (B) Light microscopy of the bone core, new bone (NB) was evident in contact with remaining graft (RG). (C) The findings of light microscopy were confirmed using B-SEM (upper panel), the gray levels indicate different degrees of mineralization. (2x)

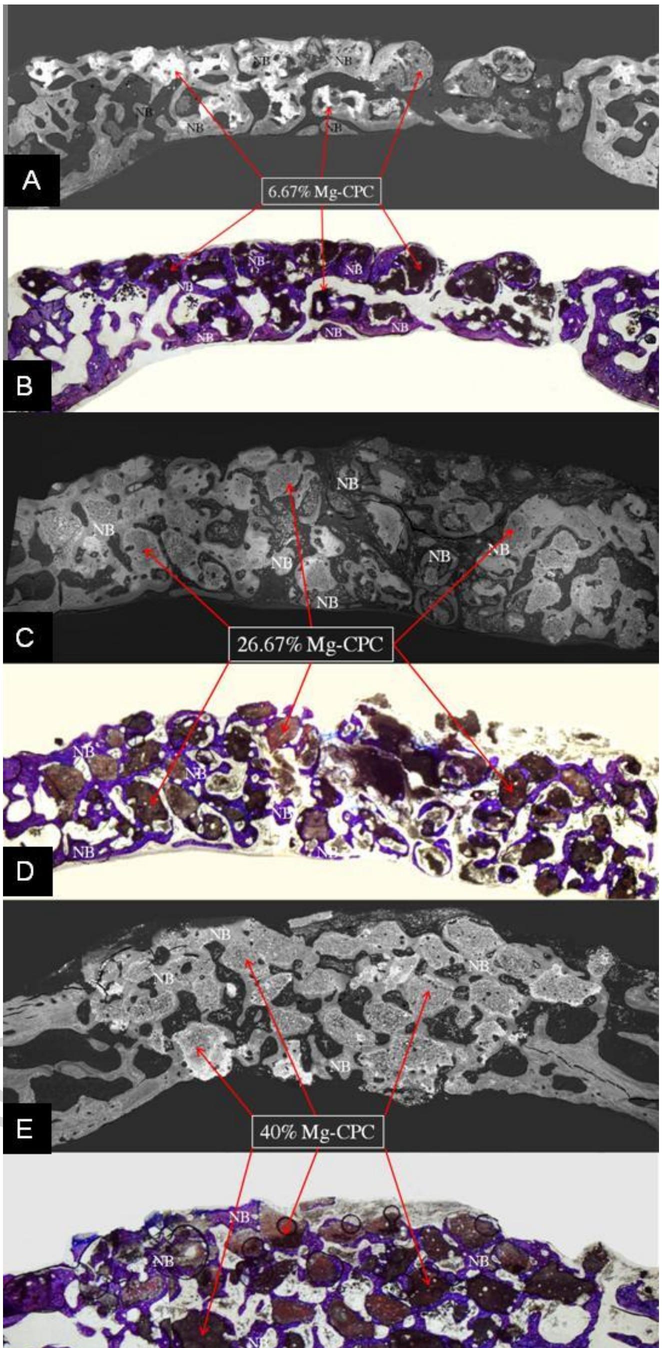


Figure 5. Section of critical defects that were fully filled with 6.67% Mg-CPC (A and B), 26.67% Mg-CPC (C and D) and 40% Mg-CPC (E and F). (2x)

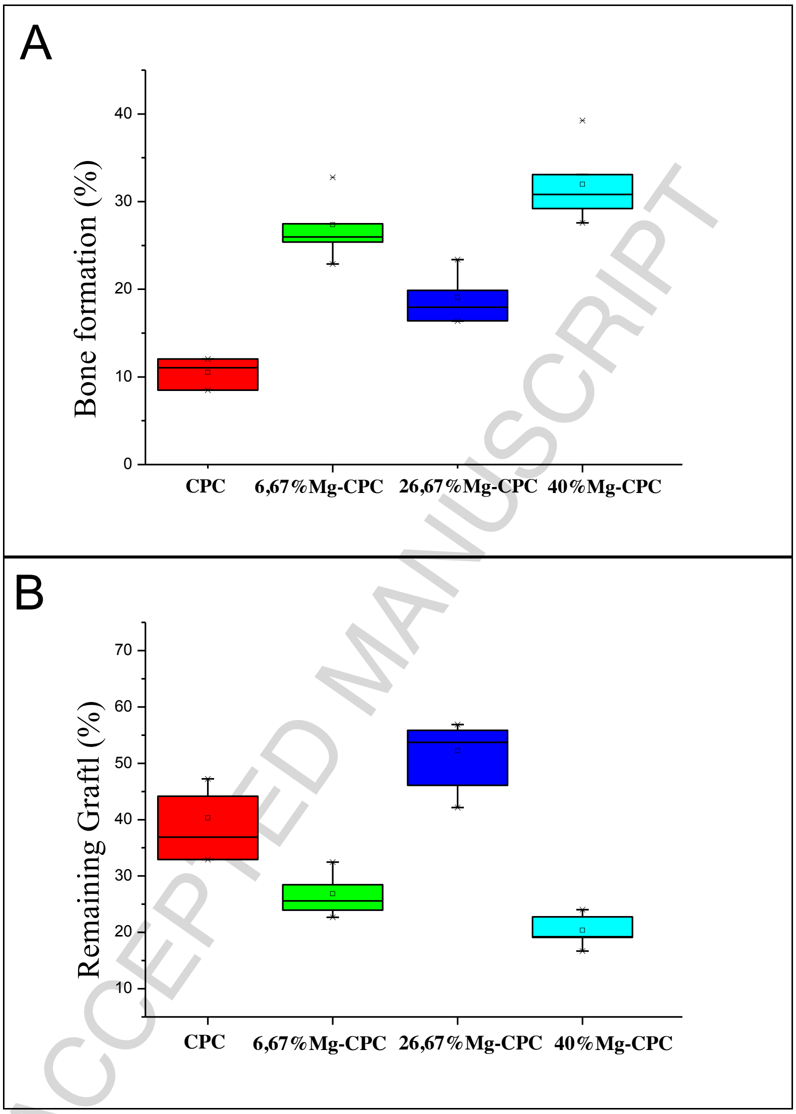


Figura 6. A) Box plot of bone formation of CPC (without Mg), and Mg-CPCs. B) Box plot of remaining graft of CPC (without Mg), and Mg-CPCs. The black thick lines indicate the median values; ▫ = mean values and the vertical black lines indicate whiskers outlier 1.5; * = maximum and minimum values

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

Samples	$CaHPO_4.2H_2O$ [mol]	$MgHPO_4.3H_2O$ [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 %

Table 2. ANOVA (Two-way) statistical analysis of the *in vitro* study ($p < 0.05$).

Source	Sum of Square	gl	Mean Square	F Value	*p* < 0.05
Material	18457.38	3	717.18	929.13	< 0.0001
Time	56819.81	3	3813.70	865.75	< 0.0001
Material * Time	9693.13	9	162.65	33.38	< 0.0001

Table 3. Statistical analysis of the bone formation ($p < 0.05$).

A. ANOVA (One-way)

Source	Sum of Square	gl	Mean Square	F Value	$p < 0.05$
Inter-group	1163.72	3	290.93	22.43	< 0.0001
Intra-gropu	350.17	27	12.97		
Total	1513.89	30			

B. Tukey test

Groups	Mean difference	$p < 0.05$
CPC - 6.67% Mg-CPC	16.84	< 0.0001
CPC - 26.67% Mg-CPC	8.57	< 0.0001
CPC - 40% Mg-CPC	21.47	< 0.0001
26.67% Mg-CPC - 6.67% Mg-CPC	-8.27	< 0.0001
26.67% Mg-CPC - 40% Mg-CPC	12.90	< 0.0001
40% Mg-CPC - 6.67% Mg-CPC	4.63	> 0.05

Table 4. Statistical analysis of the remaining graft ($p < 0.05$).

A. ANOVA (One-way)

Source	Sum of Square	gl	Mean Square	F Value	$p < 0.05$
Inter-group	5392.55	3	1348.14	37.35	< 0.0001
Intra-gropu	1119.05	27	36.10		
Total	6511.59	30			

B. Tukey test

Groups	Mean difference	$p < 0.05$
CPC - 6.67% Mg-CPC	-14.55	< 0.0001
CPC - 26.67% Mg-CPC	12.96	< 0.0001
CPC - 40% Mg-CPC	-19.95	< 0.0001
26.67% Mg-CPC - 6.67% Mg-CPC	-27.50	< 0.0001
26.67% Mg-CPC - 40% Mg-CPC	-32.91	< 0.0001
40% Mg-CPC - 6.67% Mg-CPC	-5.40	> 0.05

Highlights:

- The Mg-CPCs promote higher new bone formation than the CPC.
- The incorporation of magnesium ions in CPC improves osteoblasts proliferation.
- Mg-CPC is a bone substitute with controllable biodegradable properties.
- We suggest that the use of Mg ions could improve the clinical efficiency of CPCs.

1. Khashaba RM, Moussa MM, Mettenburg DJ, Rueggeberg FA, Chutkan NB, Borke JL. Polymeric-calcium phosphate cement composites-material properties: in vitro and in vivo investigations. Int J Biomater. doi:10.1155/2010/691452
2. Generosi A, Smirnov VV, Rau JV, Albertini YR, Ferro D, Barinov SM. Phase development in the hardening process of two calcium phosphate bone cements: an energy dispersive X-ray diffraction study. Mater Res Bull 2008;43:561–71.
3. Bohner M. Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements. Injury-Inter J Care Injured 2000;31:37–47.
4. Ohura K, Bohner M, Hardouin P, Lemaître J, Pasquier G, Flautre B. Resorption of, and bone formation from, new beta-tricalcium phosphate-monocalcium phosphate cements: an in vivo study. J Biomed Mater Res. 1996;30(2):193-200.
5. Yang S, Leong K-F, Du Z, Chua C-K. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. Tissue Eng. 2001;7(6):679–89.
6. Ginebra MP, Espanol M, Montufar EB, Perez RA, Mestres G. New processing approaches in calcium phosphate cements and their applications in regenerative medicine. Acta Biomater 2010;6(8):2863–73.
7. Alkhraisat MH, Moseke C, Blanco L, Barralet JE, Lopez-Carbacos E, Gbureck U. Strontium modified biocements with zero order release kinetics. Biomaterials 2008;29(35):4691–7.
8. Pina S, Vieira SI, Torres PMC, Goetz-Neunhoeffer F, Neubauer J, Silva O, et al. In vitro performance assessment of new brushite-forming Zn- and ZnSr-substituted beta-TCP bone cements. J Biomed Mater Res B Appl Biomater 2010;94(2):414–20.
9. Tamimi F, Torres J, Bettini R, Ruggera F, Rueda C, Lopez-Ponce M, et al. Doxycycline sustained release from brushite cements for the treatment of periodontal diseases. J Biomed Mater Res A 2008;85(3):707–14.
10. Alkhraisat MH, Rueda C, Cabrejos-Azama J, Lucas-Aparicio J, Tamimi F, Garcia-Denche JT, et al. Loading and release of doxycycline hyclate from strontium-substituted calcium phosphate cement. Acta Biomater 2010;6(4):1522–8.
11. De la Riva B, Sanchez E, Hernandez A, Reyes R, Tamimi F, Lopez E, et al. Local controlled release of VEGF and PDGF from a combined brushite chitosan system enhances bone regeneration. J Controlled Release 2010;143(1):45–52.

12. Kim SR, Lee JH, Kim YT, Riu DH, Jung SJ, Lee YJ, Chung SC, Kim YH. Synthesis of Si, Mg substituted hydroxyapatites and their sintering behaviors. Biomaterials 2003;24(8):1389–1398.
13. Rude RK. Magnesium deficiency: a cause of heterogeneous disease in humans. J Bone Miner Res. 1998;13(4):749-58.
14. Ergun C, Webster TJ, Bizios R, Doremus RH. Hydroxylapatite with substituted magnesium, zinc, cadmium, and yttrium. I. Structure and microstructure. J Biomed Mater Res. 2002;59(2):305-11.
15. Kannan S, Lemos IAF, Rocha JHG, Ferreira JMF. Synthesis and characterization of magnesium substituted biphasic mixtures of controlled hydroxyapatite/β-tricalcium phosphate ratio. J Solid State Chem. 2005;178(10):3190-3196.
16. Lagier R, Baud CA. Magnesium whitlockite, a calcium phosphate crystal of special interest in pathology. Pathol Res Pract. 2003;199(5):329-35.
17. Bigi A, Cojazzi G, Panzavolta S, Ripamonti A. Roveri N, Romanello M, Noris K, Moro L. Chemical and structural characterization of the mineral phase from cortical and trabecular bone. J Inorg Biochem 1997;68(1):45-51.
18. Raynaud S, Chsmpion E, Bernache-Assollant D, Thomas P. Calcium phosphate apatities variable Ca/P atomic ratio I. Synthesis, characterization and thermal stability of powders. Biomaterials 2002;23(4):1065-72.
19. Bertinetti L, Drouet C, Combes C, Rey C, Tampieri A, Coluccia S, Martra G. Surface characteristics of nanocrystalline apatites: effect of Mg surface enrichment on morphology, surface hydration species, and cationic environments. Langmuir. 2009;25(10):5647-54.
20. Julien M, Khairoun I, LeGeros RZ, Delplace S, Pilet P, Weiss P, Daculsi G, Bouler JM, Guicheux J. Physico-chemical-mechanical and in vitro biological properties of calcium phosphate cements with doped amorphous calcium phosphates. Biomaterials 2007;28(6):956-965
21. Alkhraisat MH, Cabrejos-Azama J, Rueda C, Blanco L, López-Cabarcos E. Magnesium substitution in brushite cements. Mater Sci Eng C 2013,33(1):475–481.
22. Ohno M, Kimoto K, Toyoda T, Kawata K, Arakawa H. Fluoride-treated Bio resorbable Synthetic Hydroxyapatite Promotes Proliferation and Differentiation of Human Osteoblastic MG-63 Cells. J Oral Implantol 2013;39(2):154-160.
23. Shapira L, Halabi A. Behavior of two osteoblast-like cell lines cultured on machined or rough titanium surfaces. Clin Oral Implants Res. 2009;20(1):50-55.
24. Qu WJ, Zhong DB, Wu PF, Wang JF, Han B. Sodium fluoride modulates caprine osteoblast proliferation and differentiation. J Bone Miner Metab. 2008;26(4):328–334.
25. Allen J, Liu Y, Kim YL, Liu Y, Kim YL, Turzhitsky VM, et al. Spectroscopic translation of cell-material interactions. Biomaterials. 2007;28(2):162–174.
26. Cai YL, Zhang S, Zeng XT, Wang YS, Qjan M, Weng WJ. Improvement of bioactivity with magnesium and fluorine ions incorporated hydroxyapatite coatings via sol–gel deposition on Ti6Al4V alloys. Thin Solid Films 2009; 517(17):5347–5351.
27. Dietrich E, Oudadesse H, Lucas-Girot A, Mami M. In vitro bioactivity of melt-derived glass 46S6 doped with magnesium. J Biomed Mater Res A. 2009;88(4):1087–1096.

28. Porter AE, Patel N, Skepper JN, Best SM, Bonfield W. Effect of sintered silicate- substituted hydroxyapatite on remodelling processes at the bone-implant interface. Biomaterials 2004;25(16):3303-14.
29. Witte F, Feyerabend F, Maier P, Fischer J, Stormer M, Blawert C, et al. Biodegradable magnesium-hydroxyapatite metal matrix composites. Biomaterials. 2007;28(13):2163–174.
30. Yamasaki Y, Yoshida Y, Okazaki M, Shimazu A, Kubo T, Akagawa Y, and Uchida T. Action of FGMgCO3Ap-collagen composite in promoting bone formation. Biomaterials 2003;24(27):4913-4920.
31. Lee JY, Kang BS, Hicks B, Chancellor TF, Chu BH, Wang HT, et al. The control of cell adhesion and viability by zinc oxide nanorods. Biomaterials. 2008;29(27):3743–3749.
32. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, Milz S. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res. 2004;24(6):3743-8
33. Frame JH. A convenient animal model for testing bone substitute materials. J Oral Surg 1980;38(3):176-180.
34. Bosch C, Melsen B, Vargervik K. Importance of the critical-size bone defect in testing bone-regenerating materials. J Craniofac Surg 1998;9(4):310-316.
35. Vikjaer D, Blom s, Hjorting-Hansen E, Pinholt EM. Effect of platelet-derived growth factor-BB on bone formation in calvarial defects: an experimental study in rabbits. Eur J Oral Sci. 1997;105(1):59-66.
36. Hollinger JO, Kleinschmidt JC. The critical size defect as an experimental model to test bone repair materials. J Craniofac Surg 1990;1(1):237-243.
37. Rabie AB, Lie Ken Jie RK. Integration of endochondral bone grafts in the presence of demineralized bone matrix. Int J Oral Maxillofac Surg 1996;25(4):311-318.
38. Driessens FCM, Planell JA, Boltong MG, Khairoun I, Ginebra MP. Osteotransductive bone cements. Proc Instn Mech Engrs [H] 1998; 212: 427-35.
39. Wei J, Jia J, Wu F, Wei S, Zhou H, Zhang H, Shin JW, Liu C. Hierarchically microporous/macroporous scaffold of magnesium-calcium phosphate for bone tissue regeneration. Biomaterials. 2010;31(6):1260-1269.
40. Yu Y, Wang J, Liu C, Zhang B, Chen H, Guo H, Zhong G, Qu W, Jiang S, Huang H. Evaluation of inherent toxicology and biocompatibility of magnesium phosphate bone cement. Colloids Surf B Biointerfaces. 2010;76(2):496-504.
41. Zeng D, Xia L, Zhang W, Huang H, Wei B, Huang Q, Wei J, Liu C Jiang X. Maxillary Sinus Floor Elevation Using a Tissue-Engineered Bone with Calcium-Magnesium Phosphate Cement and Bone Marrow Stromal Cells in Rabbits. Tissue Eng Part A 2012;18(7-8):870-81.
42. Duguy N, Petite H, Arnaud E. Biomaterials and osseous regeneration. Ann Chir Plast Esthet 2000;45:364-76.