IN VITRO EVALUATION OF A BIOMIMETIC GELATIN-CALCIUM PHOSPHATE BONE CEMENT

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Abstract: We have recently developed a new biomimetic bone cement constituted of α-tricalcium phosphate (α -TCP) and gelatin (Gel-CP). The presence of gelatin greatly improves the mechanical properties of the cement which present also a better response to the osteoblast-like cells culture. Herein we extend our investigations to primary culture of osteoblasts derived from healthy and pathological bone. Osteoblast from normal and osteopenic sheep bone were cultured up to 7 days on samples of Gel-CP, and compared to cells cultured on the control. Evaluation of cell morphology, proliferation and differentiation after 3 and 7 days was done. The results put in evidence that the presence of gelatin improves osteoblast activity and differentiation, and positively stimulates alkaline phosphatase activity, Collagen Type I and Osteocalcin production. Therefore, this biomimetic composite material could be successfully applied as bone substitute, also in the presence of osteopenic bone.

Introduction

Calcium phosphate cements are widely used as bone substitute in dentistry and orthopaedics because they possess a good biocompatibility and osteoconductivity, and can be molded into desired shape during implantation. [1]. One of the main components of these bioactive cements is α–tricalcium phosphate (α-TCP), wich hydrolizes in aqueous solutions, at physiological pH values and temperature, into calcium-deficient hydroxyapatite (CDHA). This mineral phase exhibits chemical and structural properties similar to those characteristic of the poorly crystalline carbonated apatite of bone and teeth .

We have recently developed a biomimetic bone cement constituted of α-TCP and gelatin [2]. The presence of gelatin accelerates the setting reaction and improves the mechanical properties of the cement, giving a composite material where both the organic and inorganic components resemble those of bone [3]. The remarkable improvement of the setting properties due to the presence of gelatin suggest that the biomimetic composite material could be successfully applied as bone cement. On this basis we have studied the response of osteoblast cultures onto the composite gelatin-cement, and thus compared the results with those simultaneously obtained on the cement with the same calcium phosphate composition prepared in the absence of gelatin (C-CP). To this aim we used primary cultures of osteoblasts derived from both healthy and osteopenic bone.

Materials and methods

Gelatin cements were prepared using type A gelatin from pig skin (ItalgelatineS.p.A.). α-TCP powder was added to gelatin aqueous solutions in the appropriate amount, to obtain 15 wt% of gelatin in the composite material. The solid compounds, obtained by casting the solutions, were ground and sieved (<40 μm) before mixing with 5 wt% of CaHPO4 • 2H2O (DCPD).

The cement samples (Gel-C) were prepared with bidistilled water using an L/P ratio of 0.3 ml/g. Teflon molds were used to obtain cement cylinders 6 mm in diameter and 12 mm high. Cements containing only α-TCP and DCPD were also prepared, and used as controls (C-CP).

Samples were soaked in simulated body fluid (SBF) at 37 °C for 7 days, and then submitted to X-ray diffraction (XRD), mechanical tests, and scanning electron microscopy (SEM).

Cement cylinders (12 mm diameter and 12mm high), previously immersed in SBF at 37°C for 7 days and submitted to gamma rays sterilization, were used as substrate for cell growth. To this aim, we used normal (N-OB) and osteopenic (O-OB) osteoblasts obtained from transiliac biopsies from healthy and ovariectomized mongrel sheep. A cell suspension (2.0×10^4cells/ml) was directly seeded on every sample of GEL-CP and C-CP. The same amount of cells was plated in polystyrene wells without materials as Control (C).

At the end of experimental times (3, and 7 days) the supernatant was collected from the wells and centrifuged to remove particulates, if any. Aliquots were dispensed in Eppendorf tubes for storage at -70°C and assayed for Type I Collagen (PICP, Prolagen-C enzyme Immunoassay kit, Metra Biosystem, CA, USA), and Interleukin-6 (IL-6, Human IL-6 Immunoassay kit, Biosource International, CA, USA). Alkaline

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Phosphatase activity (ALP, Sigma Kinetic method kit, St. Louis, MO, USA), and Osteocalcin (OC, Novocalcin enzyme Immunoassay kit, Metra Biosystem, CA, USA), were tested on supernatants immediately after collection. Finally, the Cell Proliferation Reagent WST-1 test was done to assess cell proliferation and viability.Samples for each material , at the end of the experimental times, were processed for Scanning Electron Micrographs (SEM): the samples were sputter-coated with platinum-palladium prior to examination.

Results and Discussion

After 7 days of soaking in SBF solution at 37°C, both the cements with and without gelatin are completely converted into hydroxyapatite, as it can be seen by x-ray powder diffraction pattern. At reaction times shorter than 7 days, the presence of gelatin increases significantly the conversion rate of α-TCP into CDHA. The close resemblance of gelatin to collagen, which is the main part of the extracellular bone matrix, suggests that gelatin can offer nucleation sites that are quite similar to those of the biological matrix. These sites can accelerate the precipitation of CDHA, with a consequent inhibition of crystal growth during the setting reaction.

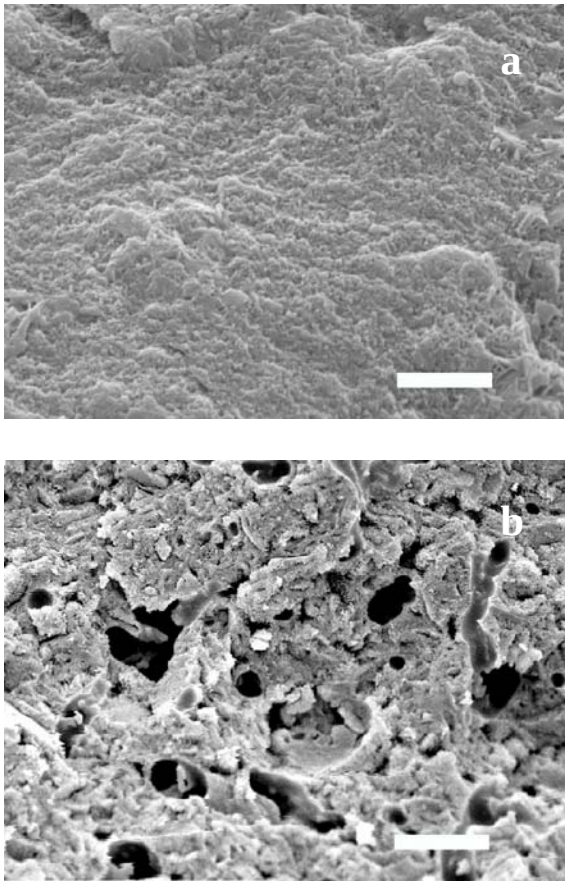

Figure 1. SEM micrographs of the fracture surfaces of Gel-CP (1a) and C-CP (1b) after 7 days of soaking in SBF.(bar = 40 micron)

SEM images reported in figure 1a and 1b show the fracture surfaces of both the cements. The gelatin containing cements display a closely packed microstructure due to the very small dimensions of the crystals (1a), while the fractured surface of C-CP (1b) is completely covered of entangled plate-like crystals. The plate-like entangled crystals do not fill all the volume, and leave small voids or pores all over the cement.

Table 1: Compressive strength (σ) and Young' s modulus (E) of the cements after different times of soaking in SBF.

Soaking time (days)	**C-CP**		**Gel-CP**	
	σ (MPa)	E (GPa)	σ (MPa)	E (GPa)
1	1.9 ± 0.3	0.08 ± 0.03	10.7 ± 0.2	1.6 ± 0.1
7	2.0 ± 0.8	0.23 ± 0.03	11 ± 2	2.6 ± 0.1

We have also tested the mechanical properties of C-CP and GEL-CP : the values of compressive strength and of Young's modulus, reported in Table 1, were quite low for the control cement, while the presence of gelatin dramatically improved the mechanical properties.

Cells were cultured for 3 and 7 days on C-CP and Gel-CP cements: after every time cell proliferation and metabolic activity were tested. Test results are reported in Table 2 and 3.

Table 2. N-OB proliferation and synthetic activity after 3 and 7 days of culture on GEL-CP, C-CP, and polystyrene (Control). Mean, n = 6 triplicates.

N-OB 3d	Gel-CP	C-CP	Control
WST1	0.48 ± 0.04	0.50 ± 0.05	0.70 ± 0.03
BALP	4.2 ± 0.1	3.9 ± 0.1	3.3 ± 0.8
CICP	6.7 ± 0.7	3.6 ± 0.7	3.8 ± 0.5
OC	8.5 ±1.7	4.0 ± 0.6	4.6 ± 0.1
IL-6	4.4 ± 0.4	4.2 ± 0.1	3.11 ± 0.01

N-OB 7d	Gel-CP	C-CP	Control
WST1	0.83 ± 0.07	0.73 ± 0.03	0.79 ± 0.04
BALP	8.2 ± 0.1	7.2 ± 0.2	6.8 ± 0.3
CICP	7.0 ± 0.3	5.7 ± 0.4	4.8 ± 0.1
OC	15 ±1	11 ± 2	4.5 ± 0.1
IL-6	3.0 ± 0.6	2.9 ± 0.1	1.8 ± 0.2

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Table 3: O-OB proliferation and synthetic activity after 3 and 7 days of culture on GEL-CP, C-CP, and polystyrene (Control). Mean, n = 6 triplicates

O-OB 3d	Gel-CP	C-CP	Control
WST1	0.49 ± 0.01	0.43 ± 0.01	0.64 ± 0.01
BALP	4.4 ± 0.1	4.0 ± 0.1	3.1 ± 0.1
CICP	5.3 ± 0.7	3.5 ± 0.5	3.9 ± 0.2
OC	7.5 ±1.7	3.0 ± 0.6	4.4 ± 0.1
IL-6	4.50 ± 0.01	5.4 ± 0.1	3.5 ± 0.3

O-OB 7d	Gel-CP	C-CP	Control
WST1	0.75 ± 0.03	0.64 ± 0.05	0.79 ± 0.04
BALP	7.8 ± 0.3	7.1 ± 0.1	7.0 ± 0.6
CICP	5.9 ± 0.3	5.0 ± 0.1	4.9 ± 0.1
OC	10 ±1	3.5 ± 0.6	4.5 ± 0.3
IL-6	3.20 ± 0.3	3.2 ± 0.2	2.74 ± 0.05

As concerns cell proliferation, at 3 days O-OB cultured on C-CP showed a significantly lower proliferation ($p<0.05$) in comparison with other groups. At 7 days O-OB cultured on C-CP remained the significantly lower value of WST1 when compared to all other groups ($p<0.005$). Moreover N-OB grown on Gel-CP showed a significantly higher proliferation in comparison to N-OB grown on C-CP and O-OB grown on Gel-CP ($p<0.05$).

After 3 days, alkaline phosphatase value of NOB/Gel-CP was significantly higher in comparison with C-CP groups ($p<0.05$) and value of O-OB/Gel-CP was higher in comparison with both N-OB and O-OB grown on C-CP ($p<0.0001$ and $p< 0.005$ respectively).

After 7 days, BALP value of both N-OB and O-OB cultured on Gel-CP were significantly higher when compared to osteoblasts cultured on C-CP.

The synthesis of collagen for N-OB and O-OB cultured on GEL-CP was significantly higher when compared to levels produced on C-CP after 3 days.

At 7 days, N-OB/Gel-CP showed a significant higher livel in comparison with all other groups, while O-OB/C-CP showed a significant lower livel .

At 3 days, osteocalcin production of both N-OB and O-OB cultured on Gel-CP was significantly higher when compared to C-CP. At 7 days OC level of N-OB/Gel-CP was significantly than all other groups, while the livel of OC produced in O-OB/C-CP group was the lower.

Finally, Interleukin 6 production was significantly stimulated in O-OB grown on C-CP in comparison with all other groups at 3 days, but at 7 days no more differences were found among all groups.

After 7 days, some samples randomly chosen were examined by SEM, in order to verify osteoblasts attachment and spreading. SEM analysis showed a quite different morphology between cells grown on Gel-CP and C-CP samples.

Figure 2 reports N-OB cultured on Gel-CP (2a) and C-CP (2b) samples: cells grown on gelatin enriched cements appear much more flattened and display more filopodia than those grown on C-CP. A similar trend has been obtained for O-OB growth.

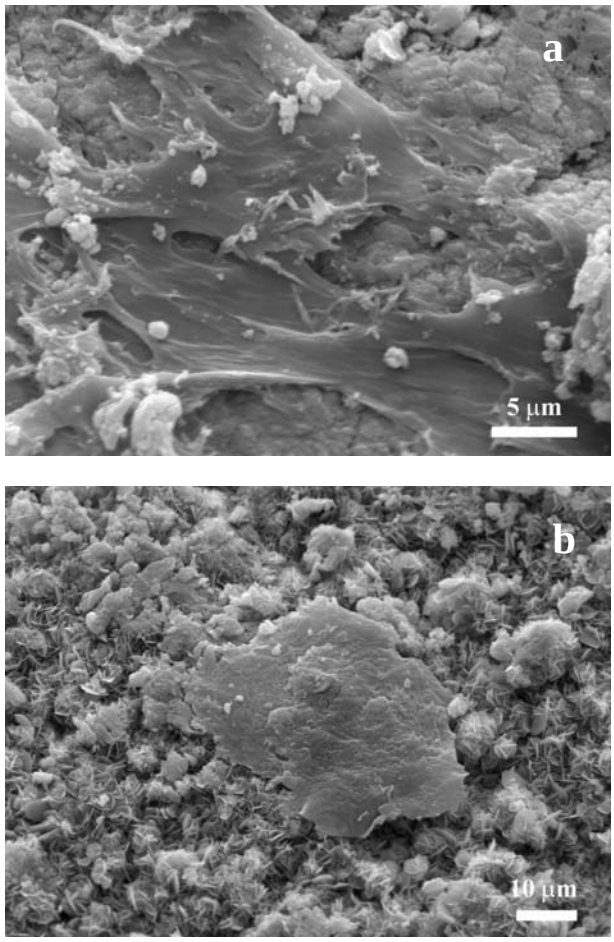

Figure 2. SEM images of normal bone derived osteoblasts after 7 days of culture on Gel-CP (2a) and C-CP (2b) samples.

Discussion

In a previous work [4] we have described the role of gelatin on the properties of this new bone cement: Gel-CP samples display a faster rate of conversion into HA, a more compact microstructure and significantly improved mechanical properties with respect to the control samples. The improvement is most likely due to the inhibition of crystal growth caused by the presence of gelatin: the apatitic crystals display reduced cristallinity and mean dimensions, which yields to a closely packed microstructure and to a reduced total porosity.

The presence of gelatin should ensure a better distribution of the mechanical load that should contribute to the improvement of the mechanical

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properties. Furthermore, biological tests previously conducted [5] by studying proliferation and differentiation of osteoblasts cell line MG63, showed that the gelatin enriched cements enhanced osteoblast activation and extra-cellular matrix mineralization process, when compared with C-CP.

In this study, we used osteoblasts derived from normal and osteopenic sheep bone, in order to evaluate the effect of this new biomaterial on pathological osteoblasts cultures.

The investigation was conducted at 3 and 7 days of culture. The osteoblast proliferation was measured by WST1. To evaluate cell activation and differentiation, BALP, CICP and OC were used, because the increase of ALP activity, of Collagen type I synthesis and of OC production are considered the expression of a more differentiated state of osteoblasts [6-8]. As a matter of fact, Collagen type I is synthesized by osteoblasts as the major organic macromolecule in the extracellular bone matrix [9-11], while Osteocalcin is produced during mineralization and is considered a late marker of osteoblasts differentiation [10,12]. The measure of Interleukin 6 is used to evaluate cytotoxicity: in fact, IL-6 is a cytokine considered an agent favoring bone resorption and a mediator of inflammation [13,14].

Data obtained showed that the highest values of IL-6 were obtained after 3 days on O-OB/C-CP, while at 7 days all the values were lower, without differences among groups. After 3 days of culturing on Gel-CP, both O-OB and N-OB showed no difference in proliferation rate, but results of ALP, CICP and OC were significantly higher with respect to those obtained on C-CP. A similar trend was observed after 7 days, demonstrating that the presence of gelatin positively influenced cell activity and differentiation in normal and in osteopenic bone derived cultures. ALP values seem less influenced by the type of osteoblasts, instead CICP and OC level on both biomaterials were lower in O-OB cultures with respect to N-OB.

This study puts in evidence the key role of gelatin, because its presence on the cement formulation positively stimulates cells activity not only in normal osteoblasts, but in osteopenic as well. The results of SEM investigation show that the cements do not alter the osteoblasts morphology, even if the cells cultured on Gel-CP appear more flattened and have much more filopodia than those grown on C-CP.

Conclusion

The results, discussed in this paper, show that the addition of gelatin to the formulation of calcium phosphate cement induces the improvement of both mechanical and biological properties of this biomaterial.

The presence of gelatin induces a higher compressive strength and favours osteoblast activation and extra-cellular matrix mineralization, if compared to C-CP. For these reasons, gelatin-containing bone cement can be regarded as a promising biomimetic material to be successfully applied as bone substitute not only in the presence of healthy bone, but also in osteopenic states of bone.

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