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Primary osteoblast cell response to sol-gel derived bioactive glass foams

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Bioactive glass macroporous structures were developed in this work to be used as scaffolds for bone tissue engineering applications. A sol-gel route was used to obtain glass foams with the introduction of a gas phase in the solution and by vigorous agitation of the sol-gel solution that contains a foam agent. Stable and homogeneous foams were formed near the gelation point, which were than dried and heat-treated. Macroporous structures with interconnected pores of up to 500 μm, porosity as high as 88% and specific surface area of 92 m^2/g were obtained. The porous glasses were tested in osteoblast cultures to evaluate adhesion, proliferation, collagen and alkaline phosphatase production. Osteoblast proliferation was higher in the presence of the foams as well as was the collagen secretion, when compared to control. The alkaline phosphatase production was not altered. Viable osteoblasts could be seen inside the foams, suggesting that the produced porous glass foams are a promising materials for bone repair, since it provides a good environment for the adhesion and proliferation of osteoblasts.

1. Introduction

Highly interconnected porous structures are one of the requirements that have to be attended when scaffolds for tissue engineering are designed [1, 2]. The high porosity (70–90%) as well as an average pore size larger than 150 μm are necessary to allow cell migration through the structure, adhesion and proliferation [3]. At the same time the material should present a controlled biodegradation rate. Bioactive glasses in the system SiO_2-CaO-P_2O_5 obtained by sol-gel method present good characteristics of osteoconduction and osteoinduction. They can be designed with controlled compositions and high specific surface area in order to be biodegradable [4, 5]. Additionally, recent findings have demonstrated that there is a genetic control of the cellular response to bioactive glass materials [6, 7]. Seven families of genes are up-regulated when primary human osteoblasts are exposed to the ionic dissolution products of bioactive glasses [7]. These findings indicate that bioactive glass materials are very interesting options for tissue regeneration and tissue engineering. The sol-gel derived bioactive glass provides high bioactivity and controlled ability to resorb [6]. The ionic products from their dissolution (Si and Ca) have the potential to control the cell cycle of the osteoblast progenitor cells and stimulate the genes in bone cells to differentiate enhancing bone regeneration [7]. Previous research has been conducted in our group to develop scaffolds based on the bioactive glasses using a sol-gel process to obtain glass foams [8]. Foaming is obtained by vigorous agitation of the sol, with the aid of surfactants. Glass foams with similar structures have also been reported [9, 10]. For bone formation, it is necessary to have viable and proliferating osteoblasts, they have to secrete collagen and this protein has to be phosphorylated by the cells [11]. Therefore, the objective of this work was to evaluate cell viability, proliferation and secretion capability of primary culture osteoblasts in the presence of the three dimensional structure of sol-gel derived bioactive glass. A description of the synthesis and of the structure of the materials obtained is presented. This study will indicate their potential as scaffolds for bone tissue engineering.

2. Materials and methods

2.1. Materials

Chemical reagents for glass preparation from different suppliers were all analytical grade. Lauryl ether sodium sulfate (LESS): PRAID-Chemical Products Ltda (São Paulo-Brazil). Fluo-3/AM: Molecular Probes (Eugene, USA). Penicillin, streptomycin, fetal bovine

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serum, Dulbecco's phosphate buffered saline, trypsin-EDTA, MTT [3(4,5dimethylthiazol-2yl) 2,5diphenyl-tetrazoliumbromide], BCIP-NBT kit: Gibco (Burlington, Ontario, Canada). Crude bacterial collagenase: Boehringer (Biberach, Germany). RPMI Cell culture medium: Sigma (St Louis, USA), SIRCOL kit: Biocolor (Newtonabbey, N Ireland) T25 culture flasks and 24 well plates: Nunc products (Naperville, USA). Glutaraldehyde, formaldehyde, alcohol: Labsynth (Diadema, Brazil). Osmium tetroxide: Pressure Chemical Co (Pittsburgh, USA).

2.2. Bioactive glass foams synthesis and characterization

Tetraethylorthosilicate (TEOS), triethylphosphate (TEP) and tetrahydrated calcium nitrate were used as the silica, phosphorous and calcium source respectively. The reagents were mixed to produce glasses with molar composition 60%SiO_2-36%CaO-4%P_2O_5.The hydrolysis was performed in the presence of acid solution of HNO_3, 2 N. The H_2O/TEOS molar ratio used was 12. Hydrofluoric acid (HF) was added in order to catalyze the gelation, allowing better control of the foam casting. The surfactant LESS was added as foaming agent. The foam was produced by vigorous stirring of the solution, followed by casting at the gel point conformed by observation. The foamed gels were aged in Teflon containers at 60 °C for 48 h and dried with a schedule ending at 170 °C. The dried samples were stabilized at 700 °C for 3 h. The samples were analyzed using Scanning Electron Microscopy, N_2 adsorption and Archimedes' density analysis. The phase composition of the glass was analyzed by X-ray diffraction (XRD, Phillips PW 1770).

2.3. Culture of osteoblasts

Osteoblasts were isolated from the calvaria of 1–5 days old neonatal Wistar rats [12]. The calvaria were dissected and freed from soft tissue, cut into small pieces and rinsed in sterile phosphate-buffered saline without calcium and magnesium. The calvaria pieces were incubated with 1% trypsin-EDTA for 5 min, followed by four sequential digestions with 2% collagenase at 37 °C for 45 min each. The supernatant of the first collagenase incubation, which contained a high proportion of periosteal fibroblasts, was discarded. The other digestions produced a suspension of cells with high proportion of preosteoblasts and osteoblasts. After centrifugation at 1000 g for 5 min, each pellet were resuspended in 5 ml of RPMI medium supplemented with 10% FBS, 1% antibiotic-antimycotic. The cells were seeded into 25 ml tissue culture flasks, and allowed to grow in a controlled 5% CO_2 95% humidified incubator at 37 °C. For experiments, only confluent cells from the 2nd passage were used.

2.4. Stimulation of osteoblasts with bioactive glass foams

For the cell culture studies bioactive glass foams were cut into 5 mm diameter and 3 mm thick discs and sterilized in ethylene oxide. Two culture procedures were used. In the first one the samples and the medium containing cell suspension were put in the wells at the same time. In this case there would be the hypothesis of cells being carried into the pores due to capillarity action. So, in the second procedure osteoblasts were plated and, after adhesion, the glass discs were gently deposited over the cells. For both procedures the density used was 5×10^4 cells/ml. After 4 days of incubation, osteoblast morphology, viability and secretion capability were tested. As control, we used cultures without samples.

2.5. Cellular viability

After incubation for the established period, osteoblast viability was evaluated by MTT assay, based on the reduction of tetrazolium salt to formazan crystals by dehydrogenase present in living cells mitochondria. We left 200 μl of culture medium in each well and added 60 μl of stock solution of MTT (5 mg/ml). Two hours later, the cell morphology was analysed by inverted optical microscopy and formazan salts were solubilized with 200 μl of SDS 10% HCl. After incubation, for 18 h, in a controlled 5% CO_2 95% humidified incubator at 37 °C, the optical density measurement was done at 595 nm [13].

2.6. Collagen secretion measurement

Collagen production was analysed by SIRCOL assay in supernatants of the cells cultures. This method is based on the selective binding property of the syrius-red dye to the [Gly-X-Y] tripeptide end sequence of mammalian collagen. The collagen present in the supernatant, precipitated by the dye, was solubilized and measured by an optical density analysis at 595 nm. The amount of collagen was calculated based on a standard curve of previously known concentrations of type I collagen and their optical density measurement [14]. As control we used cultures without samples.

2.7. Alkaline phosphatase activity

Alkaline phosphatase production was evaluated by BCIP-NBT assay. This assay is based on a chromagenic reaction initiated by the cleavage of the phosphate group of BCIP by alkaline phosphatase present in the cells. This reaction produces a proton, which reduces NBT to an insoluble purple precipitate. Briefly, the supernatant of each well was removed and the cell layer was rinsed twice with PBS. Then, 200 μl of BCIP-NBT solution, prepared as manufacturer's protocol, was added to each well. After 2 h of incubation, the cells were observed by optical microscopy and the insoluble purple precipitates were solubilized with 210 μl of SDS 10% HCl and incubated for 18 h. The optical density measurement was done at 595 nm. We used cultures without samples as control.

2.8. Cell morphology

Cell morphlogy in the culture and in the porous samples was analyzed by optical and scanning electron microscopy (SEM). For SEM analysis the samples were removed from the culture medium and fixed in

glutaraldhyde 2.5%/formaldehyde 4% in 0.1M phosphate buffer and osmium tetroxide solutions. The samples were than dehydrated in alcohol [15], dried at the critical point (Balzers Union CPD020) and coated with gold.

2.9. Statistics

Data was analyzed by Wilcoxon signed rank test, $P < 0.05$.

3. Results

The foam samples remained unbroken and completely white, after drying and stabilization treatment (Fig. 1(A)). The procedure used allowed the successful production of glasses with high porosity in the desired size range. The macropore size ranged from 100 to 500 μm and the total porosity of the samples was 88%. The foams presented high interconnection between pores. (Fig. 1(B)). Besides the macroporous structure of the foam the samples presented a mesoporous structure typical of sol-gel derived materials, as determined by N_2 adsorption. The materials presented a surface area of 92 m^2/g and average mesopore diameter of 10 nm. XRD spectrum of the glass foams showed only two broad, low intensity peaks, characterizing an amorphous solid (Fig. 2).

The cellular viability studies showed that osteoblasts were viable in the culture, in the region around the glass foam. The formazan crystals, formed after MTT metabolisation, were perfectly visualized (Fig. 3(A)). When the foam sample was sectioned after 4 days of culture, it was possible to visualize the formazan crystals inside the material, demonstrating that the cells were inside the pores and were viable (Fig. 3(B)). The viability assay and visual analysis demonstrated that, with both methods used to plate the cells, the presence of osteoblasts inside the foam were similar. The optical density measurement of the solubilized formazan crystals showed that osteoblast viability was not altered in the presence of the foams (Fig. 4). The same results were observed at day 8 and day 12 (data not shown). The collagen secretion was around 5% higher in the presence of the glass foams when compared to control (Fig. 5(A)) and the bioceramic foam did not interfere significantly with alkaline phosphatase production (Fig. 5(B)).

Osteoblast proliferation in the surface of the glass and inside the porous foam was also investigated by SEM analysis. The images showed the perfect adhesion of osteoblasts to foam surface outside and inside the pores. Adhesion structures resembling tight junctions were present. We also observed the osteoblasts elongations and their interconnection forming a cell net. It was also possible to observe the extrusion, by osteoblasts,

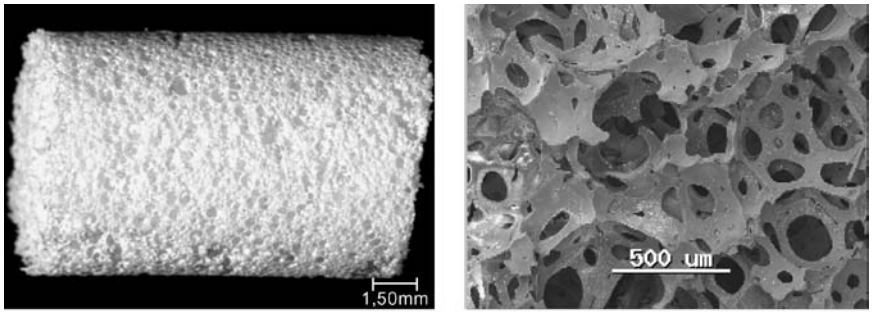


Figure 1 Optical and SEM micrographs of a representative foam. (A) Macroscopic aspect. (B) Microscopic aspect. The adequate pore size and high interconnection between pores can be observed.

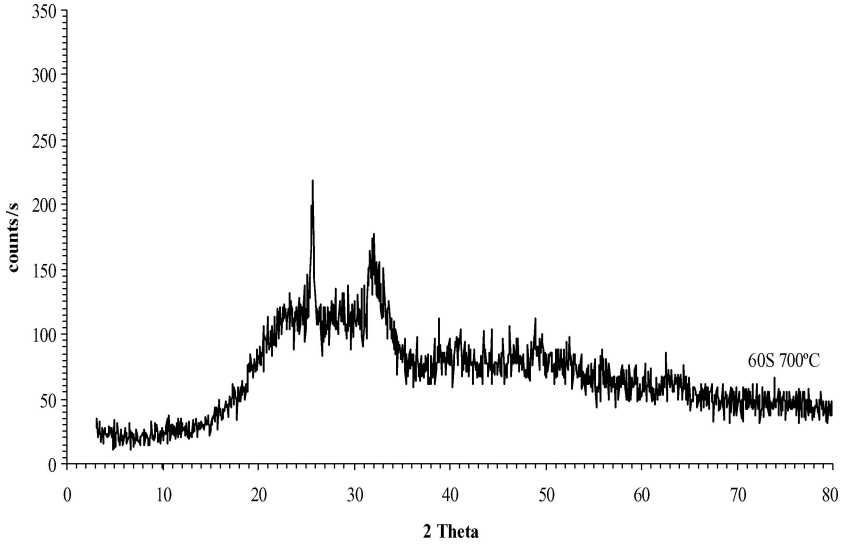
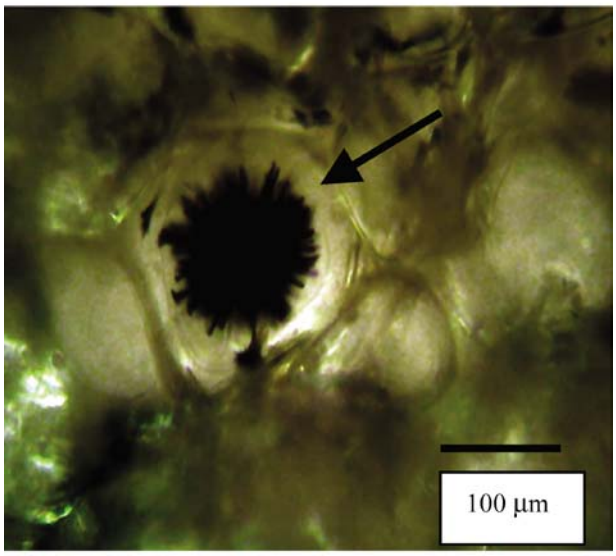


Figure 2 XRD spectrum of the glass foams.

(A)



(B)

Figure 3 Formazan crystals visualization. 1×10^5 /ml osteoblasts were plated in the presence of BG60S foam and subjected to MTT assay. The formazan crystals formation by the cells was visualized by optical microscopy (photomicrograph 400×). (A) Direct visualization of the crystals in the region around the glass foam (black area) indicating that cells were viable. Osteoblasts, metabolizing MTT, are shown by arrows. (B) The sectioned sample shows formazan crystals inside the foam indicating the presence of viable cells in this site.

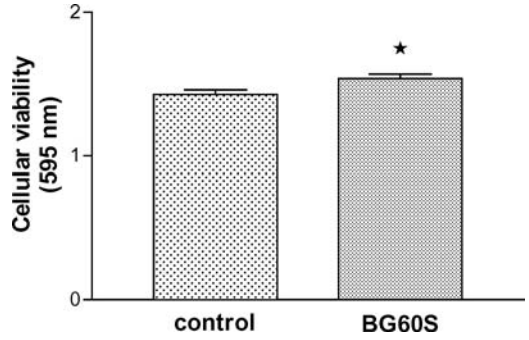


Figure 4 Osteoblast viability. Osteoblasts at 1×10^5/ml density were plated in the presence of BG60S foam. Cell viability was evaluated by MTT assay 4 days later. Optical density measurement of solubilized formazan crystals showed a slight increase in osteoblast viability in the presence of BG60S foam (*). Results represent Mean ± SD of triplicates from 4 separate experiments ($P < 0.05$).

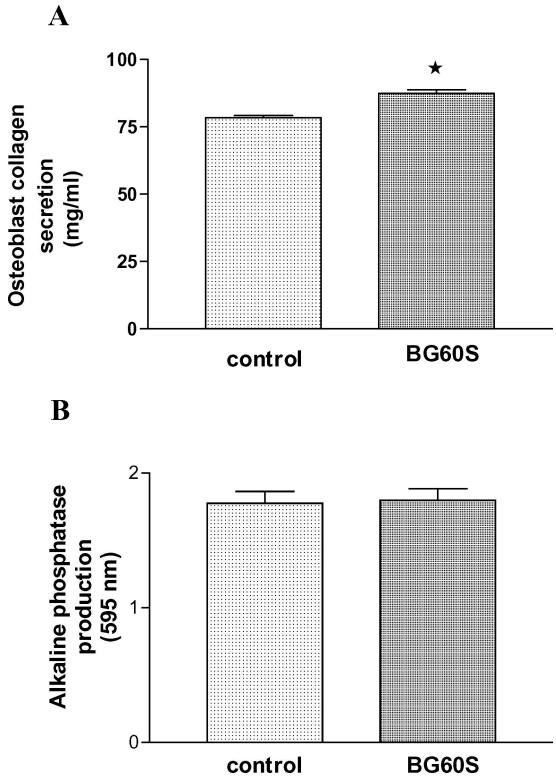


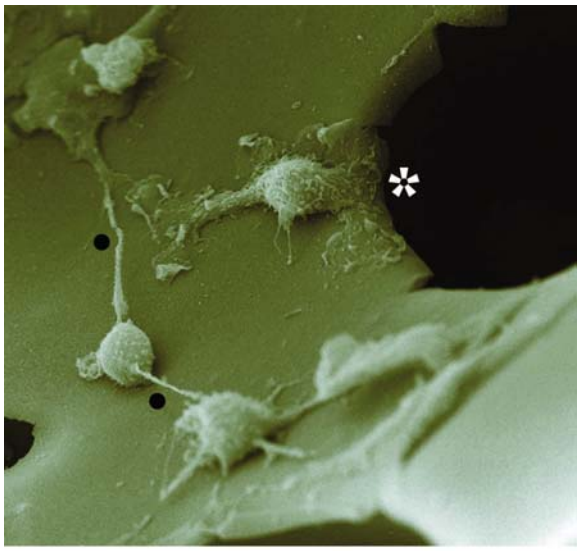
Figure 5 Collagen secretion and alkaline phosphatase production. Osteoblasts at 1×10^5/ml density were plated in the presence of BG60S foam. (A) After 4 days of incubation collagen production was evaluated by SIRCOL assay. The optical density measurement of the solubilized collagen showed higher production in the presence of BG60S (*). Result represents Mean ± SD of triplicates from 3 different experiments ($P < 0.05$). (B) After 4 days of incubation, alkaline phosphatase production was evaluated by NBT-BCIP assay. Comparing to control, there was no significant difference in this enzyme production when the osteoblasts were incubated in the presence of BG60S foam. Results represent Mean ± SD of triplicates from 4 different experiments ($P < 0.05$).

of some product suggesting secretion (Fig. 6(A) and (B)).

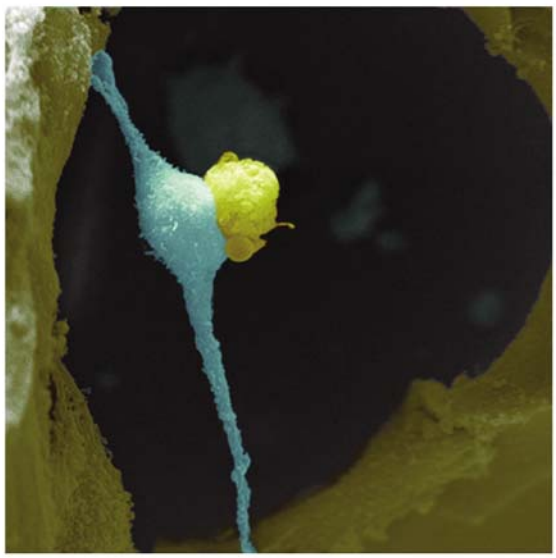
4. Discussion

Development of porous bioresorbable scaffolds of high bioactivity, for tissue engineering, has been the focus of extensive investigation [16]. The bone formation supporting capacity of any biomaterial is determined by its physico-chemical as well as surface geometrical properties [17]. However, the dissolution rate of the scaffold material may interfere, positively or negatively, with the bone tissue response. It has been demonstrated that the active ions released by the dissolution of biomaterials and their exchange with the medium provide alteration in cell proliferation and tissue repair [7, 18].

In this study, we demonstrated that BG60S scaffolds do not interfere with osteoblast viability. However, the collagen secretion by osteoblasts in the presence of BG60S foam was increased. We also demonstrated that the alkaline phosphatase production was not altered in the presence of the samples when compared to control. The results are in accord with our previous findings using the ionic products from the dissolution of BG60S powder to stimulate osteoblasts [19]. It has been demonstrated that silicilic acid is released in the medium by glass dissolution, promoting alkalosis



(A)



(B)

Figure 6 SEM images. Adhesion and interconnection of osteoblasts. (A) Large adhesion areas, suggesting tight junctions (pointed by star) and interconnections among osteoblasts (pointed by dots). (B) An adhered osteoblast in the scaffold. Osteoblast is shown extruding products.

[20, 21]. It is also known that silicilic acid enhances collagen type I production by osteoblasts and fibroblasts [22, 23]. These previous findings can be a reasonable explanation for our results.

Cell adhesion to a substrate and cell to cell attachment influence cell morphology as well as cell function, modulating proliferation and differentiation [24]. Adhesion or non-adhesion of cells to the surface of a biomaterial is one of the major factors mediating its biocompatibility [25]. The adhesion onto a scaffold surface is mediated by mechanisms involving interaction between adsorbed proteins and cell membrane receptors [26]. We demonstrated, in this study, by SEM analysis the presence of osteoblast philopodia. This result strongly suggests the presence of tight junctions and adhesion mechanisms in the scaffolds. Since osteoblasts are anchorage-dependent cells, adhesion is a prerequisite for subsequent cell functions, such as matrix secretion.

Depending on the application, we need high porosity foams suitable for cells seeding [27]. Pores were built into scaffolds to facilitate cell infiltration. Osteoblasts need a pore size of approximately 10 μm to invade the inner core of the scaffolds without consideration of the deformation and projection length, since osteoblasts sometimes deform with their expanded projections and become less likely to invade into the deeper core by adhering to the walls of pores [28]. Our microscopic studies showed that the porosity and pore interconnectivity of the BG60S scaffold were adequate for cell migration.

Another important characteristic is the osteoblast shape. It is known that cells in a rounded configuration divide at a lower rate than those flattened. Cells that attach themselves to the scaffold but spread little will show lower proliferative rates than those with greater spreading [29]. Our BG60S scaffold allowed flattening and spreading of the osteoblasts, showing adequate cell shape for proliferation and secretion functions.

5. Conclusion

The method used to foam a sol-gel solution allowed the successful production of glass foams with high, interconnected porosity in the desired size range. We concluded that the BG60S scaffold produced is a promising material for bone repair, providing a good environment for the adhesion and proliferation of osteoblasts.

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