

Borate Glass Supports the In Vitro Osteogenic Differentiation of Human Mesenchymal Stem Cells

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Bioactive ceramics have the ability to bond to surrounding bone and potentially enhance bone in-growth. Silicate based bioactive glasses and glass-ceramics, such as 45S5 bioactive glass, have been widely investigated for bone repair or as scaffolds for cell-based bone tissue engineering. Recent data have demonstrated that silica-free borate glasses also exhibit bioactive behavior and have been shown to convert to calcium phosphate at a remarkably rapid rate. Due to its relative novelty in biological applications, the cytocompatibility of borate glass is largely unknown. The objectives of this

study were to investigate the cytocompatibility of borate glass by in vitro cell culture with human mesenchymal stem cells (hMSCs) and hMSC-derived osteoblasts (hMSC-Obs). The choice of hMSCs and hMSC-Obs is based on the rationale that both cell types are preferred cell lineages attached to bone implants. Borate glass particles with diameters of 212–355 μm were loosely compacted into porous disc-shaped disks (porosity $\approx 40\%$) followed by sintering at 600°C. Partial or nearly complete conversion of the borate glass to calcium phosphate (Ca-P) material was achieved by soaking the disks for 1 day or 7 days in a 0.25 molar K_2HPO_4 solution at 37°C and at pH of 9.0. Bone marrow derived hMSCs and hMSC-Obs adhered to the pores of borate glass disks. Upon 4 week incubation of cell-seeded borate glass disks, hMSC-Obs markedly synthesized alkaline phosphatase, an early osteogenic marker. These data provide preliminary evidence of cytocompatibility of borate glass and its role in supporting the osteogenic differentiation of mesenchymal stem cells.

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1. INTRODUCTION

Certain compositions of glasses, glass-ceramics, and ceramics have been widely investigated for healing bone defects, due to their ability to enhance bone formation and to bond to surrounding tissue [1–3]. Cell-seeded bioactive ceramics are also of interest as potential scaffolds for bone tissue engineering [4–6]. Hydroxyapatite and tri-calcium phosphate ceramics, composed of the same ions as bone, are biocompatible and produce few systemic toxicity or immunological reactions. However, stoichiometric hydroxyapatite resorbs slowly or undergoes little conversion into a bonelike material after *in vivo* implantation [7–9]. In comparison to inert glass materials, bioactive glasses are osteoinductive and generally facilitate bone in-growth [6, 10]. In addition, the dissolution of bioactive glasses and conversion to calcium phosphate may affect bone cell differentiation [10]. Bone regeneration applications require gradual resorption of the implanted biomaterials and concurrent replacement of the biomaterials by host bone [6].

Bonding to bone was first demonstrated for a silicate-based bioactive glass with a typical composition of 45% SiO₂, 6% P₂O₅, 24.5% Na₂O, and 24.5% CaO (by weight), referred to as 45S5 bioactive glass [11]. Bioactive glasses based on the 45S5 composition are attractive materials because their rapid bonding to bone provides early mechanical stability, in addition to stimulating osteoprogenitor cell function, and biocompatibility [12–15]. Crystallization of the bioactive glass compositions to form glass-ceramics does not have a measurable effect on the ability to bond with bone [16]. Porous bioactive silicate glass constructs based on the 45S5 composition have been developed as possible tissue engineering scaffolds [17–18]. Glass particles, with sizes in the range of 40–70 μm, produced from a melted glass, were mixed with 2.3% CaCO₃, compacted and hot pressed at 460°C under a pressure of 50 MPa to produce disks (10 mm diameter × 2 mm thick) with 35% porosity. Cell culture experiments revealed that the porous glass can function as a template for generating mineralization *in vitro* [17].

A characteristic feature of bioactive glasses and glass-ceramics is the time-dependent modification of the surface, resulting in the formation of a calcium phosphate (Ca-P) layer that bonds with the surrounding tissue [16, 19]. The mechanisms of the Ca-P layer formation in silicate-based bioactive glasses are not well understood, but may involve the formation of a silica-rich gel on the glass surface by ion exchange reactions, followed by precipitation of calcium and phosphate ions from the surrounding fluid onto the silica-rich gel layer. Further dissolution of ions from the glass, diffusion through the silica-rich layer, and precipitation from the surrounding fluid leads to a thickening of the Ca-P layer. It has been suggested that the formation of a Ca-P layer *in vitro* is indicative of a material's bioactive potential *in vivo* [20–22].

The low chemical durability of some borate glasses has been known for decades but the potential of borate glasses in biomedical applications has not been explored until recently [23]. A

borate glass, designated 45S5B1, with the same composition as 45S5 bioactive glass but with all the SiO₂ replaced by B₂O₃, was investigated in a preliminary study by Richard [24]. A Ca-P layer was formed on borate glass surface upon immersion in a K₂HPO₄ solution at 37°C; this Ca-P layer was formed more rapidly on the borate glass than on 45S5 glass [24]. The formation of the Ca-P layer on the borate glass is believed to follow a set of dissolution-precipitation reactions similar in nature to those in the 45S5 bioactive glass system, but without the formation of a silica-rich gel layer [32]. Essentially, ions dissolve from the glass, and because Ca-P often has the lowest solubility in the system, it precipitates onto the glass surface. Further dissolution of the glass, coupled with precipitation from the surrounding fluid leads to a thickening of the Ca-P layer and the conversion of the glass to Ca-P. The ion exchange with the surrounding fluid during the conversion of the bioactive glass to Ca-P may influence cell proliferation and differentiation [23]. Because borate glass appears to undergo this conversion process at a more rapid rate than 45S5 glass, further investigation into its bioactivity must be investigated. As a first *in vivo* experiment, 45S5B1 borate glass particles (partially reacted in a K₂HPO₄ solution to produce a surface Ca-P layer) and 45S5 glass particles were separately implanted into defects (0.6–1.2 mm in diameter) in the tibia of rats [24]. Although it was sometimes difficult to distinguish between the reacted borate glass particles and the surrounding bone, histological examination after implantation for 15, 30, and 60 days indicated that the partially converted borate glass particles promoted bone in-growth more rapidly than the 45S5 glass particles. Both types of glass particles promoted sufficient bone growth for closure of the implant site after 60 days [24]. Despite this preliminary study, little is known about the fabrication of the borate glass into porous, three-dimensional constructs.

There is little ground work on the cytocompatibility of borate glass before or after its conversion to Ca-P near body temperature. Cytocompatibility usually is the first step to test whether a novel material is biocompatible. The rapid conversion of borate glass to Ca-P at near body temperature and its apparently favorable *in vivo* reaction of particles to produce bonding with bone warrant additional investigations of the value of borate glass as bone replacement materials and as scaffolds for bone tissue engineering. The objectives of this study were to fabricate novel porous, three-dimensional disks of a borate glass, and to investigate the effects of the fabricated borate glass constructs on attachment and differentiation of human mesenchymal stem cells (hMSCs) and hMSC-derived osteoblasts (hMSC-Obs).

2. MATERIALS AND METHODS

2.1. Fabrication of Porous Borate Glass Substrates

Particles of borate glass (Na₂O-CaO-B₂O₃) were prepared by melting reagent grade chemicals in a platinum crucible,

quenching the melt, and crushing the glass in a hardened steel mortar and pestle. After magnetically removing the metallic impurities, the particles were sieved through stainless steel sieves to produce sizes in the range of 212–355 μm . Porous disc-shaped disks (15 mm diameter \times 2–3 mm thickness) were produced by pouring the glass particles into vibrating graphite molds, followed by sintering for 10 min at 600°C. The structure of the porous disks was examined using X-ray diffraction and optical microscopy. The porosity was estimated from computer imaging of the optical micrographs and from density measurements.

The conversion of the porous borate glass disks to Ca-P was investigated by immersing the disks in 0.25 molar K_2HPO_4 solution with a starting pH value of 9.0 at 37°C and measuring the weight loss as a function of time. The structural characteristics of the converted material were imaged by scanning electron microscopy (SEM; Hitachi S-4700). Some glass substrates used in cell culture experiments were partially or fully converted to Ca-P to determine the most favorable condition of the borate glass for supporting cell growth and differentiation. The partially converted borate glass substrates (denoted pBG) and the fully converted substrates (denoted Ca-P) were prepared by immersing the porous constructs for 1 day and 7 days, respectively, in the K_2HPO_4 solution under the same conditions described earlier for the weight loss experiments.

2.2. Cell Culture on Porous Borate Glass Substrates

Bone marrow samples taken from healthy donors ranging from 18 to 45 years of age were purchased from AllCells (Berkeley, CA). Human bone marrow derived mesenchymal stem cells (hMSCs) were isolated using a RosetteSep kit (Stem Cell Technologies, Inc., Vancouver, BC, Canada). The hMSCs were grown in monolayer in cell culture media consisting of 89% DMEM, 10% FBS, 1% penicillin–streptomycin (basal cell culture media). After 4 days non-adherent cells were removed and the media was changed every 4 days. Cells were passaged up to four times each time upon confluency. Upon the fourth passage, 50% of the hMSCs were exposed to osteogenic-supplemented medium (basal cell culture media, 100 nM dexamethasone, 50 $\mu\text{g}/\text{mL}$ *L*-ascorbic acid-2-phosphate). Upon exposure to osteogenic supplement, hMSCs differentiated into osteoblastic cells (hMSC-Obs) [25–29], whereas the other 50% hMSCs continued incubation in DMEM complete medium without osteogenic supplement.

The hMSCs and hMSC-Obs were seeded at a density of 30,000 cells/ml by using a pipette on porous disks of the unconverted borate glass (BG), the partially converted borate glass (pBG), or the completely converted borate glass (Ca-P), and incubated for an additional 14 days. Live cell assay was then performed using Promega (Madison, WI) CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, which quantified cell viability through NADH activity using 3-(4,5-dimethyl-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). The MTS absorbance values were correlated with the number of live cells as documented in the product information sheet. Alkaline phosphatase activity (AP) was assayed by Naphthol as-biphosphate, fast red violet salt, and N,N dimethyl-formamide solution (Sigma-Aldrich Co., St. Louis, MO).

3. RESULTS AND DISCUSSION

3.1. Characteristics of Porous Borate Glass Constructs

Figure 1 shows an optical micrograph of the surface of the porous borate glass template produced by sintering. The particles are bonded at the necks between touching particles, providing enhanced strength without significant flow of the glass into the pores. Therefore, the reduction of the porosity of the disks during sintering is negligible. Computer imaging of the optical micrographs indicated that the disks had a porosity of 40–45% and a median pore size of 100–150 μm . The porosity estimated by computer imaging is in agreement with the measured density of the template relative to the density of the fully dense glass (2.58 g/cm^3). X-ray diffraction showed that the glass in the porous template remained amorphous after sintering.

The weight loss data for the porous borate glass disks during their conversion to Ca-P in K_2HPO_4 solution as a function of time are shown in Figure 2. Conversion of the glass to Ca-P, as indicated by the maximum weight loss (60–65%), is completed after approximately 6 days. As outlined earlier, the conversion of the borate glass to Ca-P is believed to involve dissolution of the glass into the surrounding fluid and precipitation of calcium and phosphate ions onto the surface of the template. Assuming

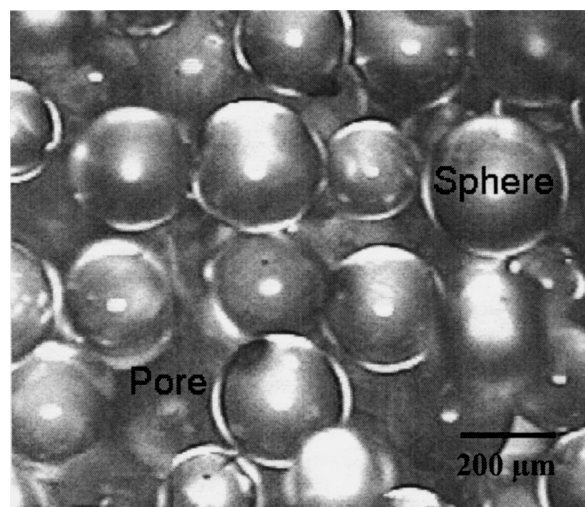


FIG. 1. Optical micrograph of the surface of a porous borate glass template produced by sintering a loosely compacted mass of particles (212–355 μm) for 10 min at 600°C.

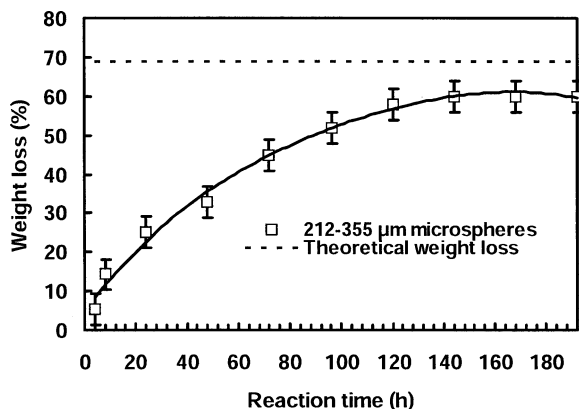


FIG. 2. Weight loss of porous borate glass disks as a function of time in 0.25 molar K_2HPO_4 solution at $37^\circ C$ and a pH value of 9.0. Conversion of the glass to a calcium phosphate (Ca-P) material in the solution is accompanied by a weight loss. The estimated theoretical weight loss is shown by the horizontal dotted line.

that all of the sodium and borate ions from the glass move into solution and all of the calcium ions go into the formation of a Ca-P material with the composition of stoichiometric hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, then the theoretical weight loss

should be 69%. The discrepancy between the maximum measured weight loss and the theoretical weight loss may be due to incomplete conversion of the glass, some calcium ions remaining in solution, the formation of a nonstoichiometric hydroxyapatite with a Ca/P ratio lower than the stoichiometric value of 1.67, or a combination of all 3 factors. Chemical analysis of the Ca-P material formed by the conversion of similar borate glasses under the same conditions indicate that Ca/P ratio is well below 1.67 [30].

The conversion of the borate glass starts at the surface and moves inward [32]. By controlling the time of reaction in the K_2HPO_4 solution, disks with different ratios of the surrounding Ca-P layer to the borate glass core can be produced. As illustrated in Figure 3, constructs reacted for 1 day consisted of an interconnected mass of composite particles with a thin surface layer of the glass, Ca-P. The thickness of the Ca-P layer, estimated from the weight loss data is 40–50 μm . Disks reacted in the K_2HPO_4 solution for 7 days are almost fully converted to Ca-P and the disks consist of an interconnected mass of Ca-P particles.

Figure 4 shows SEM micrographs of the surfaces of the three types of porous disks used in the present work in cell culture experiments. The unconverted borate glass (BG) template has

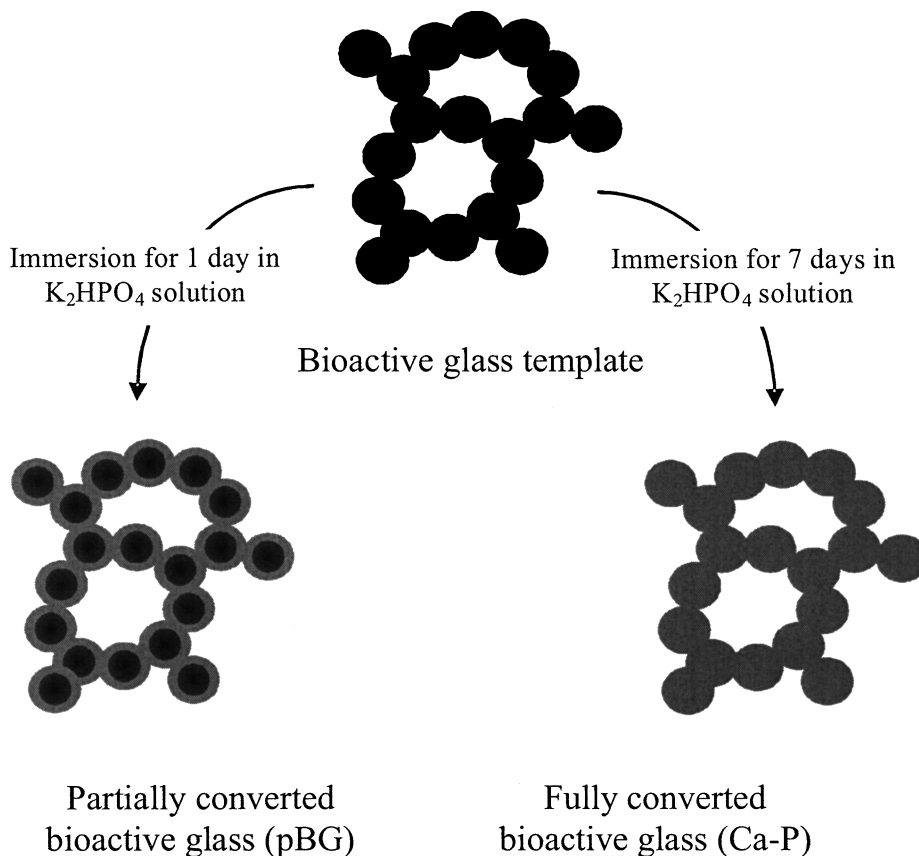


FIG. 3. Schematic diagram illustrating the controlled conversion of the borate glass (BG) to a calcium phosphate (Ca-P) material in K_2HPO_4 solution for the production of disks consisting of a partially converted borate glass (pBG) or a fully converted borate glass (Ca-P).

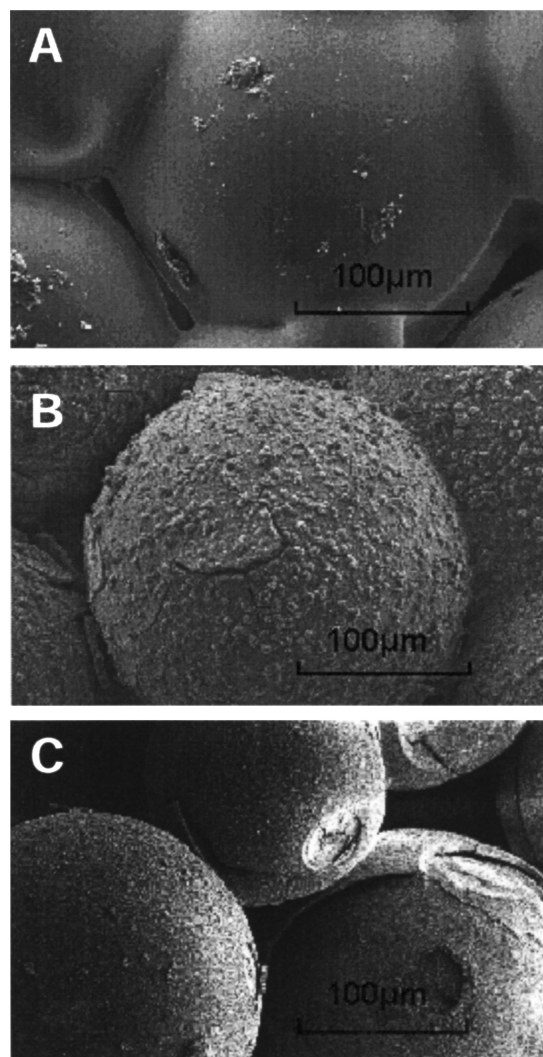


FIG. 4. SEM micrographs of the surfaces of the borate glass disks used in cell culture experiments: (A) unconverted borate glass (BG); (B) partially converted borate glass (pBG) formed by reaction for 1 day in K_2HPO_4 solution; (C) fully converted borate glass (Ca-P) formed by reaction for 7 days in K_2HPO_4 solution.

smooth surfaces characteristic of the spheroidized glass particles, whereas the constructs of the partially converted glass (pBG) and the fully converted glass (Ca-P) have less smooth surfaces. High resolution SEM, performed in related work [31], indicate that the Ca-P material is highly porous, with fine pores on the order of several tens of nanometers.

The differences in the condition of the borate glass disks may influence the interaction with cells. However, the most favorable condition of the borate glass for cellular interaction is, at present, unclear. The unconverted borate glass (BG) with its smooth surface initially may not provide favorable sites for cell attachment and significant dissolution of calcium, sodium, and borate ions will initially occur into the surrounding fluid as the glass surface reacts with the fluid. For constructs of the partially converted

glass (pBG), the porous Ca-P surfaces may provide more favorable sites for cell attachment. Dissolution of calcium, sodium, and borate ions into the surrounding fluid is still expected to occur but at a lower rate than for the unconverted glass. The fully converted constructs (Ca-P) provide surface sites similar to those of the pBG constructs, but almost no dissolution of sodium and borate ions into the surrounding fluid will occur due to the absence of any significant quantity of borate glass in the template.

3.2. Cell Culture

Whereas the unconverted borate glass disks (BG) disintegrated during cell culture experiments, presumably due to reactions of the glass with the cell culture medium, the partially converted disks (pBG) and the fully converted disks (Ca-P) remained largely intact and maintained their original shape throughout the experiments (Figure 8).

Morphological differences between the hMSCs and the hMSC-Obs were observed prior to seeding on the borate glass substrates (Figure 6A and B). The hMSCs have the typical spindle shape of mesenchymal stem cells but the hMSC-Obs appear more cuboidal, similar to the osteoblast-like cell. Alkaline Phosphatase activity staining also confirmed that cells exposed to the osteogenic supplements in the media were differentiated along the osteogenic pathway (Figure 6C and D).

Live cell number (MTS) assayed after 14 days verified the cell viability of both hMSCs and hMSC-Obs cultured on the pBG and Ca-P disks. The hMSCs seeded on the pBG disks had significantly higher cell viability than hMSCs seeded on the Ca-P disks (Figure 7). The data show a similar trend for hMSC-Obs seeded on the pBG and Ca-P disks but the difference is not significant due to the wider variability of the data for Ca-P disks. The higher cell viability of the hMSC on the pBG substrates may indicate that pBG stimulates cell function. As outlined earlier, a key difference between the pBG and Ca-P substrates is the potential for dissolution of calcium, sodium, and borate ions from the underlying borate glass core of the pBG disks into the culture medium. The mechanism by which these ions may influence cell function is not clear at present but may be important for determining the optimum condition of the borate glass disks for tissue engineering applications. For cells seeded on the pBG substrates, the data in Figure 7 also indicates that the osteogenic cells have significantly higher cell viability than hMSCs. The hMSCs seeded on pBG disks had a higher metabolic activity and cell viability than on Ca-P disks. For pBG disks, hMSC-Obs had higher cell viability than hMSCs.

Active alkaline phosphatase was produced by the cells within the borate glass disks as indicated by the dark red stain (Figure 8). Higher alkaline phosphatase activity was seen in hMSC-Ob samples (Figure 8C and F) as compared to the undifferentiated, hMSCs (Figure 8B and E). This indicates that a combination of osteogenic supplements with a bioactive borate glass substrate will have a positive effect on osteogenic differentiation. Staining

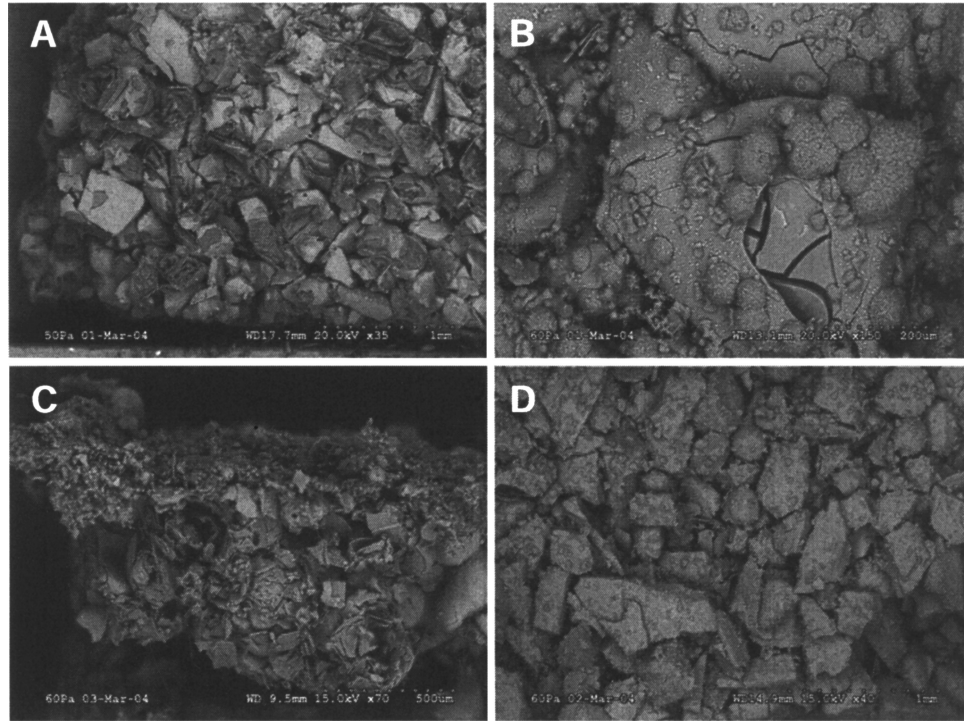


FIG. 5. SEM images of (A, B) partially converted borate glass and (C, D) fully converted borate glass immersed in culture media.

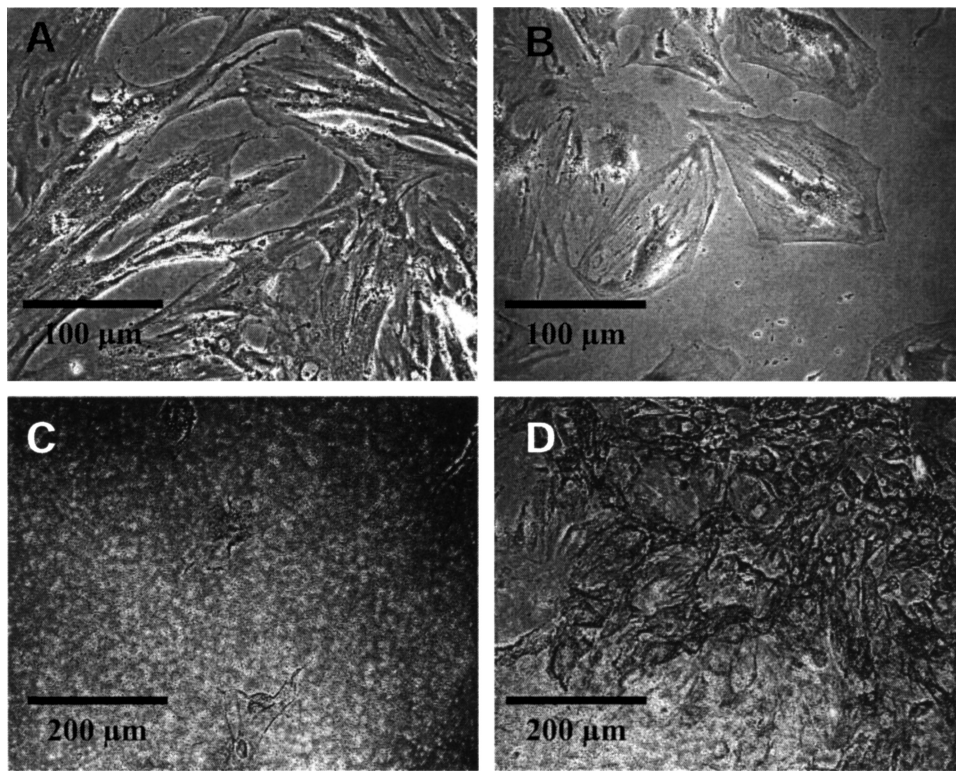


FIG. 6. Photomicrographs of (A) human bone marrow derived mesenchymal stem cells (hMSCs), (B) hMSC-derived osteoblasts (hMSC-Obs), (C) alkaline phosphatase stain micrographs of hMSCs, and (D) hMSC-Obs in culture prior to seeding on borate glass disks.

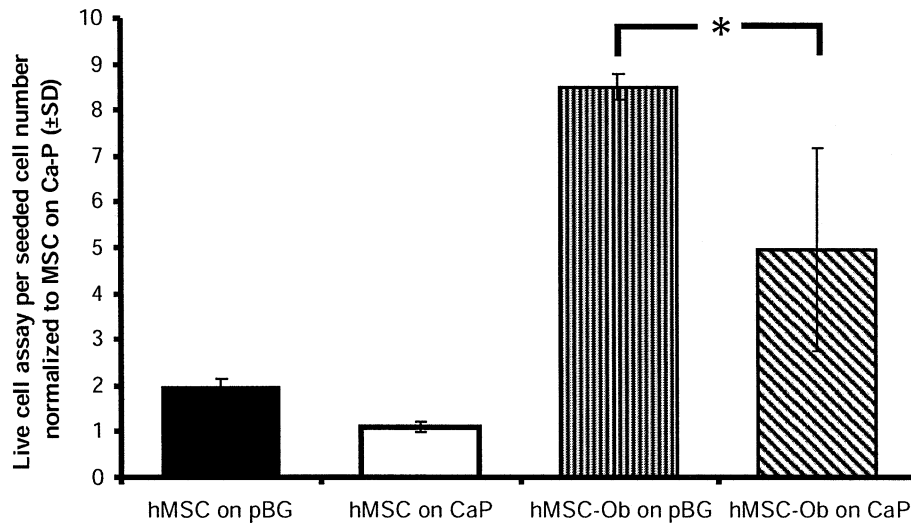


FIG. 7. Live cell assay using light absorbance as a metabolic indicator of hMSCs and hMSC-Obs on partially converted borate glass (pBG) and Ca-P substrates, n = 4, * = Students' *t*-test $p < 0.05$.

for alkaline phosphatase activity was also performed on both partially converted (pBG) and fully converted (Ca-P) borate glass samples following culture (Figure 8A and D), which did not stain red.

4. SUMMARY AND CONCLUSIONS

The present data provide initial experimental evidence of successful fabrication of borate glass into porous 3D disks, and its cytocompatibility of both human mesenchymal stem cells and their osteogenic progeny. The cytocompatibility of porous borate glass disks consisting of the unconverted glass (BG), the partially converted glass (pBG; with a thin surface layer converted to Ca-P), and the fully converted glass (Ca-P), was investigated

by in vitro cell culture with human mesenchymal stem cells (hMSCs) and hMSC derived osteoblasts (hMSC-Obs). Both cell lineages have survived up to two weeks of seeding on porous borate glass disks, suggesting that porous borate glass provides a suitable environment for cell attachment and proliferation. Previous work with silicate bioactive glass suggests the conversion to Ca-P may affect differentiation and proliferation of MSCs, and the more rapid conversion as observed with borate glass materials may produce more favorable results. In the present work, hMSCs (9,000 cells/construct) were initially seeded at a higher density than the hMSC-Obs (3,000 cells/construct) due to the fact that hMSCs proliferated more rapidly than hMSC-Obs during in vitro culture prior to seeding. The MTS absorbance

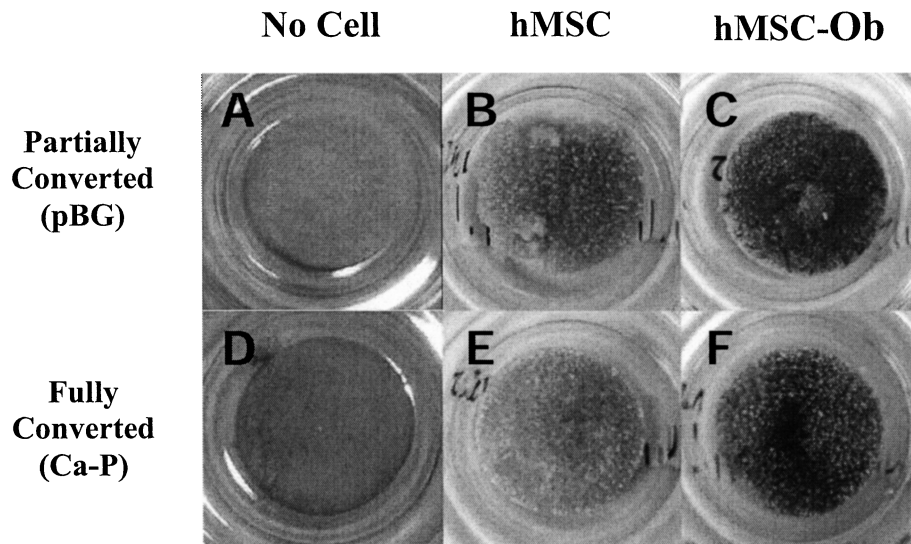


FIG. 8. Photographs of porous borate glass disks without seeded cells and with seeded human mesenchymal stem cells (hMSCs) or hMSC-derived osteoblasts following staining for alkaline phosphatase activity. A darker red indicates higher activity. Top row (A-C): partially converted borate glass (pBG); bottom row (D-F) fully converted borate glass. A and D: acellular controls, B and E: human MSCs, C and F: osteogenic human MSCs.

normalized by per seeded cell number to hMSCs grown on Ca-P indicates that both hMSCs and hMSC-Obs maintained their viability on partially converted borate glass disks. Future studies will investigate apoptosis and proliferation of MSCs and their differentiated osteoblasts on the borate glass substrates.

The present demonstration of osteogenic differentiation of human mesenchymal stem cells as evidenced by alkaline phosphatase activity on the pBG and Ca-P disks suggests that porous borate glass disks are capable of promoting the expression of this early osteogenic marker. Also, these data demonstrate the necessity of additional in vitro and in vivo investigations of the potential of bioactive borate glass as a cell-accommodating scaffold for bone tissue engineering. In particular, the pBG construct, consisting of a network of borate glass particle surrounded by a Ca-P layer, had the highest cell viability for both cell types and may represent a more favorable condition of the borate glass for bone tissue engineering. Additional qualitative and quantitative experiments should be performed, such as SEM, EDXA, and FTIR, to further ascertain the capability of porous borate glass disks in promoting both in vitro and in vivo osteogenesis. Nonetheless, the present data provide the rationale for cyto-compatibility and capacity of osteogenic promotion of human mesenchymal stem cells by porous borate glass.

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