Influence of calcium and phosphorus release from bioactive glasses on viability and differentiation of dental pulp stem cells

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Abstract

The release of ions that can significantly contribute towards cellular response is an important characteristic of bioactive glasses (BG). Here, ionic extracts of three different compositions of BG powders in the 60 mol% SiO_2, x mol% CaO (x= 28, 32 and 36) , x mol% P_2O_5 (x = 12, 8 and 4) compositional system were utilised to study their effect on the viability, differentiation and mineralization of dental pulp stem cells (DPSC) *in vitro*. ICP was applied to detect the exact ionic concentrations released from the different composition of BGs. DPSCs treated with conditioned media from the glass with 4 mol% P_2O_5 (BGCM1, media containing 44.01 ± 0.6 mg/L Si, 61.72 ± 0.1 mg/L Ca and 7.57 ± 0.01 mg/L P) were more metabolically active compared to conditioned media from the glass with 8 mol% P_2O_5 (BGCM2, media with 47.36 ± 0.7 mg/L Si, 57.4 ± 0.1 mg/L Ca and 14.54 ± 0.2 mg/L P), at all times tested, but in all cases the process was slower than the control. Cells exposed to media conditioned by the glass with 12 mol% P_2O_5 (BGCM3, 40.46 ± 0.5 mg/L Si, 61. 85 ± 0.3 mg/L Ca and 28.43 ± 0.3 mg/L P) responded differently, such that cells showed to be more metabolically active than control at day 3, but then similar to or lower than control at higher time points. Differentiation of DPSCs towards osteogenic lineage in the presence of BGCM was assessed by Alizarin red staining. Cells treated with high phosphate BGCM3 displayed a higher density of red mineralized nodules than cells treated with BGCM1 and BGCM2 after 21 days of culture in non-osteogenic medium. BGCM3 was therefore chosen for gene expression studies. Osteogenic differentiation of DPSCs in the presence and/or absence of BGCM3 or osteogenic supplements were studied by real time RT-PCR. Overall, the results demonstrated that, in the absence of osteogenic supplements, BGCM3 group showed a significantly higher mRNA expression levels for alkaline phosphatase at day 7, osteopontin and osteonectin at days 7 and 14, and a high level of collagen I at day 14, compared to negative control group (BM-). Overall the results obtained from BGCM3 group are beneficial for the design and manufacture of scaffolds or particulates with tailored ion release for a range of bone repair applications.

Keywords: Bioactive glasses, bone tissue engineering, dental pulp stem cells, osteogenic differentiation

1. Introduction

Bioglass was Larry Hench's seminal discovery and bioactive glasses (BGs) (1-3) are used as synthetic bone grafts for bone regeneration. They have proven to be advantageous over other bioactive ceramics due to their strong bonding with the host bone, biodegradation (4-6) and because they stimulate more rapid bone repair in comparative studies(1-7). This is because they bond with bone but also stimulate cell activity due to the release of soluble silica and calcium ions (7-9). The latter is Larry Hench's third key discovery. His second was that bioactive glasses can be made by sol-gel derived route. The SiO_2–CaO–P_2O_5 system is one of the most extensively studied for bioactive sol–gel glasses (10). Previous *in vitro* utilizing bone cells, whether primary or cell-lines, have shown that the cells grow well with enhanced production of bone matrix on or in the presence of BGs (11-13). Numerous studies have also evaluated BGs for *in vivo* applications and in almost all cases BG was found to successfully lead to the regeneration of bone defect (7, 14-19).

It is well accepted that bone induction by BGs may occur through direct contact between substrate and cells as well as through soluble ions released by these materials during their degradation, which have shown to stimulate bone repair and regeneration (19, 20). A full understanding of the effect of BG ionic dissolution products on cellular behavior *in vitro* (21, 22) would enable fabrication of advanced scaffolds with tailored ion release that would be advantageous for tissue engineering applications. Calcium and phosphorus are the main components in bone tissue, so it is believed that the release of these ions from BG would contribute in new bone formation. It is well documented that phosphate plays a key role in extracellular matrix (ECM) mineralisation and also contributes to calcium deposition *in vitro* (23, 24). Silicon is known to be important in calcification and formation of extracellular matrix and also it has been shown that soluble silica contributes to osteoblast activity (9, 25). In addition, in previous studies high levels of Si contents were detected in early stages of bone matrix calcification (26, 27). The great sum of results on the biological performances of BGs presented in the literature (28) confirms the hypothesis that the ionic dissolution products stimulate the genes of cells towards a path of regeneration and self-repair (29).

However, regardless of the osteogenic ability of BGs, the mode of action of their dissolution products on different types of stem cells has not been fully determined and has recently evoked interest. Many studies have shown that when cells, mostly osteosarcoma cell line (30, 31) or

osteoblasts (9, 32, 33), are exposed to BG ionic dissolution products, with no direct contact to the BG surface, osteogenic differentiation is stimulated (28, 32, 34, 35). From literature, very limited data exists on the response of mesenchymal stem cells (MSCs) to BG ionic dissolution products. The 45S5 (32, 33, 36) or 58S (5, 6, 37) compositions have been more extensively evaluated.

To date, little information is available concerning the effects of BGs on the osteogenic differentiation of MSCs *in vitro* (11, 38-40). In a study by Reilly *et al.*, no significant difference in levels of alkaline phosphatase activity was seen between human MSCs grown in the presence of 45S5 BG or tissue culture plastic, hence they reported that positive effect of BG on bone growth is not correlated with the accelerated differentiation of MSC. Labbaf *et al.* (40) evaluated the osteogenic differentiation potential of 85 mol% SiO_2-15 mol% CaO BG nanoparticles following uptake by adipose and bone marrow MSCs and found that BG particles did not stimulate nor inhibited the differentiation process in the presence and/or absence of osteogenic supplements. This could be due to the low calcium and zero phosphate content. When mouse embryonic stem cells were cultured in osteogenic media (containing dexamethasone and β-glycerophosphate) conditioned with 58S (60 mol% SiO_2, 36 mol% CaO, 4 mol% P_2O_5) sol–gel glass dissolution products, the number of mineralised bone nodules increased as the concentration of dissolution products increased, in a dose-dependent manner, but there was little evidence for stem cell differentiation without the supplements (35). With changes in BG composition and consequently ion release levels, and also cell type, the results are also bound to be different (33, 35, 37, 41). Therefore, further investigations utilizing various cell sources and glass compositions are required. Here, SiO_2-P_2O_5-CaO ternary BGs with different compositions, prepared by the sol-gel method, were utilised to study their effect on viability, differentiation and mineralization of DPSCs *in vitro*. DPSCs have the potent capacity of self-renewing and multi-lineage differentiation and have also shown good osteogenic potential (42). DPSCs are an attractive cell source for tissue regeneration applications because of their relative ease of isolation and expansion from dental pulp of molars and their differentiation potential. They can become chondroblastic, neuronal, endothelial odontoblast and osteoblast cells (43-46). DPSCs are well established and extensively characterized, demonstrating osteoblastic potential both *in vitro* and *in vivo*, highlighting a certain similarity with bone marrow-derived mesenchymal stromal cells (47). It is strongly believed that the current study will have great contribution towards the use of optimised BG composition for a range of bone tissue applications.

2. Materials and Methods

2.1 Powder synthesis

Bioactive glass powders were prepared through sol-gel processing route, as previously described by Jones *et al* (36), in the 60 mol% SiO_2, x mol% CaO (x= 28, 32 and 36) , x mol% P_2O_5 (x = 12, 8 and 4) (Table 1), based on 58S composition (60 mol% SiO_2, 36 mol% CaO and 4 mol% P_2O_5) (10). All reagents were purchased from Sigma-Aldrich (Dorset, UK). Sol was made by hydrolysis of tetraethyl orthosilicate (TEOS). The TEOS was added to DI water (molar ratio of water to TEOS (R) was 12) and 2 N nitric acid (ratio of 2N nitric acid to deionised water was 1:6 in a screw top 1 L PTFE jar under continuous stirring. After 1 h, triethyl phosphate (TEP) was added. After 1 h, calcium nitrate tetrahydrate was added. The jar was sealed and left at room temperature for 1 h before aging at 60^{o}C for 72 h. The screw top was then loosened for drying with a three stage heating schedule (dwell for 20 h at 60^{o}C; heating to 90^{o}C at 0.1^{o}C/min, holding for 24 h; heating to 130^{o}C at 0.1^{o}C/min and holding for 40 h). After drying, the dried gel was transferred into an alumina crucible and stabilisation carried out at 700^{o}C following a three stage heating schedule: 1 ^{o}C/min to 100 ^{o}C; 0.5 ^{o}C/min to 300 ^{o}C; 2 h dwell; 1 ^{o}C/min to 700 ^{o}C holding for 5 h. The glass was ground using a planetary ball mill (FRITSCH Planetary Micro Mill PULVERISETTE 7 premium line). Two weight balanced grinding cups were placed in the mounts, each cup containing 9 alumina grinding balls (15 mm in diameter) and glass granules. A sieve shaker was used to separate the powders smaller than 38 μm from the resulting powder.

2.2 Preparation of BG conditioned medium

Bioactive glass conditioned medium (BGCM) containing ionic dissolution products of BG were prepared as described by Bielby *et al.* (41): $1.5x10^{-3}$ g/mL BG particulates (<38 μm in size) were UV sterilized for 2 h and then incubated for 24 h at 37˚C in Dulbecco's Modified Eagle Medium (DMEM-HG, GibcoBRL, NY, USA) supplemented with 1% penicillin-streptomycin (GibcoBRL, NY, USA). Following 24 h incubation, particulates were removed through centrifugation followed by filtration through a 0.22 μm filter (Millipore, Billerica, MA). Filtered extracts were then used for cell studies as BGCM1, BGCM2, BGCM3 (Table 1).

2.3 Powder characterization

Scanning Electron Microscopy (SEM, LEO 435 VP, UK) was applied to visualize the morphology of BG particulates before and after conditioning in DMEM. For this purpose, powders were spread on carbon tape and coated by gold sputtering. To study the surface reactions of BG and possible formation of hydroxyl-carbonated apatite, infrared spectroscopy was applied using a FTIR (Bruker Tensore 27) in the range between 4000 and 400 cm^{-1} for powders before and after preconditioning. Filtered extracts of BGs were analysed using inductive coupled plasma optical emission spectroscopy (ICP-OES, Thermo iCAP 6300). Samples were diluted with 9 ml of 2 M nitric acid. If concentration levels were outside of the 0-40 µg ml^{-1} then samples were further diluted at a ratio of 1:9 with 2 M nitric acid.

2.4 Isolation and cell culture of hDPSC

Intact human impacted third molars with immature roots were collected from healthy patients (3 donors aged 28 years) in the Dental Clinics at Isfahan Hospital, under the approved guidelines by the Ethical committee of Royan institute (48). All experiments were conducted after patients had signed informed consent forms. The pulp was separated from the crowns and roots, minced into small pieces, and digested in 3 mg/mL collagenase type I (Sigma-Aldrich). The cell suspensions were collected and seeded onto a culture plate containing DMEM with 5% fetal bovine serum (Gibco, USA), 1% penicillin (Gibco, USA), and 1 % streptomycin (Gibco, USA) at 37°C in 5% CO_2. The cells were detached with 0.25% trypsin-EDTA (Gibco, USA) and were expanded when 70%–80% confluency was reached (49, 50). The cells from the same passage (passage 3) were used in the same experiment.

2.5 Cytotoxicity and cell viability

The alamarBlue® cytotoxicity/proliferation assay (ThermoFisher Scientific, UK) was performed according to the manufacturer's protocol to determine whether BGCM were cytotoxic. For this purpose, all BGCMs were supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, and 1% non-essential amino acid. The hDPSCs were seeded in a 24 well-plate at a seeding density of 5,000 cells/cm^2 and incubated for 24 h to allow cell attachment. Following cell attachment, hDPSCs were cultured in the presence of basal cell culture medium (BM) or BGCM 1, 2 and 3 for a period of 1, 3, 5, 7 and 14 days. At the end of each time point, medium was removed and 500 µl of Alamar blue solution (1:10 culture medium: Alamar blue

reagent) was added to each well and incubated for 2 h. Optical density was measured at 570 nm using the microplate spectrophotometer multi-well plate reader (Bio-Rad, USA). The results represent the mean values ± SD of two individual experiments each in quadruplicate.

2.6 DAPI staining

The hDPCs were seeded at 12,000 cells per well in a 24-well plate and incubated in the presence of BM and BGCM 1, 2 and 3 for 24 h. Following 24 h, cells were fixed with 4% (w/v) paraformaldehyde in phosphate buffer solution (PBS) at 37 °C for 1 h and then washed with PBS. 500 µL of DAPI (4',6-diamidino-2-phenylindole, Sigma, USA) solution (1:10 DAPI:PBS) was added to each well and incubated for 5 min, and then viewed with Olympus fluorescent microscope (Optica microscopy XDS-3FL4, Italy) to check for possible apoptotic cells.

2.7 Matrix mineralization

The hDPCs were seeded at 5,000 cells/cm^2 in a 24-well plate and cultured in the presence of BM, BGCM 1, 2, 3 for up to 21 days and the medium was changed every three days. At the end of 21 days of culture, cells were washed with PBS and fixed with methanol for 10 min at room temperature. 500 µl of the prepared alizarin red solution (2 g of alizarin red dissolved in 100 ml distilled water with pH adjusted to 4.1-4.3 with 0.1% NH_4OH) was added to each well for 20 min. The dye was then drained and the cells were washed extensively with distilled water and viewed under a light microscope (Olympus CKX41SF, Philippines).

2.8 Real time RT-PCR analysis

For this part of the study, only BGCM3 was selected, based on results of the Alizarin red staining. For RT-PCR, cells were seeded at 5,000 cells/cm^2 in 6-well plates and cultured with basal medium (BM) with and without osteogenic supplements (C+ and C-) or with BGCM3 with and without osteogenic supplements (BGCM3+ and BGCM3-) for 2, 4, 7, 14 and 21 days. Osteogenic supplements were 10 mmol/L β-glycerophosphate,100 nmol/L dexamethasone and 50µg/ml ascorbic acid. Total RNA was extracted from cultured cells by the RNeasy Mini Kit (Qiagen) and samples were treated with DNaseI (Fermentas) to remove contaminating genomic DNA. cDNA synthesis was carried out using a random hexamer and the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas). Real time (SYBR Green, TAKaRa) PCR was performed in a thermal cycler Rotor gene 6000 (Corbett) as noted by the protocol (TaKaRa). The

PCR mixture contained 10 µl Rotor-Gene SYBR Green PCR Master Mix (TaKaRa). The thermal cycling conditions were as follows, 40 cycles of 95°C for 10 s and 60°C for 30 s and 70°C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for the quantification of gene relative expression. The expressions of the differentiation and mineralization markers in the hDPCs, namely, alkaline phosphatase (ALP), collagen type I (Col I), osteopontin (OPN), and osteonectin (ON), were monitored. All measurements were performed in duplicate. Real time RT-PCR specific primer pairs were designated by the Beacon designer (Version7.2, USA) as obtained from Metabion Company (Germany). The forward and reverse primers used for real time RT-PCR were designed according to the complementary DNA sequences available in GenBank (Table 2). The experiment was triplicated and the fold change was defined as the relative expression compared with the value of the control group (C-) on day 2. Real time RT-PCR data were assessed and reported according to the ΔΔCt method.

2.9 Qualitative analysis of extracellular matrix production

The hDPCs were seeded at a density of 5,000 cells/cm^2 in a 12-well plate and were cultured in the presence of BM and BGCM3 for 28 days. The medium was changed every three days. After 28 days in culture, cells were fixed with 2.5% gluteraldehyde (Sigma, UK) in PBS for 40 min at 4 °C as previously described (51). Cells were dehydrated through a series of increasing concentration of ethanol (0, 25, 50, 75 and 100%). Samples were sputter coated with gold and viewed using scanning electron microscope (SEM, Philips XL30) using an accelerating voltage of 5 kV.

2.10 Statistical analysis

The data are expressed as the mean values and standard deviation (SD) of two individual experiments each performed in triplicate. Differences between groups were determined by student's t test with values of $p<0.05$ considered significant. For the RT-PCR analysis the data are expressed as the mean values of three experiments (each treatment in duplicate) and the RT-PCR was run three times for each sample. Differences between groups were determined by SPSS, one-way analysis of variance(ANOVA) with $p < 0.05$ considered significant.

3.0 Results and Discussion

One major aim of the current study was to determine an ionic concentration of Si, Ca and P that would stimulate osteogenic differentiation of DPSC. The initial Si, Ca and P concentrations in the DMEM following incubation with BG particles were detected using ICP (Fig. 1). Ca release occurred due to ion exchange of cations from the glass (from Si-OCa non bridging bonds) with H^+ from the DMEM. Dissolution of the silica network (breaking Si-O-Si bonds) then occurs. Ca and P can combine in solution and deposit onto silanol bonds on the glass surface, nucleating a hydroxycarbonate apatite layer (4, 36). As this layer forms, Ca and P content in solution drops. Among the three groups studied, the Ca concentrations of 61.72 ± 0.1mg/L, P 7.57 ± 0.1 mg/L and Si of 44.01 ± 0.6 mg/L were detected for BG1, producing BGCM1. The lowest Ca (57.4 ± 0.1 mg/L) and highest Si (46.63 ± 0.7 mg/L) with P of 14.5 ± 0.2 mg/L concentration were observed for BG2 group (BGCM2). For BGCM3, Ca (61.85 ± 0.3 mg/L) was similar to BG1, P (28.43 ± 0.3 mg/L) was highest and Si (40.46 ± 0.5 mg/L) the lowest ionic concentration. The release of ionic products from all groups confirmed dissolution of BGs within 24 h of incubation in DMEM.

The Si level in BGCM3 was found to be lower than other glasses since calcium acts a silica network modifier and it can affect the silica network formation during glass synthesis and BG3 had the lowest amount of calcium (BG3) and therefore the most connective silica network.

The P level of the medium was found to decrease following 24 h of immersion for both BGCM1 and BGCM2 due to migration of phosphate to BG surface. The phosphate combines with some Ca ions from the glass and from the media to nucleate calcium phosphate layer deposition (4). The phosphorous level in media exposed to BGCM3 had a similar P level to the control media because phosphate was released from the glass, replenishing that lost due to deposition. Si was also detected in all three groups, implying that silica network breakup had occurred following incubation.

Calcium-phosphate deposition on the surface of glass particles was detected by FTIR. FTIR spectra of all glass compositions showed significant changes after incubation in DMEM in comparison to the spectrum of the unreacted glass (Fig. 2). The bands for untreated glass powders were typically showing Si-O vibrational modes: Si-O stretch, Si-O bend (52, 53), with the non-bridging oxygen (NBO Si-O) band at 920 cm^{-1} disappearing. In the as-synthesised

glasses, twin P-O bend bands were present in BG1 and BG2 spectra at 560 and 600 cm^{-1} but the spectrum of BG3 showed a single P-O bending band. The twin P-O bands are related to apatite or other calcium orthophosphates, including octacalcium phosphate. This orthophosphate phase formed during synthesis. In BG3, instead a single P-O bend band at 560–600 cm^{-1}indicates amorphous calcium phosphate. After immersion in DMEM for 24 h, all glasses showed a single, broad P-O bending band at 570–600 cm^{-1}, taken as an indication of the presence of either amorphous calcium phosphates or calcium orthophosphates (including apatite) of poor crystallinity, suggesting that the process of apatite formation had started. The calcium phosphate deposition on the glasses is the reason that BGCM1 and BGCM2 had enhanced Ca and Si content but lower P compared to the control DMEM. Fig. 3 shows the changes in morphology and surface texture of BGs after 24 h incubation in DMEM. The surface of BG particulates appeared rough with the appearance of nucleation on the surface; further confirming degradation and surface reaction of BGs.

In vitro, the differentiation of hDPSCs into osteogenic lineage and thus bone formation is a steady process by a sequential expression of genes and is categorized by three principal periods: proliferation, extracellular matrix production and maturation and mineralization (42, 54), with peak mRNA levels defining the transition between periods. BGs show a burst release of ions following incubation in media; hence the study on the effects of their dissolution products on cellular behavior is crucial. The changes in the nuclear morphology of the cells following 24 h incubation with BG ionic dissolution products were examined using DNA-specific fluorochrome DAPI (Fig. 4). DAPI staining was also used to study apoptotic cell (fragmentation of cell nuclei). Chromatin condensation and nuclear fragmentation were the criteria used to demonstrate apoptosis. Based on the images presented in Fig.4, the nucleus morphology for both control and treated cells remained intact with no detectable or visible changes.

Cell viability and metabolic activity of DPSCs were determined using alamarBlue® (Fig. 5). Cells were cultured with BM, BGCM-1, 2 and 3 for 1, 3, 5, 7 and 14 days. Time points over 14 days were not considered since the cultures had reached confluence. At days 3 and 5 the metabolic activity of BGCM 1 was found to be significantly lower than the control group. At days 1, 3 and 5 BGCM2 cell's metabolic activity were found to be significantly lower than control group. The metabolic activity and growth of DPSCs was affected by BGCM3 at day 7

only which suggests that cells treated with this group had entered a differentiation phase (34, 42, 47). Interestingly, at day 3, cells treated with BGCM2 and 3 had a significantly lower metabolic activity than BGCM3 suggesting that the high level of P in BGCM3, which is almost three time higher than BGCM1 and two times higher than BGCM2, may have positively affected cell behavior in particular their metabolic activity, but this requires in depth study to further confirm the role of P. Overall, the cells appeared to be more metabolically active in the group treated with BGCM1 than for BGCM2 at all times tested, but in all cases the activity was seen to be slower than the control group. This phenomenon may be caused by the difference in ionic concentrations of Ca, P and Si in BGCM, which may have an inhibitory effect on cell proliferation (9, 38, 39). The differences in the ionic levels of the conditioned medium may directly or indirectly have an effect on cellular behavior and hence different compositions of BGs may show different effects on cellular behavior. Bielby *et al.* (35, 41) found that dissolution products of 58S increased the differentiation of mouse embryonic stem cells in a dose-dependent manner, but there was little evidence for stem cell differentiation without the supplements. BGCM 3 produced a higher level of P in the conditioned medium and provoked different biological effects compared to BGCM1 and 2. Recently, the addition of P (310 mg/L) to culture medium was found to stimulate expression of matrix Gla protein (MGA), an important regulator for bone formation, in osteoblastic cells (55, 56). Interestingly, the slow cell metabolic activity at days 5 and 7 observed in BGCM3 group may greatly suggest that P at the given concentration, in conjunction with the Ca and Si levels, plays a crucial role in osteogenic differentiation of DPSCs since cell metabolic activity level and hence proliferation rate of cells remain constant or reduce when cells enter differentiation phase (4, 34, 36, 57). To further study this observation, differentiation of DPSCs toward osteogenic lineage was assessed by Alizarin red staining and gene expression for differentiation-related genes.

Fig. 6 shows the effect of dissolution products in the absence of osteogenic supplements on DPSC differentiation, as assessed by Alizarin red staining. Cells treated with BGCM3 displayed a higher density (mineralized area per field) of red mineralized nodules than cells treated with BGCM1 and BGCM2. On day 21, no red staining was observed in the control group, low and moderate positive red nodules staining were observed in BGCM1 and BGCM2 groups, respectively. This could again suggest that phosphate may have a greater role than calcium in

stem cell differentiation behavior (56). Therefore, based on this finding the group treated with BGCM3 was used for further study using RT-PCR.

The effect of BGCM3 with and without osteogenic supplements on hDPSC differentiation was assessed by the transcription profile of a panel of osteoblast markers using RT-PCR. Collagen I, ALP, osteonectin and osteopontin markers were studied. For this purpose, mRNA expression for cells treated in four different conditioned media of BM only (BM-), BM plus osteogenic supplement (BM+), BGCM in the presence (BGCM+) and absence (BGCM-) of osteogenic supplements at various time points of 2, 4, 7, 14 and 21 d. Generally, in cultures that demonstrate collagen-based apatite formation, collagen I is expressed during the early stages of differentiation and before mineral deposition, followed closely or concurrently with ALP (36, 58). ALP is a marker for detection of early osteogenic differentiation of cells (59, 60) and is also an ectoenzyme that plays a role in the degradation of pyrophosphate to release phosphate for mineralization (61).

Here, hDPSCs cultured in BGCM3+ medium expressed a higher level of ALP compared to other groups up to 4 d, after which ALP and Collagen I level dropped, suggesting the onset of extracellular matrix deposition and mineralisation. It must be noted that both ALP and Collagen I are normally the first major phenotypical markers of osteoblasts and their expression declines with the progression of differentiation (36). hDPSCs cultured with BGCM3- expressed a significantly higher level of ALP at 7 d compared to other groups. This result correlates well with Fig. 4, which presented a significantly lower metabolic activity compared to control group, confirming that hDPSCs had entered differentiation phase. Generally, directed differentiation of stem cells into osteogenic lineage is often enhanced by a combination of osteogenic supplements such as ascorbic acid, β-glycerophosphate, and dexamethasone that are widely used (62). β-glycerophosphate enhances mineralization by the osteoblasts formed, and dexamethasone (at a concentration of 10 nM) enhances proliferation and differentiation of the concerned cells (62, 63). β-glycerophosphate is promptly hydrolyzed by alkaline phosphatase that leads to a high level of phosphate ions, providing an environment suitable for mineral deposition (63). It is suggested that phosphate greatly contributes towards deposition and mineralization of extracellular matrix but the exact concentration of phosphate is also considered important since a high level can lead to dystrophic calcification, as opposed to the desired bone-like mineralization

(55-57, 64, 65). Here, the phosphate from the glass has taken the role of the phosphate usually supplied by β-glycerophosphate, as BGCM3- expressed a high level of ALP and Collagen I up to 7 d followed by a drop (36). This also explains the lower gene expression level of osteonectin and osteopontin for BGCM3+ at all times (64, 66). At the beginning of matrix mineralization, genes for proteins such as osteonectin and osteopontin are expressed and once mineralization is completed, calcium deposition can be visualized using alizarin red, as shown in Fig. 6.

Osteopontin is a recognized osteogenic marker that regulates the formation of mineral nodules and leads to osteogenesis. Hence, osteopontin expression levels are often correlated with the mineralization ability of the cells (32, 63, 67). Interestingly BGCM3- group showed a significantly higher mRNA expression level for both osteonectin and osteopontin at 7, 14 and 21 days compared to other groups, indicating bone matrix mineralization. ALP and collagen I are typically the first major phenotypical markers of osteoblasts and their expression declines with the progression of differentiation (32, 36). The expression levels of BGCM3+ group did not behave as predicted at the time points where differentiation was likely to occur when compared to BGCM3- group. The high concentration of P in the medium from both BGCM3 and β-glycerophosphate in BGCM3+ group may have led to dystrophic calcification. Overall, based on the RT-PCR data, BGCM group in the presence of osteogenic supplement did not show a positive effect in the long term, only at day 4 ALP and Col were up-regulated but for the late markers this effect was not seen to be beneficial. Also, in the absence of osteogenic supplements, alkaline phosphatase production was affected by the presence of BGCM, indicating accelerated osteogenic differentiation.

Osteogenic differentiation and subsequent bone formation is a gradual and well-organised process associated with the three-stage paradigm of proliferation, extracellular matrix production and maturation and matrix mineralization (68-70). Collagen I is expressed during the initial period of proliferation and ECM biosynthesis and this phenomena correlates well with the RT-PCR results obtained in this study. RT-PCR results demonstrated that ALP activity, an early marker of osteoblastic differentiation and collagen I are significantly higher in BGCM3-. at day 7 (71). The expression of osteopontin and osteonectin occurs later during the third period of matrix mineralization. In particular, expression of osteopontin indicates the initial growth of hydroxyapatite crystals and their ability to bind and potentially catalyse mineralization process

(68, 72-75) and the high expression of osteonectin gene suggests the formation of a bone-associated protein (68, 76). This is also observed in the SEM images in Fig. 8, suggesting that the release of ions from BGs have the potential to stimulate differentiation of DPSC *in vitro*.

Fig. 8 represents SEM images of DPSCs cultured in BM and BGCM3- following 28 days of culture. Images show matrix production that appears to cover the surface and around the cells treated with BGCM3-. This finding further verifies the above observations.

Conclusion

Overall BGCM 1, 2 and 3 had no cytotoxic effect on hDPSC's and they appeared to initiate mineralization. From Alizarin red staining BGCM3 group showed a great amount of deposition compared to the other groups. Further analysis using RT-PCR for the osteogenic differentiation of hDPSC showed to be positively effected by BGCM3 in the absence of supplements.

Compliance with Ethical Standards:

The authors declare that they have no conflict of interest.

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Figure captions:

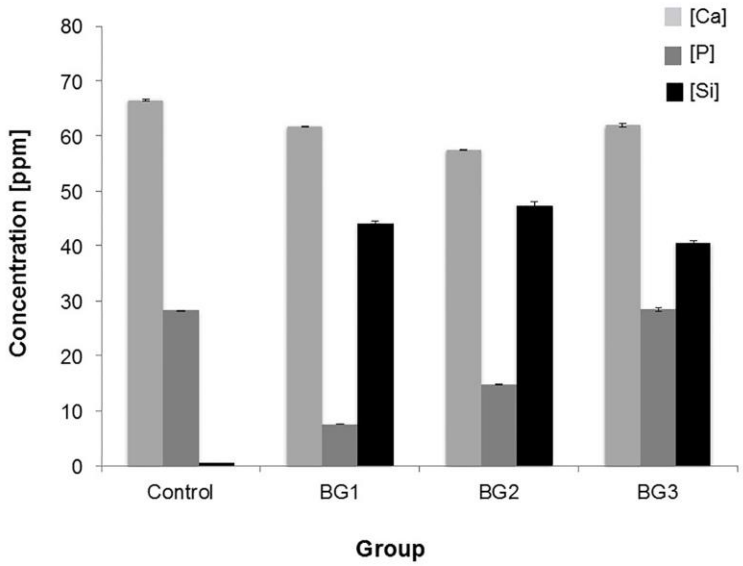

Fig. 1. Elemental concentrations of the culture medium (DMEM) following 24 h incubation with BG1 60 mol% SiO_2 36 mol% CaO 4 mol% P_2O_5, BG2 60 mol% SiO_2 32 mol% CaO 8 mol% P_2O_5 and BG3 60 mol% SiO_2 28 mol% CaO 12 mol% P_2O_5, measured in triplicate by ICP.

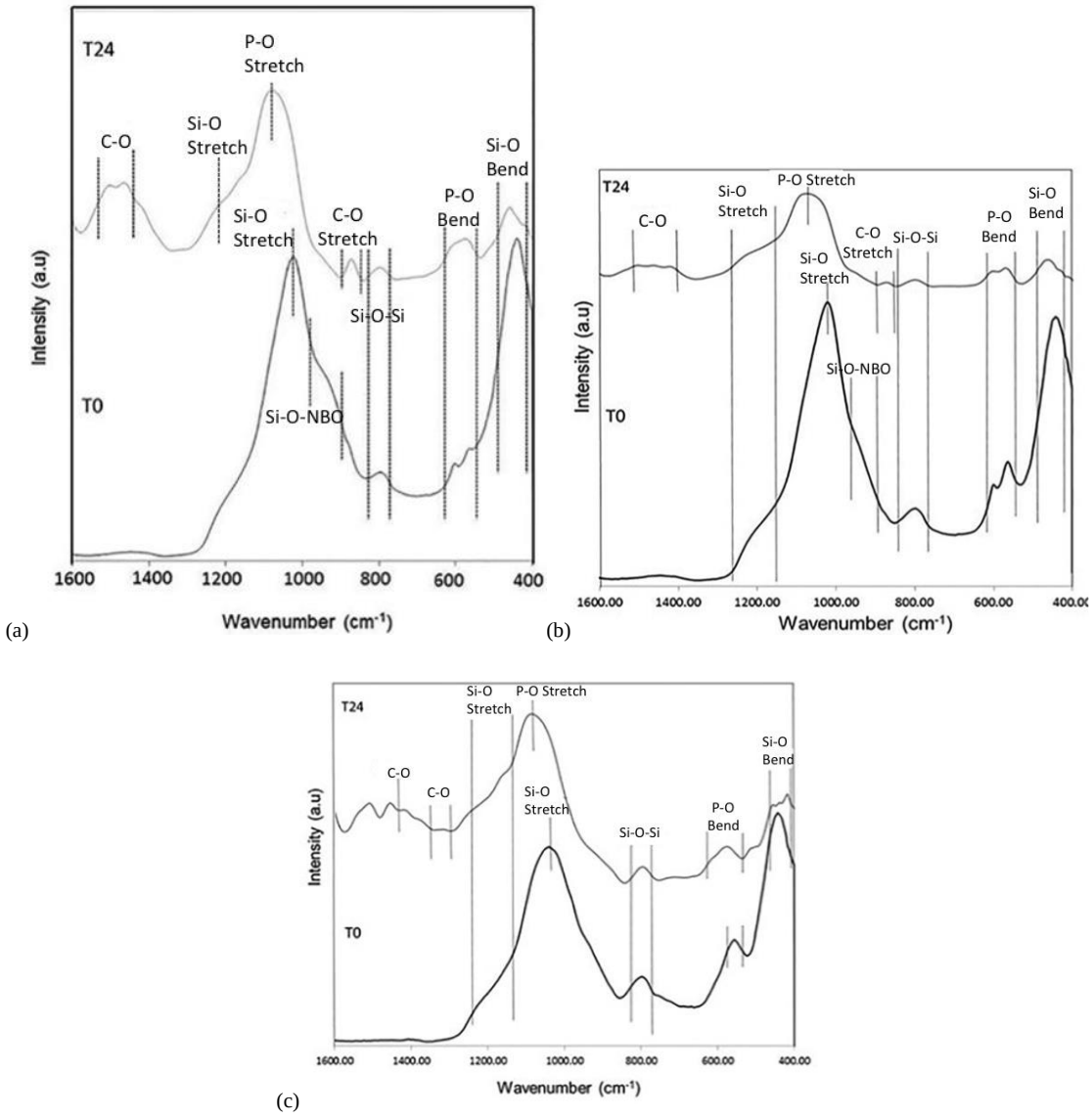

Fig. 2: FTIR spectra of a) BG1, b) BG2 and c) BG3 before (T0) and after (T24) immersion in DMEM for 24 h.

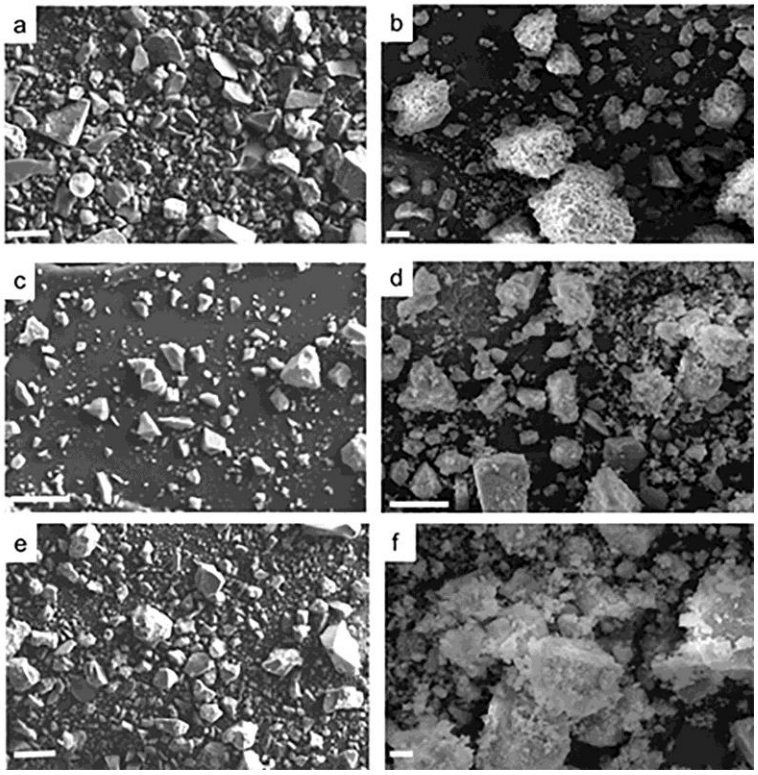

Fig. 3. SEM micrographs of a,b) BG1, c,d) BG2 and e,f) BG3 before (a,c,e) and after (b, d, f) 24 h incubation in DMEM. Scale bar 50 μm.

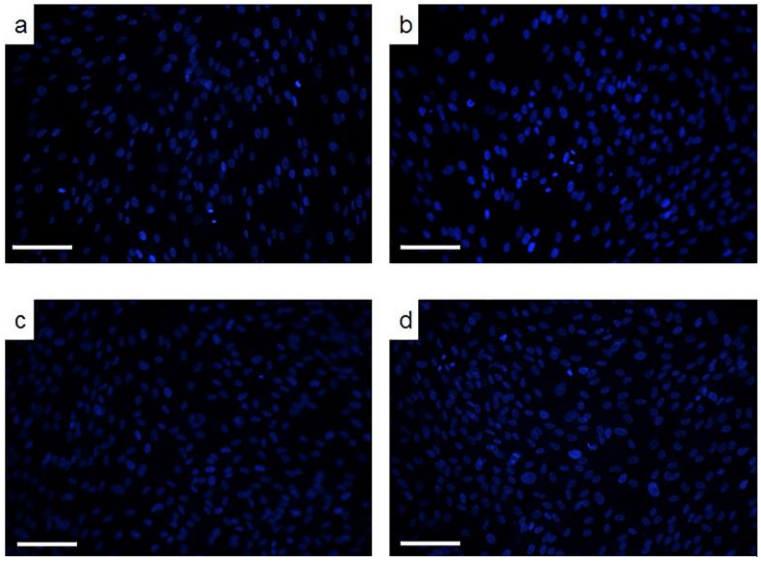

Fig. 4: Fluorescence images of DAPI stained DPSCs cultured in the presence of a) BM b) BGCM1 c) BGCM2 d) BGCM3 following 24 h incubation. Scale bar 100 μm.

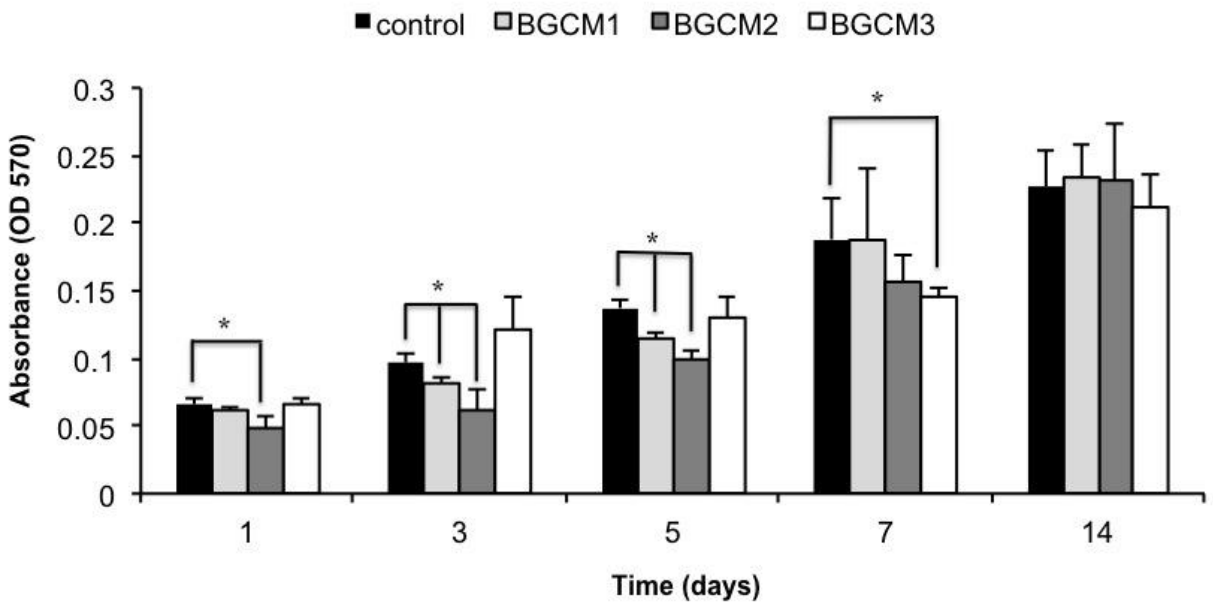

Fig. 5. Cytotoxicity assays. Cell viability was monitored using Alamar blue. All assays were performed following cell treatment with conditioned medium following 1, 3, 5 and 7 and 14 days post seeding. Values represent the mean SD of two individual experiments each performed in triplicate (*) indicates the statistical significant difference (*$p < 0.05$ relative to control).

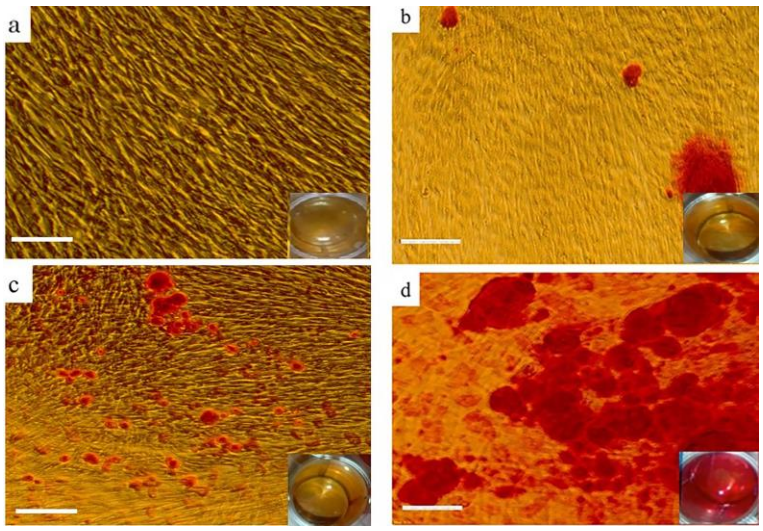

Fig. 6: Mineralization assay of hDPCs cultured in BG extractions. Alizarin S staining of mineralized nodules of hDPCs cultured in a) BM b) BGCM 1, c) BGCM 2 and D) BGCM 3 on day 21, in the absence of osteogenic supplements. Scale bar 100 µm.

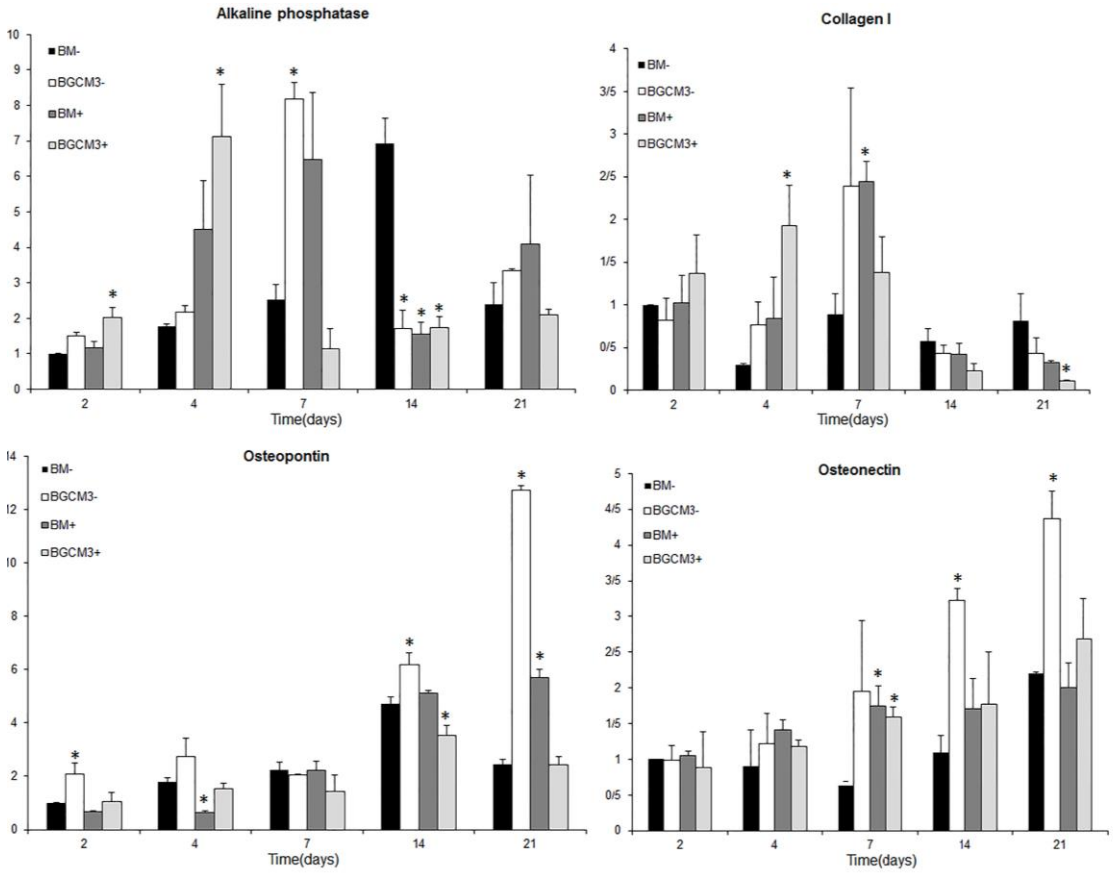

Fig. 7: Gene expression profile of DPSC cultured in the presence of BM with (BM+) and without (BM-) osteogenic supplements and BGCM3 only, and BGCM3 with supplement (BGCM+). Data are mRNA levels of the genes of interest and are represented as fold difference relative to BM day 2 and have been normalised to the expression of the housekeeping gene GAPDH. The data are the mean values of 3 experiments (each treatment in duplicate) and the real time PCR was run three times for each sample (*$p < 0.05$ relative to cells cultured with BM).

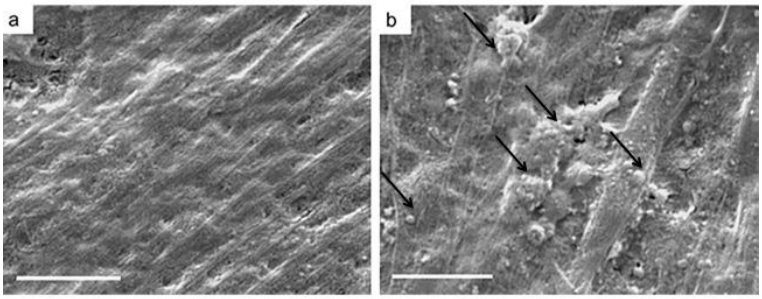

Fig. 8: SEM micrographs of DPSCs following 28 days of culture in a) BM and b) BGCM3 in the absence of osteogenic supplements. Arrows show areas of matrix formation. Scale bar 50 µm.

Table captions

Table 1: Bioactive glass compositions.

Bioactive Glass	SiO_2 (mol%)	P_2O_5 (mol%)	CaO (mol%)
BG1	60	4	36
BG2	60	8	32
BG3	60	12	28

Table 2: Primers used for real time RT-PCR.

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	F:CCACTCCTCCACCTTTGACG	R :CCACCACCCTGTTGCTGTAG
Collagen type I	F: GTGGTGACAAGGGTGAGACAG	R: CAACAGGACCAGCATCACCAG
Alkaline phosphatase	F: CTGATGTGGAGTATGAGAGT	R :AGTGGGAGTGCTTGTATC
Osteonectin	F: GCAAAGGGAAGTAACAGA CAC	R:GAAAGGTAAAGGAGGAAA TGG
Osteopontin	F: GCC GAG GTG ATA GTG TGG TT	R: TGA GGT GAT GTC CTC GTC TG