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# Substituted Borosilicate Glasses with Improved Osteogenic Capacity for Bone Tissue Engineering

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**Abstract**

Borosilicate bioactive glasses (BBGs) have shown capacity to improve the new bone formation when compared to silicate bioactive glasses. Herein, we assessed the capacity of BBGs to induce the osteogenic differentiation of bone marrow mesenchymal stem cells (BM-MSCs), as a function of their substituted divalent cations ($Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$). To this purpose, we synthesized BBG particles by melt quench. The cell viability, proliferation and morphology, (i.e. PrestoBlue®, PicroGreen®, and DAPI and Phalloidin stainings, respectively) as well as protein expression (of ALP, osteopontin and osteocalcin) of BM-MSCs in contact with the BBGs were evaluated for 21 days. We observed an enhanced expression of bone-specific proteins (ALP, OP and OC) and high mineralization of BM-MSCs under BBG-Mg and BBG–Sr conditioned osteogenic media for concentrations of 20 and 50 mg/ml with low cytotoxic effects. Moreover, BBG-Sr at a concentration of 50 mg/ml was able to increase the mineralization and expression of bone-specific proteins even under basal media conditions. These results indicated that the proposed BBGs improved the osteogenic differentiation of BM-MSCs. Therefore, showing their potential as relevant biomaterials for bone tissue regeneration, not only by bonding to bone tissue, but also by stimulating new bone formation.

**Key words:** Borosilicate glasses, strontium, mineralization, osteogenic induction, BM-MSCs

## 1. Introduction

The properties of bioactive glasses (BGs) support their key relevance of in clinical applications associated to bone tissue repair and regeneration.(1, 2) They are part of a tissue engineering-based strategy that can overcome the drawbacks of the traditionally used autologous bone grafts (e.g. lack of adequate amount and quality of bone, donor site morbidity). BGs are considered relevant for bone tissue repair since they: (i) promote osteointegration (forming a bone-like hydroxyapatite (HA) layer on their surface); (ii) are biocompatible; and (iii) their degradation shows positive biological effects after implantation.(2-5) There are however drawbacks associated with conventional BGs including in vitro cytotoxicity related to the release of $Na^+$ ions, and interest in modified compositions has increased in recent years.(6)

Recently, borosilicate bioactive glasses (BBGs) have attracted interest in bone tissue engineering.(1, 7, 8) BBGs have shown capability to improve the new bone formation when compared to silicate-based BGs.(9, 10) They present controllable degradation rates and have a high compositional flexibility that potentially allows BBGs to be tailored with enhanced osteogenic and angiogenic properties, as well as with antibacterial capacity.(11, 12) On one hand, as shown by Huang et al., a glass network composed of borosilicate's have more controllable conversion rates to HA.(13) This has also been demonstrated to occur in vivo.(14) As a matter of fact, the addition of borate to the glass network can also be beneficial for bone healing, as well as formation, and maintenance of new bone, while supporting cell osteogenic differentiation.(15) Frequently, it has been associated with the increase in bone resistance to fracture.(16, 17) On the other hand, by exploiting the compositional flexibility of BBGs, inorganic divalent cations, such as $Mg^{2+}$, $Ca^{2+}$ and $Sr^{2+}$ can be incorporated and play a key role in bone metabolism. For instance, $Mg^{2+}$ increases bone formation rate as well as stimulates

bone cell adhesion increasing their stability.(18, 19) The $Ca^{2+}$ is known to be essential during the apatite formation process, being also favorable to osteoblast proliferation, differentiation and the mineralization of the extracellular matrix (ECM).(20) $Sr^{2+}$ also has bone therapeutic potential. Different studies evidenced its beneficial effects on bone cells and bone formation in vivo,(21, 22) being even used for the treatment of osteoporosis.(23)

BGs are known to be osteoinductive materials, capable of stimulating the function and osteogenic differentiation of bone and stem cells without any additional supplementation. Findings from Fu et al. suggested that the borosilicate 13-93B1 scaffolds supported the proliferation and function of osteogenic Murine Osteocyte-like Cell Line MLO-A5,(15) while Gentleman et al. demonstrated that $Sr^{2+}$-substituted bioactive glasses stimulated osteoblast metabolic activity promoting cell proliferation and ALP activity.(24) More significantly, Santocildes et al. demonstrated that Sr-containing BGs appeared to be capable of promoting osteoblastic differentiation in a proportion of bone marrow mesenchymal stem cells (BM-MSCs) that were in some way pre-committed to this lineage.(25) Liang et al. showed that borate glasses support the attachment and differentiation of human bone marrow derived mesenchymal stem cells and human mesenchymal stem cell derived osteoblasts.(26)

The present study aims to fabricate three substituted BBGs to be used in bone tissue repair and regeneration field. The incorporation of different divalent cations (i.e. $Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$) into the BBGs was tested due to the fact that the release of these cations might induce specific effects on BM-MSCs, such as: in their proliferation, differentiation and the mineralization of the ECM, as well as stimulating bone cells and promoting bone formation.(11) BM-MSCs are a cell type of especial interest for bone tissue research and therapy because of their ease and reproducibility of isolation as well

as their ability to differentiate into mesodermal lineage cells such as osteoblasts, osteoclasts and osteocytes.(27) Therefore, we aimed to investigate the effects of BBGs biological activity over viability, proliferation, and differentiation of BM-MSCs, providing novel biocompatible BBGs that promote osteointegration and cell differentiation for bone tissue engineering.

## 2. Experimental

### 2.1. Preparation of BBGs

The BBGs of general formula $0.05Na_2O \cdot xMgO \cdot yCaO \cdot (0.35\text{-}x\text{-}y)SrO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$ (molar ratio, where x, y = 0.35 or 0.00, and $x \neq y$) were synthesized by melt-quenching. The suitable amounts of, silica ($SiO_2$, Macherey-Nagel, Germany), boron oxide ($B_2O_3$, Alfa Aesar, Germany), sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, Australia), and magnesium oxide (MgO, Sigma-Aldrich, Portugal), or calcium carbonate ($CaCO_3$, Sigma-Aldrich, Portugal), or strontium carbonate ($SrCO_3$, Sigma-Aldrich, Portugal) were thoroughly mixed with the addition of ethanol in a porcelain pestle with the help of a mortar, vacuum dried overnight and transferred to a platinum crucible. After entirely dried, each batch was heated to 1450 ˚C in air for 1 h and subsequently the melt was quickly poured into cold water to form the glass frit. Afterwards, the as-quenched glasses were ground in an Agate mortar (RETSCH, Germany) and sieved to a particle size <63 μm. Before the in vitro tests BBG-Mg ($0.05Na_2O \cdot 0.35MgO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$), BBG-Ca ($0.05Na_2O \cdot 0.35CaO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$) or BBG-Sr ($0.05Na_2O \cdot 0.35SrO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$) were weighted, dried and sterilized at 160 ºC for at least 2 h.

### 2.2. Morphology and chemical composition of BBGs

The morphology of the synthesized BBGs was observed by scanning electron microscopy (SEM, model S360, Leica Cambridge, UK) equipped with energy dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of the surface chemical composition.

### 2.3. Isolation and expansion of mesenchymal stem cells

Bone marrow mesenchymal stem cells (BM-MSCs) were isolated from bone marrow of 4-5 week-old male Wistar rats according to the method established by Maniatopoulos et al. (28) and recently proposed by Santocildes et al..(25) BM-MSCs were expanded in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK), supplemented with 100 U/ml penicillin (Sigma,-Aldrich, UK) and 1 mg/ml streptomycin (Sigma-Aldrich, UK). Cells were cultured at 37 ºC in an atmosphere of 5% $CO_2$.

Prior to the in vitro studies, BM-MSCs, at passage 2, were harvested and seeded into 24 well plates, at a density of $2\times10^4$ cells per well. Cells were cultured in the presence of the BBGs at concentrations of 20 and 50 mg/ml, for 7, 14 and 21 days under static conditions. The BM-MSCs cultured in the absence of BBGs were used as negative control and in the presence of 45S5 bioglass® as positive control. The BBGs at the desired concentrations and 45S5 bioglass® were deposited on top of the cells, in cell culture inserts with porous membranes (0.4 µm ThinCerts™ Cell Culture Inserts; Greiner, Germany), as used in previous works (29). The ThinCerts™ were used as an inert platform to support the glass particles under cell culture conditions. All conditions were cultured in basal and osteogenic differentiation media (basal medium supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and $10^{-8}$ M dexamethasone). Figure 1 presents a schematic of the experimental design.

**Figure 1.** Schematic of the experimental design.

### 2.4. Potential cytotoxic effect of BBGs dissolution on BM-MSCs

**Cell viability and proliferation (PrestoBlue® and PicoGreen® assays).** The PrestoBlue® reagent (Fisher Scientific, UK) is a resazurin-based solution that is reduced to resorufin by viable cells which can be detected fluorimetrically. The cell viability assay was executed according to the manufacturer's instructions. In brief, the PrestoBlue® reagent was added to a final concentration of 10% to the wells and the cells were incubated for 1 h at 37 $^{o}$C. Afterwards, 200 µl samples of the culture medium were removed and placed in 96-well plates and the resorufin fluorescence was quantified spectrophotometrically using a plate reader (Tecan Infinite M200). The fluorescence was determined at an excitation wavelength of 560 nm and emission wavelength of 590 nm. The metabolic activity was presented in fluorescence values and compared with the control (cell cultured in the absence of glass particles under basal medium conditions).

The PicoGreen® dsDNA reagent (Invitrogen, USA) is an ultrasensitive fluorescent nucleic acid dye for quantification of double-stranded DNA (dsDNA) in solution. This assay enables the measurement of cell proliferation. After each culturing period, the cell monolayers were washed with PBS and then incubated at 37 ºC for 3 h followed by a freezing step at -80 ºC for at least over night in ultra-pure water (1 ml) to ensure cell lysis. The assay was performed according to the manufacturer's protocol. And the fluorescence was determined at an excitation wavelength of 485 nm and emission wavelength of 528 nm. The DNA concentration was presented in µg/ml and compared with the control (cell cultured in the absence of glass particles under basal medium conditions).

**Cell morphology and distribution**. After each culturing period the cell grown in tissue culture coverslips were washed with PBS and fixed with 4% formalin solution (0.5 ml) for 15 min at room temperature (RT). The cell layers were then washed with PBS, containing 0.2% Triton X, for 2 min. After the fixation and permeation steps, the cell monolayers were washed again with PBS and stained with 4,6-diamidino-2-phenyindole dilactate (1:1000 DAPI, Sigma, UK) for 2 min at RT, and phalloidin-tetramethylrhodamine B isothiocyanate (Sigma, UK) for 1 h at RT. Finally, the cells were washed and observed using an Axioplan 2 imaging fluorescent microscope with a digital camera QIC AM 12-bit (Zeiss, UK).

## 2.5. Osteogenic capacity of BBGs on BM-MSCs

**Alkaline phosphatase quantification**. The concentration of alkaline phosphatase (ALP) was determined for all the culture time periods, using the lysates used for DNA quantification. Briefly, the ALP quantity was assessed using the Alkaline Phosphatase, Diethanolamine Detection kit (Sigma-Aldrich, UK) in which p-nitrophenyl phosphatase (pNPP) solution is hydrolyzed by ALP to yellow free p-nitrophenol. In brief, a buffered pNPP solution was prepared and equilibrated at 37 ºC. Afterwards, 2% (v/v) of sample or control were added. Immediately after mixing the absorbance was read at 405 nm in a plate reader (Tecan Infinite M200) for ≈ 5 min. An ALP standard solution was used as control and buffer as blank. The units were calculated according to the following equation: $\frac{(\Delta A_{405nm}/\min\ Test - \Delta A_{405nm}/\min\ Blank)\times df \times V_F}{18.5 \times V_E}$. Where df = dilution factor; $V_F$ = Volume of final solution; 18.5 = millimolar extinction coefficient of pNPP at 405 nm and $V_E$ = Volume of samples/ALP standard solution. ALP activity was calculated by normalizing ALP concentration per DNA concentration for each condition and time point.

**Alizarin red staining.** After 21 days culture, the cells grown in tissue culture coverslips were fixed in 70% ice-cold methanol at -20 °C at least for 30 min. The cell layers were then washed with PBS and dried overnight. Afterwards, cells were stained with alizarin red solution for 10 min [342 mg of alizarin red, (Sigma-Aldrich, UK) in 25 ml of distilled water and the pH was adjusted to 4.1 with 10% ammonium hydroxide (Sigma-Aldrich, UK)] for 10 min. The coverslips were then, washed in distilled water, dehydrated in acetone/xylene (Sigma-Aldrich, UK) solution and mounted using an aqueous mountant. The stained constructs were observed under an optical microscope (BX51, Olympus Corporation, UK) and images captured by a digital camera (DP70, Olympus Corporation, UK). The BM-MSCs morphology and mineral deposition was

also observed using SEM (model S360, Leica Cambridge, England) equipped with energy dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of the surface chemical composition.

**Immunodetection of bone-specific proteins.** Osteopontin (OP) and osteocalcin (OC) protein expression of BM-MSCs was assessed by immunoassay technique to evaluate their osteoblastic differentiation. The procedures were executed according to the manufacturer's instructions. The concentrations of OP and OC were determined for all the culture time periods, using the lysates used for DNA quantification. The OP quantitative determination was performed using Mouse/Rat Osteopontin Quantikine ELISA Kit (R&D Systems, UK). In brief, 50 µl of assay diluent RD1W and 50 µl of standard (2500 to 39 pg/ml), control and samples were added and the plate incubated for 2 h at RT. After 4 washing steps and perfectly dried, 100 µl of Mouse/Rat OP Conjugated were added and incubated for 2 h at RT. The sandwich complex was washed 4 times and allowed to react with 100 µl of substrate solution before adding 100 µl of stop solution. Finally, the optical density was determined at 450 nm and the concentration of OP obtained from a standard curve plot. OC quantitative determination was performed by the use of Rat Gla-Osteocalcin High Sensitive EIA kit (Takara Clontech, Japan). In brief, 100 µl of samples and standard solution (16 to 0.25 ng/ml) were incubated for 1 h at 37 ºC with the capture-antibody, rat osteocalcin C-terminus-specific antibody. After OC capture and 3 washing steps, 100 µl of the enzyme-labelled antibody (GlaOC4-30) specific to Gla-OC was incubated for 1 h at RT. The sandwich complex was washed 4 times and allowed to react with 100 µl of substrate solution for 10-15 min. Finally, after adding the stop solution the optical density was determined at 450 nm and the concentration of OC obtained from a standard curve plot. OP and OC

content was calculated by normalizing OP or OC concentration per DNA concentration for each condition and time point.

### 2.6. Statistical analysis

Results are expressed as mean ± standard deviation with n = 3 for each sample. Error bars represent standard deviations. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple Comparison test. $^{***}$ $p<0.001$; $^{**}$ $p<0.01$; $^{*}$ $p<0.05$ in respect to the control.

## 3. Results and discussion

### 3.1 Morphology of BBGs and their chemical composition

BBGs' frits were successfully obtained by melt quenching and ground in a controlled manner. Figure 2a, 2b and 2c show the SEM/EDS analysis of BBGs, which exhibited an angular shape with low sphericity and confirmed the successful incorporation of the different modifier divalent cations (i.e. $Mg^{2+}$, $Ca^{2+}$ and $Sr^{2+}$). The composition of BBGs was confirmed in a prior work by the use of X-ray fluorescence and follows the general formula $0.05Na_2O{\cdot}xMgO{\cdot}yCaO{\cdot}(0.35\text{-}x\text{-}y)SrO{\cdot}0.20B_2O_3{\cdot}0.40SiO_2$ (molar ratio, where x, $y = 0.35$ or 0.00, and $x \neq y$). (30) Moreover, studies after immersion in SBF, showed that the studied BBGs are bioactive due to the formation of bone-like apatite structures onto their surface, and the constant release of ions to the reaction media over time. (29)

**Figure 2.** SEM/EDS micrographs of BBGs, a) BBG-Mg, b) BBG-Ca and c) BBG-Sr. SEM images are shown as insets, displaying the morphology of the glass particles. The specific modifier divalent cation is highlighted in yellow for each BBG.

### 3.2 In Vitro biological evaluation

Osteoblast differentiation can be divided into three stages: cell proliferation, ECM synthesis and maturation, and ECM mineralization, each with a cellular characteristic behavior.(31)

#### 3.2.1 Potential cytotoxic effect of BBGs leachables on BM-MSCs

The effect of BBGs' concentration on cell viability and proliferation was studied on a previous work (29, 30). It showed that a concentration between 20 and 50 mg/ml did not significantly affect cell viability and proliferation. On the other hand, Romero et al. (32) studied the osteogenic response of BM-MSCs to strontium-substituted bioactive glasses (SrBG) and observed that 20 mg of Sr50BG promoted the osteoblastic differentiation of BM-MSCs. Based on these results, we decided on the BBGs concentrations of 20 and 50 mg/ml.

Herein, we cultured BM-MSCs in basal and osteogenic differentiation media for 7, 14 and 21 days under static conditions, in the presence and absence of BBGs, in order to evaluate their biological activity and capacity to maintain their structural integrity. The cellular metabolism and proliferation was evaluated by quantifying the conversion of resazurin-to resorufin by viable cells; the amount of double stranded DNA (live cells) in the culture wells; and their morphology. The data was analyzed in comparison with the BM-MSCs (without any BBGs, i.e. control experiment) to evaluate the impact of BBGs in the cell behavior.

Fluorescence microscopy images showed the morphology of BM-MSCs in culture containing BBGs or 45S5 bioglass® (Figure 3). The adhered BM-MSCs exhibited a well-spread morphology, exhibiting cell-to-cell contacts in a comparable manner on the BBGs conditioned cultures as in the control experiment. While BM-MSCs cultured with

45S5 bioglass® presented a round shape for the last time point (21 days), suggesting cell death. At the same timepoint, especially for cultures under osteogenic differentiation media (e.g. BBG-Sr), there was distinguishable well-spread polygonal shape cells, suggesting osteoblast-like morphology.

**Figure 3.** BM-MSCs morphology observed by fluorescence microscopy, after 7, 14 and 21 days culture with BBGs either under basal or osteogenic culture medium. Each sample was incubated at two different concentrations (20 and 50 mg/ml). Cells cultured with basal and osteo medium were used as negative control and 45S5 bioglass® incubated with medium was used as positive control. Nuclei stained blue by DAPI; Actin stained green by Phalloidin.

**Figure 4.** Metabolic activity (PrestoBlue® assay) and proliferation (PicoGreen® assay) of BM-MSCs cultured either in basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a, b), BBG-Ca (c, d) and BBG-Sr (e, f). The 45S5 bioglass® (g, h) was used as control. Standard culture medium was used as negative control. Results are expressed as mean ± standard deviation with n = 3 for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple Comparison test. $^{***}$ $p<0.001$; $^{**}$ $p<0.01$; $^{*}$ $p<0.05$ in respect to the control. The statistical significance is represented relative to the control of BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

From the PrestoBlue® data (Figure 4a, 4c, 4e and 4g) it is possible to observe that the metabolic activity of BM-MSCs increased over the 21 days of culture. In the first 7 days of culture, the BM-MSCs under conditioned cultures presented reduced viability in respect to the cells culture under basal condition (negative control), especially for BBG-Ca culture conditions, which may be due to the initial burst release of ions to the media. In contrast, from day 14, BM-MSC cultures under osteogenic media displayed a reduction of metabolic activity with respect to the control (absence of glass particles and cell cultured under basal medium). It is noteworthy that BM-MSC cultures at day 21 with 45S5 bioglass® under osteogenic media showed very low metabolic activity when compared with the control experiment (osteogenic media), being consistent with the cell death observed by fluorescent microscopy (Figure 3). Complementary to the viability analysis, the cellular proliferation was also assessed by measuring the total cell DNA (Figure 4b, 4d, 4f, 4h). The PicoGreen® data showed an increase on the number of BM-MSCs over time. However, under osteogenic media, the cells presented lower proliferation rates than cultures under basal media from day 14. Noteworthy, is the prominent reduction of BM-MSCs for cultures with BBG-Mg (osteogenic and basal media) and 45S5 bioglass® (osteogenic media) at day 21. To emphasize, BBG-Sr and 45S5 bioglass® under osteogenic media showed a significant reduction on the cell proliferation at day 14 when compared with the cells cultured under basal media, suggesting an alteration of biological behavior.(33) Regarding the effect of concentration, there was no potential toxic effects with time except in the case of BM-MSCs cultured in the presence of BBG-Mg (osteogenic and basal media) and 45S5 bioglass® (osteogenic media) at day 21, where a large reduction in cell number was observed in relation to the control (basal media).

### 3.2.2 Alkaline phosphate quantification

It is commonly accepted that ALP is a key player in the process of osteogenesis, being ALP known to be involved in early stages of normal and pathological calcification. (34-36) In general, an increase of the ALP activity is correlated with osteogenesis, increasing during the bone formation stage.(37) Not surprisingly, the ALP quantification data (Figure 5) showed a significantly higher ALP activity when cells were cultured under osteogenic media rather than basal media.(38) However, at day 21 the levels of ALP activity on BBG-Mg, -Sr and 45S5 bioglass® cultured under osteogenic media are significantly higher than the cultures without glass addition (e.g. at day 21, under osteogenic media, the addition of 20 mg of BBG-Sr (c) induced a highly significant ($p<0.001$) enhanced ALP activity in relation to the cells cultured in the absence of glass particles). BBG-Mg, -Sr and 45S5 bioglass® were not capable of inducing the ALP protein expression alone, however, they were capable to increase the ALP expression during the differentiation process of BM-MSCs into osteoblasts under osteogenic media (for 21 days of cell culture). It is relevant to point out that previous studies quantified the chemical species released from BBGs in solution, demonstrating a higher concentration of $Mg^{2+}$ and $Sr^{2+}$ ions (2-fold) when compared with $Ca^{2+}$ (from BBG-Mg, BBGs-Sr and BBG-Ca, respectively). (29, 30) The presence of higher concentration of specific ions (i.e. $Mg^{2+}$ and $Sr^{2+}$) might facilitate cell differentiation. It is also relevant to highlight the fact that along with the increase of ALP activity for the case of BBG-Mg and -Sr glasses there was observed an increase of cell proliferation. In contrast, the viability and proliferation data, presented a reduction of viable and live cells for the BM-MSCs cultured in the presence of these BBG-Ca and 45S5 bioglass®. Remarkably, BBG-Sr promoted the increase of ALP activity for both concentrations of glass particles, i.e. 20 and 50 mg/ml.

**Figure 5.** ALP activity of BM-MSCs (cultured either in basal or osteogenic media in the presence of different concentrations (0, 20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as mean ± standard deviation with n = 3 for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple Comparison test. *** $p<0.001$; ** $p<0.01$; * $p<0.05$ in respect to the control. The statistical significance is represented relative to the control of BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

### 3.2.3 Matrix mineralization

Similar results were found at day 21 after alizarin red staining for mineral deposits (Figure 6). In agreement with ALP activity data, cells cultured under osteogenic media yielded more bone-like nodules, resulting from ECM mineralization.(35) The intense red spots dispersed in the cell culture correspond to the mineralized nodules. Of importance, and corroborating with ALP activity data, mineral deposits were more evident with BBG-Mg, -Sr and 45S5 bioglass® (Figure 6a, 6c and 6d) and an increase of red nodules with the increase of concentrations can be observed in the case of the cells cultured in the presence of BBG-Sr (**Error! Reference source not found.**c, osteo 20 mg/ml and 50 mg/ml). Higher concentrations of BBG-Sr and 45S5 bioglass® show the presence of red nodules even in cells cultured in basal medium. However, ALP activity data, suggests that BBG-Sr and 45S5 bioglass® (Figure 6c and 6d basal for a concentration of 50 mg/ml) are capable to induce ECM mineralization by themselves. Therefore, the combination of ALP activity and mineralization results suggest the use of BBG-Sr to promote osteogenesis.(39)

**Figure 6.** Alizarin red staining of BM-MSCs cultured during 21 days, either in basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control.

**Figure 7.** SEM micrographs of BM-MSCs in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c) after 21 days of cell culture either in basal or osteogenic media. Higher SEM magnifications are shown as insets, highlighting the formed apatite-like structures. The 45S5 bioglass® was used as a control.

In Figure 7 we presented SEM micrographs of BM-MSCs cultured for 21 days in the presence of BBGs and 45S5 bioglass®. In the images it is possible to observe the deposition of minerals over the dense layer of cells, when they were cultured in the presence of BBG-Mg, -Sr and 45S5 bioglass® (Figure 7a, 7c and 7d). The presence of these glass compositions in the culture medium promoted a mineralization typical to occur while BM-MSCs differentiate into osteoblasts.(38) This mineralization is in agreement with the alizarin red data where the mineral deposits were more evident in the cells cultured in the presence of BBG-Mg, -Sr and 45S5 bioglass® (Figure 6a, 6c and 6d). In these culture conditions, it is observed in the SEM/EDS images calcium phosphate deposits over the dense cellular layer. Once more, higher concentrations of BBG-Sr and 45S5 bioglass® under basal culture conditions promoted the deposition of a higher amounts of calcium phosphate structures, suggesting that BBG-Sr and 45S5 bioglass® (Figure 7c and 7d basal for a concentration of 50 mg/ml) are capable of

391 inducing ECM mineralization by itself, which could be beneficial for bone regeneration
392 (39).

393 ### 3.2.4 Protein expression (OP and OC)

394 Complementary to the reported biological data, the differentiation level of BM-MSC,
395 cultured in the presence (20 and 50 mg/ml) and absence of BBG-Mg, -Ca, -Sr and 45S5
396 bioglass® (either in basal or osteogenic media) was assessed by the quantification of the
397 expression level of two major bone-specific proteins, i.e. OP and OC. The relative
398 expression of these proteins was normalized in relation to the number of cells, i.e.
399 amount of dsDNA. It is well known that osteoblasts are differentiated cells that
400 mineralize the bone matrix. OP is a phosphoprotein synthesized by bone forming cells,
401 which present calcium-binding domains and is responsible for cell attachment,
402 proliferation, and ECM mineralization.(40) In the case of OC, it is a bone-specific
403 glycoprotein capable of binding to calcium, which promotes ECM calcification.(40) Not
404 surprisingly, the OP and OC quantification data (Figure 8 and Figure 9) showed a
405 significantly higher protein expression when BM-MCSs were cultured under osteogenic
406 media rather than basal media.(41) In the case of OP, as expected, a delay in the protein
407 synthesis is observed (Figure 8). At day 7 there was no significant difference of OP
408 expression in relation to the control (absence of glass particles and cell culture in basal
409 medium). However, at day 14 there is a high expression peak by BM-MSCs cultured in
410 osteogenic medium (in the presence of BBG-Mg, -Sr and 45S5 bioglass®), which
411 determines the decay of the matrix deposition phase and the beginning of the
412 mineralization phase. Moreover, BBG-Sr and 45S5 bioglass® continue to induce a
413 significant overexpression of OP over time (e.g. at day 21), supporting the
414 mineralization demonstrated by ALP and alizarin red analysis (**Error! Reference
415 source not found.Error! Reference source not found.**Figure 5 and 6, respectively). In

the OC case there was a high protein expression up to day 14, indicating bone ECM maturation (Figure 9).(42) At day 7 there is a significant difference in OP expression in relation to the control (cell culture in basal medium and in the absence of glass particles). After day 7 there was a reduction of OC expression, consistent with matrix mineralization. Noteworthy is the observation that BBG-Sr under basal medium induced the BM-MSCs to exhibit a peak of OC expression at day 14. This data suggested that the BBG-Sr glass particles (at a concentration of 50 mg/ml) induced the OC protein expression, which is in agreement with the ALP and alizarin red data. Also, BBG-Sr and 45S5 bioglass® prolonged the OC overexpression over the 21 days of culture. In addition, the 45S5 bioglass® promoted a high deposition of OC at day 21 (Figure 4h) compared with high BM-MSCs density in the case of BBG-Sr (Figure 4f). However, it is important consider that in the case of the cultures in the presence of 45S5 bioglass® a very low BM-MSCs cell density was observed, which might be related with the cytotoxicity of 45S5 bioglass®.(43) Therefore, and overall our data suggests that the BBG-Sr glass particles are able to induce the BM-MSCs to express higher levels of OP and OC, while maintaining the BM-MSCs cell density.

**Figure 8.** OP protein content of BM-MSCs cultured either with basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as mean ± standard deviation with n = 3 for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple Comparison test. $^{***}$ $p<0.001$; $^{**}$ $p<0.01$; $^{*}$ $p<0.05$ in respect to the control. The statistical significance is represented relative to the control of BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

**Figure 9.** OC protein content of BM-MSCs cultured either with basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as mean ± standard deviation with n = 3 for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple Comparison test. $^{***}$ $p<0.001$; $^{**}$ $p<0.01$; $^{*}$ $p<0.05$ in respect to the control. The statistical significance is represented relative to the control of BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

Hence, combining the obtained biological data, i.e. viability, proliferation, mineralization and protein expression analysis (ALP, OC and OP), we were able to identify the BBG-Mg, -Sr (at concentrations of 20 to 50 mg/ml) as relevant promoters of the osteogenic differentiation of BM-MSCs. Several authors reported that $Mg^{2+}$ ions significantly enhanced osteoblast adhesion by the altering cell-matrix interactions, which modulate the function of integrins related with cell differentiation. (19, 44) Our results suggest that BBG-Mg also promoted BM-MSCs differentiation, which might be related with the presence of $Mg^{2+}$ in the culture medium. Remarkably, BBG-Sr (at a concentration of 50m/ml and 21 days of culture) presented the capacity to induce osteogenic response in BM-MSCs in the absence of osteogenic medium. Other authors reported Sr containing glasses to stimulate osteoblast metabolic activity, inhibiting osteoclast differentiation, as well as promoting the increment of ALP activity. (24) For instance, Hurtel-Lemaire et al. (45) have shown that Sr does induce osteoclast apoptosis

at concentrations higher than 9 mM. This is in accordance with our previous studies (29) that demonstrated a concentration of Sr in the reaction media about to 20 mM after 3 days of culture. The ALP activity results showed the shift of BM-MSCs to a more differentiated state, while the alizarin red analysis demonstrated that the cells in the presence of BBG-Mg and BBG-Sr glass particles demonstrate intense and dispersed red spots in the cell culture, corresponding to the mineralization promoted by the BM-MSCs. Santocildes et al. (25) demonstrated that the dissolution of Sr-containing glasses stimulated the upregulation of genes associated with the process of osteogenic differentiation, such as Bglap (osteocalcin) and Spp1 (osteopontin). In agreement with this data, we also observed that BBGs-Mg an BBG-Sr might also influence the ECM maturation and mineralization, through the promotion of the OP and OC protein overexpression, which suggests that these glass compositions may be effective in inducing and sustaining the osteoblastic phenotype. (46)

## 4. Conclusion

BBGs with different substituted divalent cations ($Ca^{2+}$, $Sr^{2+}$ or $Mg^{2+}$) were successfully synthesized by melt quenching. In vitro studies demonstrated that the studied BBGs exhibit the capability to improve the osteogenic differentiation of BM-MSCs with no deleterious effects over cell viability and proliferation. Specially, BBG-Mg and BBG-Sr (at 20 and 50 mg/ml) provided favorable conditions for BM-MSCs to differentiate to osteoblast-like cells and induce the formation of a high amount of mineralized nodules. The phenotypic expression of two major bone-specific proteins, namely, OP and OC confirmed the osteogenic potential of the BBGs.

Finally, the findings that the BBGs may promote in vitro cell differentiation into an osteogenic lineage, support their potential application in regenerative medicine. Based on these promising results we propose the incorporation of these BBGs into

biomaterials for bone regeneration. From our results, BBGs are able to enhance the osteogenic potential of the implants and promote the osteogenic differentiation of BM-MSCs. The proposed BBGs are relevant candidates for further in vivo evaluation.


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## Author Disclosure Statement

No competing financial interests exist.

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