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Substituted Borosilicate Glasses with Improved Osteogenic Capacity for Bone Tissue Engineering

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24 **Abstract**

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

42

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

45

1. Introduction

The properties of bioactive glasses (BGs) support their key relevance 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

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

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

88 The present study aims to fabricate three substituted BBGs to be used for bone tissue
89 regeneration. We evaluated the impact of incorporating different divalent cations (i.e.
90 Mg^{2+}, Ca^{2+}, Sr^{2+}) into the BBGs on their ability to induce the BM-MSCs (known to
91 differentiate into the mesodermal lineage cells, such as osteoblasts, osteoclasts and
92 osteocytes (27)) to proliferate, differentiate and mineralize the ECM, while promoting
93 bone formation. (11)

94 ## 2. Experimental

95 ### 2.1. Preparation of BBGs

96 The BBGs of general formula $0.05Na_2O \cdot x MgO \cdot y CaO \cdot (0.35\text{-}x\text{-}y)SrO \cdot 0.20B_2O_3 \cdot$
97 $0.40SiO_2$ (molar ratio, where *x*, *y* = 0.35 or 0.00, and $x \neq y$) were synthesized by melt-
98 quenching. The suitable amounts of, silica (SiO_2, Macherey-Nagel, Germany), boron
99 oxide (B_2O_3, Alfa Aesar, Germany), sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich,
100 Australia), and magnesium oxide (MgO, Sigma-Aldrich, Portugal), or calcium carbonate
101 ($CaCO_3$, Sigma-Aldrich, Portugal), or strontium carbonate ($SrCO_3$, Sigma-Aldrich,
102 Portugal) were thoroughly mixed with the addition of ethanol in a porcelain pestle with
103 the help of a mortar, vacuum dried overnight and transferred to a platinum crucible. After
104 entirely dried, each batch was heated to 1450 ˚C in air for 1 h and subsequently the melt
105 was quickly poured into cold water to form the glass frit. Afterwards, the as-quenched
106 glasses were ground in an Agate mortar (RETSCH, Germany) and sieved to a particle
107 size <63 μm. Before the *in vitro* tests BBG-Mg ($0.05Na_2O \cdot 0.35MgO \cdot 0.20B_2O_3 \cdot$
108 $0.40SiO_2$), BBG-Ca ($0.05Na_2O \cdot 0.35CaO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$) or BBG-Sr ($0.05Na_2O$
109 $\cdot 0.35SrO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$) were weighted, dried and sterilized at 160 ºC for at least
110 2 h.

111 ### 2.2. Morphology and chemical composition of BBGs

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

116 ### 2.3. Isolation and expansion of mesenchymal stem cells

117 BM-MSCs were isolated from bone marrow of 4-5 week-old male Wistar rats according
118 to the method established by Maniatopoulos *et al.* (28) and recently proposed by
119 Santocildes *et al*. (25) BM-MSCs were expanded in basal medium consisting of
120 Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK), supplemented with
121 100 U/ml penicillin (Sigma,-Aldrich, UK) and 1 mg/ml streptomycin (Sigma-Aldrich,
122 UK). Cells were cultured at 37 ºC in an atmosphere of 5% CO_2.

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

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

136 ### 2.4. Potential cytotoxic effect of BBGs dissolution on BM-MSCs

137 **Cell viability and proliferation (PrestoBlue® and PicoGreen® assays).** The
138 PrestoBlue® reagent (Fisher Scientific, UK) is a resazurin-based solution that is reduced
139 to resorufin by viable cells which can be detected fluorimetrically. The cell viability assay

140 was executed according to the manufacturer's instructions. In brief, the PrestoBlue®
141 reagent was added to a final concentration of 10% to the wells and the cells were
142 incubated for 1 h at 37 ºC. Afterwards, 200 µl samples of the culture medium were
143 removed and placed in 96-well plates and the resorufin fluorescence was quantified
144 spectrophotometrically using a plate reader (Tecan Infinite M200). The fluorescence was
145 determined at an excitation wavelength of 560 nm and emission wavelength of 590 nm.
146 The metabolic activity was presented in fluorescence values and compared with the
147 control (cell cultured in the absence of glass particles under basal medium conditions).

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

157 **Cell morphology and distribution**. After each culturing period the cell grown in tissue
158 culture coverslips were washed with PBS and fixed with 4% formalin solution (0.5 ml)
159 for 15 min at room temperature (RT). The cell layers were then washed with PBS,
160 containing 0.2% Triton X, for 2 min. After the fixation and permeation steps, the cell
161 monolayers were washed again with PBS and stained with 4,6-diamidino-2-phenyindole
162 dilactate (1:1000 DAPI, Sigma, UK) for 2 min at RT, and phalloidin-
163 tetramethylrhodamine B isothiocyanate (Sigma, UK) for 1 h at RT. Finally, the cells were

164 washed and observed using an Axioplan 2 imaging fluorescent microscope with a digital
165 camera QIC AM 12-bit (Zeiss, UK).

166 **2.5. Osteogenic capacity of BBGs on BM-MSCs**

167 **Alkaline phosphatase quantification**. The concentration of alkaline phosphatase (ALP)
168 was determined for all the culture time periods, using the lysates used for DNA
169 quantification. Briefly, the ALP quantity was assessed using the Alkaline Phosphatase,
170 Diethanolamine Detection kit (Sigma-Aldrich, UK) in which p-nitrophenyl phosphatase
171 (pNPP) solution is hydrolyzed by ALP to yellow free p-nitrophenol. In brief, a buffered
172 pNPP solution was prepared and equilibrated at 37 ºC. Afterwards, 2% (v/v) of sample or
173 control were added. Immediately after mixing the absorbance was read at 405 nm in a
174 plate reader (Tecan Infinite M200) for ≈ 5 min. An ALP standard solution was used as
175 control and buffer as blank. The units were calculated according to the following
176 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 =
177 Volume of final solution; 18.5 = millimolar extinction coefficient of pNPP at 405 nm and
178 V_E = Volume of samples/ALP standard solution. ALP activity was calculated by
179 normalizing ALP concentration per DNA concentration for each condition and time point.
180 **Alizarin red staining.** After 21 days of culture, the cells grown in tissue culture
181 coverslips were fixed in 70% ice-cold methanol at -20 °C at least for 30 min. The cell
182 layers were then washed with PBS and dried overnight. Afterwards, cells were stained
183 with alizarin red solution [342 mg of alizarin red, (Sigma-Aldrich, UK) in 25 ml of
184 distilled water and the pH was adjusted to 4.1 with 10% ammonium hydroxide (Sigma-
185 Aldrich, UK)] for 10 min. Afterwards, the coverslips were washed with distilled water,
186 dehydrated in an acetone/xylene (Sigma-Aldrich, UK) mixture and mounted using an
187 aqueous mountant. The stained constructs were observed under an optical microscope

188 (BX51, Olympus Corporation, UK) and images were captured by a digital camera (DP70,
189 Olympus Corporation, UK). The BM-MSCs morphology and mineral deposition was also
190 observed using SEM (model S360, Leica Cambridge, UK) equipped with energy
191 dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of the
192 surface chemical composition.

193 **Immunodetection of bone-specific proteins.** Osteopontin (OP) and osteocalcin (OC)
194 protein expression of BM-MSCs was assessed by immunoassay technique to evaluate
195 their osteoblastic differentiation. The procedures were executed according to the
196 manufacturer's instructions. The concentrations of OP and OC were determined for all
197 the culture time periods, using the lysates used for DNA quantification. The OP
198 quantitative determination was performed using Mouse/Rat Osteopontin Quantikine
199 ELISA Kit (R&D Systems, UK). In brief, 50 µl of assay diluent RD1W and 50 µl of
200 standard (2500 to 39 pg/ml), control and samples were added and the plate incubated for
201 2 h at RT. After 4 washing steps and perfectly dried, 100 µl of Mouse/Rat OP Conjugated
202 were added and incubated for 2 h at RT. The sandwich complex was washed 4 times and
203 allowed to react with 100 µl of substrate solution before adding 100 µl of stop solution.
204 Finally, the optical density was determined at 450 nm and the concentration of OP
205 obtained from a standard curve plot. OC quantitative determination was performed by the
206 use of Rat Gla-Osteocalcin High Sensitive EIA kit (Takara Clontech, Japan). In brief, 100
207 µl of samples and standard solution (16 to 0.25 ng/ml) were incubated for 1 h at 37 ºC
208 with the capture-antibody, rat osteocalcin C-terminus-specific antibody. After OC capture
209 and 3 washing steps, 100 µl of the enzyme-labelled antibody (GlaOC4-30) specific to
210 Gla-OC was incubated for 1 h at RT. The sandwich complex was washed 4 times and
211 allowed to react with 100 µl of substrate solution for 10-15 min. Finally, after adding the
212 stop solution the optical density was determined at 450 nm and the concentration of OC

213 obtained from a standard curve plot. OP and OC content was calculated by normalizing
214 OP or OC concentration per DNA concentration for each condition and time point.

215 **2.6. Statistical analysis**

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

220 **3. Results and discussion**

221 **3.1 Morphology of BBGs and their chemical composition**

222 BBGs' frits were successfully obtained by melt quenching and ground in a controlled
223 manner. **Figure** 2a, 2b and 2c show the SEM/EDS analysis of BBGs, which exhibited an
224 angular shape with low sphericity and confirmed the successful incorporation of the
225 different modifier divalent cations (i.e. Mg^{2+}, Ca^{2+} and Sr^{2+}). The composition of BBGs
226 was confirmed in a prior work by the use of X-ray fluorescence and follows the general
227 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,
228 y = 0.35 or 0.00, and $x \neq y$). (30) Moreover, studies after immersion in SBF, showed that
229 the studied BBGs are bioactive due to the formation of bone-like apatite structures onto
230 their surface, and the constant release of ions to the reaction media over time. (29)

231

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

235 **3.2 *In Vitro* biological evaluation**

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

239 **3.2.1 Potential cytotoxic effect of BBGs leachables on BM-MSCs**

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

247 Herein, we cultured BM-MSCs in basal and osteogenic differentiation media for 7, 14
248 and 21 days under static conditions, in the presence and absence of BBGs, in order to
249 evaluate their biological activity. The cellular metabolism and proliferation was evaluated
250 by quantifying the conversion of resazurin to resorufin by viable cells and the amount of
251 double stranded DNA (live cells) in the culture wells; as well as their morphology.

252 Fluorescence microscopy images showed the morphology of BM-MSCs in culture
253 containing BBGs or 45S5 bioglass® (Figure 3). The adhered BM-MSCs exhibited a well-
254 spread morphology, exhibiting cell-to-cell contacts in a comparable manner on the BBGs
255 conditioned cultures as in the control experiment. While BM-MSCs cultured with 45S5
256 bioglass® presented a round shape for the last time point (21 days), suggesting cell death.
257 At the same timepoint, especially for cultures under osteogenic differentiation media (e.g.

258 BBG-Sr), there was distinguishable well-spread polygonal shape cells, suggesting
259 osteoblast-like morphology.

260

261 **Figure 3.** BM-MSCs morphology observed by fluorescence microscopy, after 7, 14 and
262 21 days culture with BBGs either under basal or osteogenic culture medium. Each sample
263 was incubated at two different concentrations (20 and 50 mg/ml). Cells cultured with
264 basal and osteo medium were used as negative control and 45S5 bioglass® incubated with
265 medium was used as positive control. Nuclei stained blue by DAPI; Actin stained green
266 by Phalloidin.

267

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

277

278 From the PrestoBlue® data (Figure 4a, 4c, 4e and 4g) it is possible to observe that the
279 metabolic activity of BM-MSCs increased over the 21 days of culture. In the first 7 days
280 of culture, the BM-MSCs under conditioned cultures presented reduced viability in
281 respect to the cells culture under basal condition (negative control), especially for BBG-

282 Ca culture conditions, which may be due to the initial burst release of ions to the media.
283 In contrast, from day 14, BM-MSC cultures under osteogenic media displayed a reduction
284 of metabolic activity with respect to the control (absence of glass particles and cell
285 cultured under basal medium). It is noteworthy that BM-MSC cultures at day 21 with
286 45S5 bioglass® under osteogenic media showed very low metabolic activity when
287 compared with the control experiment (osteogenic media), being consistent with the cell
288 death observed by fluorescent microscopy (Figure 3). Complementary to the viability
289 analysis, the cellular proliferation was also assessed by measuring the total cell DNA
290 (Figure 4b, 4d, 4f, 4h). The PicoGreen® data showed an increase on the number of BM-
291 MSCs over time. However, under osteogenic media, the cells presented lower
292 proliferation rates than cultures under basal media from day 14. Noteworthy, is the
293 prominent reduction of BM-MSCs for cultures with BBG-Mg (osteogenic and basal
294 media) and 45S5 bioglass® (osteogenic media) at day 21. To emphasize, BBG-Sr and
295 45S5 bioglass® under osteogenic media showed a significant reduction on the cell
296 proliferation at day 14 when compared with the cells cultured under basal media,
297 suggesting an alteration of biological behavior. (33) Regarding the effect of
298 concentration, there was no potential toxic effects with time except in the case of BM-
299 MSCs cultured in the presence of BBG-Mg (osteogenic and basal media) and 45S5
300 bioglass® (osteogenic media) at day 21, where a large reduction in cell number was
301 observed in relation to the control (basal media).

302 **3.2.2 Alkaline phosphate quantification**

303 It is commonly accepted that ALP is a key player in the process of osteogenesis, being
304 ALP known to be involved in early stages of normal and pathological calcification. (34-
305 36) In general, an increase of the ALP activity is correlated with osteogenesis, increasing
306 during the bone formation stage. (37) Not surprisingly, the ALP quantification data

307 (Figure 5) showed a significantly higher ALP activity when cells were cultured under
308 osteogenic media rather than basal media. (38) However, at day 21 the levels of ALP
309 activity on BBG-Mg, -Sr and 45S5 bioglass® cultured under osteogenic media are
310 significantly higher than the cultures without glass addition (e.g. at day 21, under
311 osteogenic media, the addition of 20 mg of BBG-Sr (c) induced a highly significant
312 (p<0.001) enhanced ALP activity in relation to the cells cultured in the absence of glass
313 particles). BBG-Mg, -Sr and 45S5 bioglass® were not capable of inducing the ALP
314 protein expression alone, however, they were capable to increase the ALP expression
315 during the differentiation process of BM-MSCs into osteoblasts under osteogenic media
316 (for 21 days of cell culture). It is relevant to point out that previous studies quantified the
317 chemical species released from BBGs in solution, demonstrating a higher concentration
318 of Mg^{2+} and Sr^{2+} ions (2-fold) when compared with Ca^{2+} (from BBG-Mg, BBGs-Sr and
319 BBG-Ca, respectively). (29, 30) The presence of higher concentration of specific ions
320 (i.e. Mg^{2+} and Sr^{2+}) might facilitate cell differentiation. It is also relevant to highlight the
321 fact that along with the increase of ALP activity for the case of BBG-Mg and -Sr glasses
322 there was observed an increase of cell proliferation. In contrast, the viability and
323 proliferation data, presented a reduction of viable and live cells for the BM-MSCs
324 cultured in the presence of these BBG-Ca and 45S5 bioglass®. Remarkably, BBG-Sr
325 promoted the increase of ALP activity for both concentrations of glass particles, i.e. 20
326 and 50 mg/ml.

327

328 **Figure 5.** ALP activity of BM-MSCs (cultured either in basal or osteogenic media) in the
329 presence of different concentrations (0, 20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b)
330 and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as
331 mean ± standard deviation with n = 3 for each bar. The data was analyzed by non-

332 parametric statistics: Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple
333 Comparison test. $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$ in respect to the control, i.e. BM-MSCs
334 culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).
335

336 **3.2.3 Matrix mineralization**

337 Similar results were found at day 21 after alizarin red staining for mineral deposits (Figure
338 6). In agreement with ALP activity data, cells cultured under osteogenic media yielded
339 more bone-like nodules (intense red spots dispersed in the cell culture), resulting from
340 ECM mineralization. (35) Of importance, and corroborating with ALP activity data,
341 mineral deposits were more evident with BBG-Mg, -Sr and 45S5 bioglass® (Figure 6a,
342 6c and 6d) and an increase of red nodules with the increase of concentrations can be
343 observed in the case of the cells cultured in the presence of BBG-Sr (Figure 6c, osteo 20
344 mg/ml and 50 mg/ml). Higher concentrations of BBG-Sr and 45S5 bioglass® show the
345 presence of red nodules even in cells cultured in basal medium. However, ALP activity
346 data, suggests that BBG-Sr and 45S5 bioglass® (Figure 6c and 6d basal for a concentration
347 of 50 mg/ml) are capable to induce ECM mineralization by themselves. Therefore, the
348 combination of ALP activity and mineralization results suggest the use of BBG-Sr to
349 promote osteogenesis. (39)
350

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

354

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

359 In Figure 7 we presented SEM micrographs of BM-MSCs cultured for 21 days in the
360 presence of BBGs and 45S5 bioglass®. In the images it is possible to observe the
361 deposition of minerals over the dense layer of cells, when they were cultured in the
362 presence of BBG-Mg, -Sr and 45S5 bioglass® (Figure 7a, 7c and 7d). The presence of
363 these glass compositions in the culture medium promoted a mineralization typical to
364 occur while BM-MSCs differentiate into osteoblasts. (38) This mineralization is in
365 agreement with the alizarin red data where the mineral deposits were more evident in the
366 cells cultured in the presence of BBG-Mg, -Sr and 45S5 bioglass® (Figure 6a, 6c and 6d).
367 In these culture conditions, it is observed in the SEM/EDS images calcium phosphate
368 deposits over the dense cellular layer. Once more, higher concentrations of BBG-Sr and
369 45S5 bioglass® under basal culture conditions promoted the deposition of a higher
370 amounts of calcium phosphate structures, suggesting that BBG-Sr and 45S5 bioglass®
371 (Figure 7c and 7d basal for a concentration of 50 mg/ml) are capable of inducing ECM
372 mineralization by themselves, which could be beneficial for bone regeneration. (39)

373 ### 3.2.4 Protein expression (OP and OC)

374 Complementary to the reported biological data, the differentiation level of BM-MSC,
375 cultured in the presence (20 and 50 mg/ml) and absence of BBG-Mg, -Ca, -Sr and 45S5
376 bioglass® (either in basal or osteogenic media) was assessed by the quantification of the
377 expression level of two major bone-specific proteins, i.e. OP and OC. The relative

378 expression of these proteins was normalized in relation to the number of cells, i.e. amount
379 of dsDNA. It is well known that osteoblasts are differentiated cells that mineralize the
380 bone matrix. OP is a phosphoprotein synthesized by bone forming cells, which present
381 calcium-binding domains and is responsible for cell attachment, proliferation, and ECM
382 mineralization. (40) In the case of OC, it is a bone-specific glycoprotein capable of
383 binding to calcium, which promotes ECM calcification. (40) Not surprisingly, the OP and
384 OC quantification data (Figure 8 and Figure 9) showed a significantly higher protein
385 expression when BM-MCSs were cultured under osteogenic media rather than basal
386 media. (41) In the case of OP, as expected, a delay in the protein synthesis is observed
387 (Figure 8). At day 7 there was no significant difference of OP expression in relation to
388 the control (absence of glass particles and cell culture in basal medium). However, at day
389 14 there is a high expression peak by BM-MSCs cultured in osteogenic medium (in the
390 presence of BBG-Mg, -Sr and 45S5 bioglass®), which determines the decay of the matrix
391 deposition phase and the beginning of the mineralization phase. Moreover, BBG-Sr and
392 45S5 bioglass® continue to induce a significant overexpression of OP over time (e.g. at
393 day 21), supporting the mineralization demonstrated by ALP and alizarin red analysis
394 (Figure 5 and 6, respectively). In the OC case there was a high protein expression up to
395 day 14, indicating bone ECM maturation (Figure 9). (42) At day 7 there is a significant
396 difference in OP expression in relation to the control (cell culture in basal medium and in
397 the absence of glass particles). After day 7 there was a reduction of OC expression,
398 consistent with matrix mineralization. Noteworthy is the observation that BBG-Sr under
399 basal medium induced the BM-MSCs to exhibit a peak of OC expression at day 14. This
400 data suggested that the BBG-Sr glass particles (at a concentration of 50 mg/ml) induced
401 the OC protein expression, which is in agreement with the ALP and alizarin red data.
402 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, i.e. 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, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

427

428 Hence, combining the obtained biological data, i.e. viability, proliferation, mineralization
429 and protein expression analysis (ALP, OC and OP), we were able to identify the BBG-
430 Mg, -Sr (at concentrations of 20 to 50 mg/ml) as relevant promoters of the osteogenic
431 differentiation of BM-MSCs. Several authors reported that Mg^{2+} ions significantly
432 enhanced osteoblast adhesion by the altering cell-matrix interactions, which modulate the
433 function of integrins related with cell differentiation. (19, 44) Our results suggest that
434 BBG-Mg also promoted BM-MSCs differentiation, which might be related with the
435 presence of Mg^{2+} in the culture medium. Remarkably, BBG-Sr (at a concentration of 50
436 mg/ml and 21 days of culture) presented the capacity to induce osteogenic response in
437 BM-MSCs in the absence of osteogenic medium. Other authors reported Sr containing
438 glasses to stimulate osteoblast metabolic activity, inhibiting osteoclast differentiation, as
439 well as promoting the increment of ALP activity. (24) For instance, Hurtel-Lemaire *et al.*
440 (45) have shown that Sr induces osteoclast apoptosis at concentrations higher than 9 mM.
441 This is in accordance with our previous studies (29) that demonstrated a concentration of
442 Sr in the culture media of about 20 mM after 3 days of culture. The ALP activity results
443 showed the shift of BM-MSCs to a more differentiated state, while the alizarin red
444 analysis demonstrated that the cells in the presence of BBG-Mg and BBG-Sr glass
445 particles present intense and dispersed red spots in the cell culture, corresponding to the
446 mineralization promoted by the BM-MSCs. Finally, Santocildes *et al.* (25) demonstrated
447 that the dissolution of Sr-containing glasses stimulated the upregulation of genes
448 associated with the process of osteogenic differentiation, such as *Bglap* (OC) and *Spp1*
449 (OP). In agreement with this data, we also observed that BBGs-Mg an BBG-Sr might also
450 influence the ECM maturation and mineralization, through the promotion of the OP and

451 OC protein overexpression, which suggests that these glass compositions may be
452 effective in inducing and sustaining the osteoblastic phenotype. (46)

453 **4. Conclusion**

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

462 The findings that the BBGs are able to promote *in vitro* cell differentiation into an
463 osteogenic lineage, support their potential application in regenerative medicine. Based on
464 these promising results we propose the incorporation of these BBGs into biomaterials for
465 bone regeneration. The proposed BBGs are also relevant candidates for further *in vivo*
466 evaluation.

467

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482 No competing financial interests exist.

483 **References**

484 1. Rahaman, M.N. 3 - Bioactive ceramics and glasses for tissue engineering. In:
485 Boccaccini A.R., Ma P.X., eds. Tissue Engineering Using Ceramics and Polymers
486 (Second Edition): Woodhead Publishing; 2014. pp. 67.
487 2. Rabiee, S.M., Nazparvar, N., Azizian, M., Vashaee, D., and Tayebi, L. Effect of ion
488 substitution on properties of bioactive glasses: A review. Ceram Int **41**, 7241, 2015.
489 3. Hench, L. The story of Bioglass. Journal of Materials Science: Materials in Medicine
490 **17**, 967, 2006.
491 4. Bhakta, S., Faira, P., Salata, L., de Oliveira Neto, P., Miller, C., van Noort, R., Reaney,
492 I., Brook, I., and Hatton, P. Determination of relative in vivo osteoconductivity of
493 modified potassium fluorrichterite glass–ceramics compared with 45S5 bioglass. Journal
494 of Materials Science: Materials in Medicine **23**, 2521, 2012.
495 5. Gomes, F.O., Pires, R.A., and Reis, R.L. Aluminum-free glass-ionomer bone cements
496 with enhanced bioactivity and biodegradability. Materials Science and Engineering: C
497 **33**, 1361, 2013.

498 6. Wallace, K.E., Hill, R.G., Pembroke, J.T., Brown, C.J., and Hatton, P.V. Influence of
499 sodium oxide content on bioactive glass properties. J Mater Sci: Mater Med **10**, 697.
500 7. Pires, R.A., Abrahams, I., Nunes, T.G., and Hawkes, G.E. Multinuclear magnetic
501 resonance studies of borosilicate glasses for use in glass ionomer cements: incorporation
502 of CaO and Al2O3. Journal of Materials Chemistry **16**, 2364, 2006.
503 8. Pires, R.A., Abrahams, I., Nunes, T.G., and Hawkes, G.E. The role of alumina in
504 aluminoborosilicate glasses for use in glass-ionomer cements. Journal of Materials
505 Chemistry **19**, 3652, 2009.
506 9. Pan, H.B., Zhao, X.L., Zhang, X., Zhang, K.B., Li, L.C., Li, Z.Y., Lam, W.M., Lu,
507 W.W., Wang, D.P., Huang, W.H., Lin, K.L., and Chang, J. Strontium borate glass:
508 potential biomaterial for bone regeneration. J R Soc Interface **7**, 1025, 2010.
509 10. Rahaman, M.N., Day, D.E., Sonny Bal, B., Fu, Q., Jung, S.B., Bonewald, L.F., and
510 Tomsia, A.P. Bioactive glass in tissue engineering. Acta Biomaterialia **7**, 2355, 2011.
511 11. Hoppe, A., Güldal, N.S., and Boccaccini, A.R. A review of the biological response to
512 ionic dissolution products from bioactive glasses and glass-ceramics. Biomaterials **32**,
513 2757, 2011.
514 12. Lakhkar, N.J., Lee, I.-H., Kim, H.-W., Salih, V., Wall, I.B., and Knowles, J.C. Bone
515 formation controlled by biologically relevant inorganic ions: Role and controlled delivery
516 from phosphate-based glasses. Advanced Drug Delivery Reviews **65**, 405, 2013.
517 13. Huang, W., Day, D., Kittiratanapiboon, K., and Rahaman, M. Kinetics and
518 mechanisms of the conversion of silicate (45S5), borate, and borosilicate glasses to
519 hydroxyapatite in dilute phosphate solutions. Journal of Materials Science: Materials in
520 Medicine **17**, 583, 2006.
521 14. Yao, A., Deping Wang, and Huang, W. In Vitro Bioactive Characteristics of Borate-
522 Based Glasses with Controllable Degradation Behavior. J Am Ceram Soc **1**, 303, 2006.

523 15. Fu, Q., Rahaman, M.N., Bal, B.S., Bonewald, L.F., Kuroki, K., and Brown, R.F.
524 Silicate, borosilicate, and borate bioactive glass scaffolds with controllable degradation
525 rate for bone tissue engineering applications. II. In vitro and in vivo biological evaluation.
526 JOURNAL OF BIOMEDICAL MATERIALS RESEARCH A | OCT 2010 **95A**2010.
527 16. Shen, Y., Liu, W., Wen, C., Pan, H., Wang, T., Darvell, B.W., Lu, W.W., and Huang,
528 W. Bone regeneration: importance of local pH-strontium-doped borosilicate scaffold. J
529 Mater Chem **22**, 8662, 2012.
530 17. Chapin, R., Ku, W., Kenney, M., and McCoy, H. The effects of dietary boric acid on
531 bone strength in rats. Biol Trace Elem Res **66**, 395, 1998.
532 18. Yamasaki, Y., Yoshida, Y., Okazaki, M., Shimazu, A., Uchida, T., Kubo, T.,
533 Akagawa, Y., Hamada, Y., Takahashi, J., and Matsuura, N. Synthesis of functionally
534 graded MgCO3 apatite accelerating osteoblast adhesion. Journal of Biomedical Materials
535 Research **62**, 99, 2002.
536 19. Zreiqat, H., Howlett, C.R., Zannettino, A., Evans, P., Schulze-Tanzil, G., Knabe, C.,
537 and Shakibaei, M. Mechanisms of magnesium-stimulated adhesion of osteoblastic cells
538 to commonly used orthopaedic implants. Journal of Biomedical Materials Research **62**,
539 175, 2002.
540 20. Maeno, S., Niki, Y., Matsumoto, H., Morioka, H., Yatabe, T., Funayama, A., Toyama,
541 Y., Taguchi, T., and Tanaka, J. The effect of calcium ion concentration on osteoblast
542 viability, proliferation and differentiation in monolayer and 3D culture. Biomaterials **26**,
543 4847, 2005.
544 21. Marie, P.J., Ammann, P., Boivin, G., and Rey, C. Mechanisms of Action and
545 Therapeutic Potential of Strontium in Bone. Calcif Tissue Int **69**, 121, 2001.
546 22. Marie, P.J. Strontium ranelate: A physiological approach for optimizing bone
547 formation and resorption. Bone **38**, 10, 2006.

548 23. Wu, C., Fan, W., Gelinsky, M., Xiao, Y., Simon, P., Schulze, R., Doert, T., Luo, Y.,
549 and Cuniberti, G. Bioactive SrO–SiO2 glass with well-ordered mesopores:
550 Characterization, physiochemistry and biological properties. Acta Biomaterialia **7**, 1797,
551 2011.
552 24. Gentleman, E., Fredholm, Y.C., Jell, G., Lotfibakhshaiesh, N., O'Donnell, M.D., Hill,
553 R.G., and Stevens, M.M. The effects of strontium-substituted bioactive glasses on
554 osteoblasts and osteoclasts in vitro. Biomaterials **31**, 3949, 2010.
555 25. Santocildes-Romero, M.E., Crawford, A., Hatton, P.V., Goodchild, R.L., Reaney,
556 I.M., and Miller, C.A. The osteogenic response of mesenchymal stromal cells to
557 strontium-substituted bioactive glasses. Journal of Tissue Engineering and Regenerative
558 Medicine **9**, 619, 2015.
559 26. Liang, W., Rahaman, M.N., Day, D.E., Marion, N.W., Riley, G.C., and Mao, J.J.
560 Bioactive borate glass scaffold for bone tissue engineering. Journal of Non-Crystalline
561 Solids **354**, 1690, 2008.
562 27. Brooke, G., Cook, M., Blair, C., Han, R., Heazlewood, C., Jones, B., Kambouris, M.,
563 Kollar, K., McTaggart, S., Pelekanos, R., Rice, A., Rossetti, T., and Atkinson, K.
564 Therapeutic applications of mesenchymal stromal cells. Seminars in Cell &
565 Developmental Biology **18**, 846, 2007.
566 28. Maniatopoulos, C., Sodek, J., and Melcher, A.H. Bone formation in vitro by stromal
567 cells obtained from bone marrow of young adult rats. Cell Tissue Res **254**, 317, 1988.
568 29. Fernandes, J.S., Martins, M., Neves, N.M., Fernandes, M.H.F.V., Reis, R.L., and
569 Pires, R.A. Intrinsic Antibacterial Borosilicate Glasses for Bone Tissue Engineering
570 Applications. ACS Biomaterials Science & Engineering 2016.

571 30. Fernandes, J.S., Gentile, P., Moorehead, R., Crawford, A., Miller, C.A., Pires, R.A.,
572 Hatton, P.V., and Reis, R.L. Design and Properties of Novel Substituted Borosilicate
573 Bioactive Glasses and Their Glass-Ceramic Derivatives. Cryst Growth Des 2016.
574 31. Stein, G.S., and Lian, J.B. 2 - MOLECULAR MECHANISMS MEDIATING
575 DEVELOPMENTAL AND HORMONE-REGULATED EXPRESSION OF GENES IN
576 OSTEOBLASTS: An Integrated Relationship of Cell Growth and Differentiation. In:
577 Noda M., ed. Cellular and Molecular Biology of Bone. San Diego: Academic Press; 1993.
578 pp. 47.
579 32. Romero, M. The osteogenic response of mesenchymal stromal cells to strontium-
580 substituted bioactive glasses. Journal of Tissue Engineering and Regenerative Medicine
581 2015.
582 33. Lian, J.B., and Stein, G.S. Development of the osteoblast phenotype: molecular
583 mechanisms mediating osteoblast growth and differentiation. The Iowa Orthopaedic
584 Journal **15**, 118, 1995.
585 34. Malaval, L., Liu, F., Roche, P., and Aubin, J.E. Kinetics of osteoprogenitor
586 proliferation and osteoblast differentiation in vitro. Journal of Cellular Biochemistry **74**,
587 616, 1999.
588 35. Huang, S.-C., Wu, B.-C., and Ding, S.-J. Stem cell differentiation-induced calcium
589 silicate cement with bacteriostatic activity. Journal of Materials Chemistry B **3**, 570,
590 2015.
591 36. Golub, E.E., and Boesze-Battaglia, K. The role of alkaline phosphatase in
592 mineralization. Current Opinion in Orthopaedics **18**, 444, 2007.
593 37. Sabokbar, A., Millett, P.J., Myer, B., and Rushton, N. A rapid, quantitative assay for
594 measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone and Mineral
595 **27**, 57, 1994.

596 38. Martins, A., Duarte, A.R.C., Faria, S., Marques, A.P., Reis, R.L., and Neves, N.M.
597 Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release
598 functionality. Biomaterials **31**, 5875, 2010.
599 39. Bosetti, M., and Cannas, M. The effect of bioactive glasses on bone marrow stromal
600 cells differentiation. Biomaterials **26**, 3873, 2005.
601 40. Karsenty, G. The genetic transformation of bone biology. Genes & Development **13**,
602 3037, 1999.
603 41. Amorim, S., Martins, A., Neves, N.M., Reis, R.L., and Pires, R.A. Hyaluronic
604 acid/poly-l-lysine bilayered silica nanoparticles enhance the osteogenic differentiation of
605 human mesenchymal stem cells. Journal of Materials Chemistry B **2**, 6939, 2014.
606 42. Mizuno, M., and Kuboki, Y. Osteoblast-Related Gene Expression of Bone Marrow
607 Cells during the Osteoblastic Differentiation Induced by Type I Collagen. Journal of
608 Biochemistry **129**, 133, 2001.
609 43. Chen, Q.Z., Xu, J.L., Yu, L.G., Fang, X.Y., and Khor, K.A. Spark plasma sintering
610 of sol–gel derived 45S5 Bioglass®-ceramics: Mechanical properties and biocompatibility
611 evaluation. Materials Science and Engineering: C **32**, 494, 2012.
612 44. Chen, X., Liao, X., Huang, Z., You, P., Chen, C., Kang, Y., and Yin, G. Synthesis
613 and characterization of novel multiphase bioactive glass-ceramics in the CaO-MgO-SiO2
614 system. Journal of Biomedical Materials Research Part B: Applied Biomaterials **93B**,
615 194, 2010.
616 45. Hurtel-Lemaire, A.S., Mentaverri, R., Caudrillier, A., Cournarie, F., Wattel, A.,
617 Kamel, S., Terwilliger, E.F., Brown, E.M., and Brazier, M. The Calcium-sensing
618 Receptor Is Involved in Strontium Ranelate-induced Osteoclast Apoptosis: NEW
619 INSIGHTS INTO THE ASSOCIATED SIGNALING PATHWAYS. Journal of
620 Biological Chemistry **284**, 575, 2009.

621 46. Habibovic, P., and de Groot, K. Osteoinductive biomaterials—properties and
622 relevance in bone repair. Journal of Tissue Engineering and Regenerative Medicine **1**, 25,
623 2007.