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Angiogenic effects of ionic dissolution products released from a boron-doped 45S5 bioactive glass

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Abstract

In regenerative medicine of vascularized tissues, there is great interest in the use of biomaterials able to stimulate angiogenesis, a process necessary for rapid revascularization to allow the transport and exchange of oxygen, nutrients, growth factors and cells that take part in tissue repair and/or regeneration. An increasing number of publications have shown that bioactive glasses stimulate angiogenesis. Since it has been established that boron (B) may play roles in angiogenesis, the aim of this study was to assess the *in vivo* angiogenic effects of the ionic dissolution products from a bioactive glass (BG) in the 45S5 system doped with 2 wt% B_2O_3 (45S5.2B). The pro-angiogenic capacity of 45S5.2B BG was assessed on the vasculature of the embryonic quail chorioallantoic membrane (CAM). Ionic dissolution products from 45S5.2B BG increased angiogenesis, quantitatively evidenced by the greater expression of integrin $\alpha_v\beta_3$ and higher vascular density in the embryonic quail CAM. The response observed at 2 and 5 days post-treatment was equivalent to that achieved by applying 10 μg/mL of basic fibroblast growth factor. This results show that the ionic dissolution products released from the bioactive glass 45S5.2B stimulate angiogenesis *in vivo*. The effects observed are attributed to the presence the ionic dissolution products, which contained 160±10 μM borate.

Keywords: Angiogenesis, boron, bioactive glass, biomaterials, therapeutic ions

1. Introduction

In regenerative medicine of vascularized tissues (such as bone and heart muscle), there is great interest in the use of biomaterials able to stimulate angiogenesis, a process necessary to achieve a rapid revascularization to allow the transport and exchange of oxygen, nutrients, growth factors and cells involved in tissue repair and regeneration.[1-4] Biomaterials have been categorized as first-, second- and third-generation biomaterials, according to the biological behavior they are able to trigger. First-generation materials have inert (non-bioactive) behavior, whereas second- and third-generation materials have bioactive properties.[5] Non-bioactive materials determine the formation of a capsule of non-adherent fibrous connective tissue around the implant material, whereas bioactive materials have been defined as those able to trigger a specific biological response at the level of the interface that determines the integration of the material to the tissues.[5] The new generation of bioactive materials, known as third-generation materials, comprises all those bioreabsorbable and/or biodegradable materials able to stimulate specific cellular responses at the molecular level as a result of the release of ions.[5] A growing number of publications have shown experimental evidence about the biological effects of the ionic dissolution products released from bioceramic materials.[6-10] Within this context, it is important to mention the progress made with bioactive glasses (BGs).[6,8-10] The most widely used BGs are amorphous solids based on a silicate matrix.[11-14] Although a growing number of publications have shown that the BGs in the 45S5 system (in weight %: 45% SiO_2, 24.5% Na_2O, 24.5% CaO, 6% P_2O_5 (Bioglass®)) or related compositions stimulate angiogenesis[15], the specific mechanisms that accelerate neovascularization in the presence of the ions released by the dissolution of BGs of different chemical composition have not yet been elucidated.

The rationale for incorporating boron (B) to BGs as biomaterials for osseous implants and bone tissue-engineering scaffolds is the emerging scientific evidence that B has well-defined biological effects such as stimulation of bone healing *in vivo* and angiogenesis *in vitro*.[16-18] Noteworthy, Brown *et al.*[19] reported that with increasing B_2O_3 content, greater than ~1/3 of the SiO_2 in the 45S5 BG system, the glasses produced a greater inhibition of cell proliferation, particularly in static culture conditions.

We have previously demonstrated that microparticles of a boron-modified 45S5 composition, containing 2 wt% B_2O_3 (45S5.2B), enhance bone formation more than 45S5 glass particles when implanted into the intramedullary canal of rat tibiae.[16] Moreover, human umbilical vein endothelial cells (HUVECs) possess greater proliferative and migratory response, greater tubule formation capacity *in vitro* and greater secretion of pro-angiogenic cytokines (IL-6 and bFGF) when stimulated with the dissolution products from 45S5.2B BG.[18] Therefore, the aim of the present study was to assess the *in vivo* angiogenic effects of the ionic dissolution products released from 45S5.2B BG prepared by partially replacing the SiO_2 content of 45S5 BG with 2 wt% of B_2O_3.

2. Materials and methods

2.1. Obtention and characterization of ionic dissolution products from bioactive glasses

We used the following bioactive glasses (BGs): 45S5 and 45S5 doped with B (45S5.2B), previously developed in our laboratory.[16,18] The base composition selected for the preparation of these BGs complies with the following characteristics: a) it is bioactive and b) it belongs to the

SiO_2-CaO-Na_2O-P_2O_5 system. The BGs were prepared from the following raw materials: SiO_2, $CaCO_3$, Na_2CO_3, $CaHPO_4.2H_2O$, and $B_4Na_2O_7.10H_2O$. The raw materials were mixed in the relationship necessary to obtain the desired composition in a base 45S5 BG containing 45% (in weight %) SiO_2, 24.5% Na_2O, 24.5% CaO, and 6% P_2O_5, and to which 2% of B_2O_3 (45S5.2B) was added to partially replace SiO_2.[16,18] Mixing was carried out in a platinum crucible at 1350°C using an electric oven (Carbolite RHF 17/6S, Carbolite Ltd, England) holding the temperature for 3 h to allow the fusion of the components and the homogenization of the glass. The molten glass was quenched on graphite plates to allow fast cooling and thus prevent its crystallization. Considering that the use of particles smaller than or equal to 5 µm allows a fast in vitro release of ions from 45S5 BG[20], in the present study, the ionic dissolution products were obtained by incubating 1 mg/mL of 45S5 BG or 45S5.2B BG particles (<5 µm) in a Ca and Mg free Hanks balanced salt solution (HBSS) at 37°C on an orbital shaker for 24 h. The ionic dissolution products were buffered (pH 7) and then filtered through a 0.22-µm filter to be used in the different experiments. The B, Si, P, Ca, and Na ions leached from the BGs were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 3100 XL, Perkin Elmer, Wellsley, MA, USA) (Table 1).

Table 1. Elemental concentration determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (mean ± SD).

	B (µM)	**Si (µg/mL)**	**P (µg/mL)**	**Ca (µg/mL)**	**Na (µg/mL)**
HBSS	Bld	Bld	20 ± 0.6	Bld	3451 ± 51
HBSS + 45S5	Bld	Bld	4.5 ± 0.08	12.8 ± 0.3	3467 ± 102
HBSS + 45S5.2B	160 ± 10	Bld	4.4 ± 0.11	12 ± 0.3	3498 ± 40

SD: standard deviation; Bld: below the limit of detection.

2.2. Evaluation of the angiogenic response

The angiogenic effects were evaluated on the vasculature of the chorioallantoic membrane (CAM) of quail (*Coturnix coturnix japonica*) embryos according to the method described by Parsons-Wingerter *et al*.[21] Quail egg culture and experimental methods were approved by the institutional ethics committee of the School of Dentistry, University of Buenos Aires, Argentina. Humane care of the animals were carried out according to the Guide for the Care and Use of Laboratory Animals, National Research Council (USA), 2010.

Fertilized eggs were incubated *in ovo* for 3 days at 38°C and 60% relative humidity. Subsequently, their content was poured in six-well culture plates. After 7 days of total incubation, either 0.5 mL HBSS enriched with ionic dissolution products (HBSS+45S5; HBSS+45S5.2B) or 0.5 mL HBSS enriched with ionic dissolution products supplemented with 10 µg/mL of basic fibroblast growth factor (bFGF) (HBSS+45S5/bFGF; HBSS+45S5.2B/bFGF) was added on the CAMs. HBSS supplemented with bFGF (HBSS+bFGF) was used as a positive control, whereas

HBSS with no ionic products or bFGF was used as a negative control. We also evaluated the effect of HBSS enriched with a final borate concentrations of 5 μM, 50 μM and 150 μM. Each experiment included ten embryos per group and was repeated twice. At 2 and 5 days post-treatment, embryos were killed by freezing (-20°C), fixed in 10% formalin buffer and the external morphological characters described by Ainsworth *et al*[22] evaluated with the aim to determine the embryonic stage reached. CAMs were then processed for further analysis according to the technical protocol for each of the procedures described below:

(a) Evaluation of the expression of integrin $\alpha_v\beta_3$ as an indicator parameter of angiogenesis

ELISA

An ELISA was conducted to determine the endogenous levels of integrin $\alpha_v\beta_3$. At 2 days post-treatment, CAMs were homogenized in PBS, and 10 μg of total protein (as determined by the Bradford assay) was used to sensitize 96-well plates, in triplicate. The plates were dried at 37°C, blocked with 5% BSA in PBS, then incubated with the anti-$\alpha_v\beta_3$ monoclonal antibody (Millipore LM609 1 μg/mL), washed three times with PBS and incubated with rabbit anti-mouse IgG antibody conjugated to peroxidase (Sigma A9044 - 2ML), which was revealed with 3,3',5,5' - tetramethyl-benzidine (Sigma T4444). The optical density was determined in an ELISA reader at 492 nm.

Polyacrylamide gel electrophoresis

To analyze the expression levels of the β_3 subunit of integrin $\alpha_v\beta_3$, CAMs were homogenized in 1 mL of radio-immunoprecipitation assay lysis buffer [100 mM Tris pH 7.5, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 2 mM phenylmethanesulfonyl fluoride and the phosphatase inhibitors NaF (500 mM) and vanadate (1 mM)] and centrifuged for 15 min at 15000 g and the amount of total protein determined by the Bradford assay. Equivalent amounts of protein were heated (at 90°C for 5 min) in loading buffer (50 mM Tris-HCl, pH 6.6, 2% SDS, 10% glycerol and 0.05% bromophenol blue) and loaded in an 8% mini-polyacrylamide gel. Electrophoresis was developed in 0.025 M Tris buffer, 0.192 M glycine, 0.1% SDS, pH 8.3 at a constant current of 35 mA/gel in an electrophoresis cell (Mini - Protean® II, Bio-Rad).

Western Blot

The polyacrylamide gels were balanced in transfer buffer (25 mM Tris-HCl, pH 8.3, 150 mM glycine, 20% (v/v) methanol) for 15 min and the proteins transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences, 0.45 μm RPN303D) at 100 V for 1 h at 4°C in an electrophoretic transfer cell (Mini-Trans-Blot, Bio-Rad). Proteins were then stained in a solution with 0.2% Ponceau S in 0.5% acetic acid to visualize the total proteins and verify the effectiveness of the transfer in all lanes. The membranes were blocked with 5% skim milk powder and 0.05% Tween-20 in PBS (PBS-L-T) overnight at 4ºC, washed three times with 0.05% Tween-20 in PBS and incubated for 24 h with the anti-β_3 IgG polyclonal primary antibody produced in rabbit in a 1:500 dilution (Santa Cruz Biotechnology sc-14009) in PBS-L-T at 4°C. CAMs were then washed and detection was carried out by incubating for 2 h with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase in a 1:2000 dilution (Santa Cruz

Biotechnology sc-2004) in blocking buffer at room temperature. CAMs were then washed and incubated for 1 min with a chemiluminescent substrate for peroxidase (ECL™, Amersham Biosciences). The result was visualized by autoradiography of the chemiluminescent reaction.

(b) Quantification of angiogenesis

CAMs corresponding to 2 and 5 days post-treatment were fixed *in situ* in 4% paraformaldehyde/ 2% glutaraldehyde in PBS. After 3 days, CAMs were resected and analyzed under a stereomicroscope maintained at one focal plane. Images were taken from three different areas of the CAMs delimited with Whatman filter paper discs (5 mm in diameter). The vascular density was evaluated with ImageJ according to the method described by Brooks *et al*[23], which consists in quantifying the number of blood vessel branch points in the area of the CAM (˜20 mm^2) confined to each disk.

3. Statistical analysis

The results were statistically analyzed assuming α=0.05 and β=0.10. For all the data, we calculated the mean ± standard deviation. The results were analyzed by analysis of variance (ANOVA) and Bonferroni was used as a post-hoc test.

4. Results

(a) Evaluation of the expression of integrin $\alpha_v\beta_3$

The ELISA showed that the levels of expression of integrin $\alpha_v\beta_3$ in CAMs 2 days after the treatment with ionic dissolution products from the bioactive glass 45S5.2B were 2.5-3-fold higher than in those treated with HBSS (Fig. 1). In addition, the Western blot showed greater expression of the β_3 subunit of integrin $\alpha_v\beta_3$ (Fig. 2).

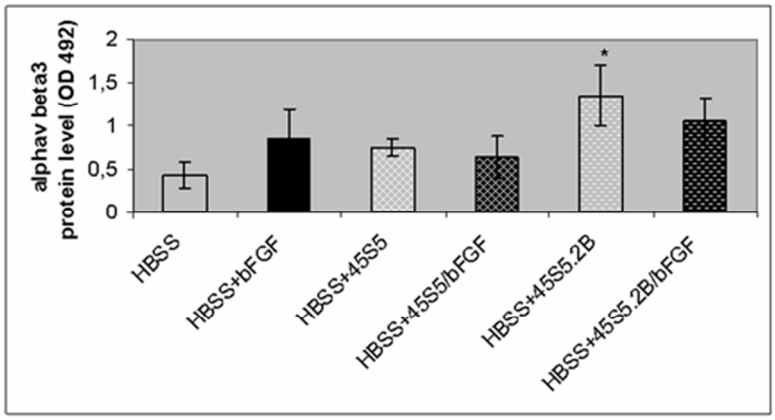
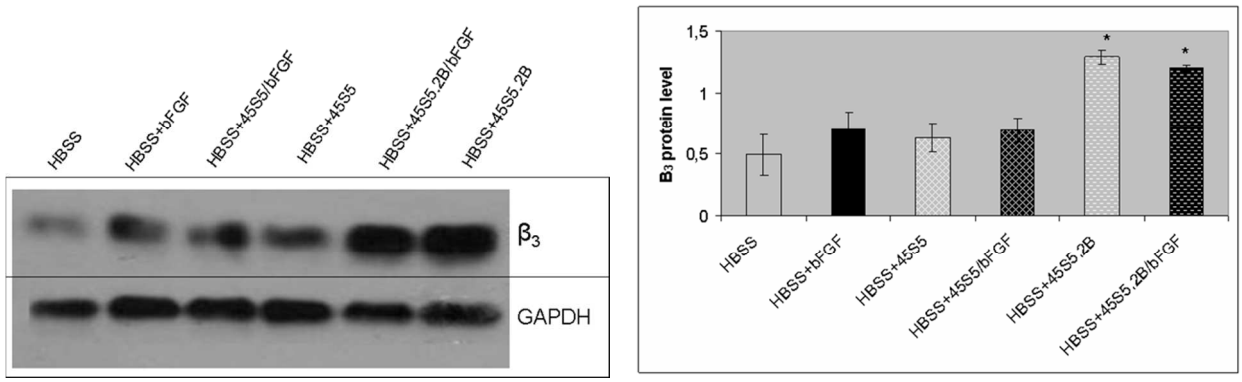


Fig. 1 Endogenous expression levels of integrin αvβ3 in CAMs at two days post-treatment (*p<0.05). OD: optical density.



a **b**

Fig. 2 Western blot (a) and relative expression of the β3 subunit of integrin αvβ3 (b). (*p<0.05 with respect to HBSS and HBSS+bFGF).

(b) Quantification of the angiogenic response

Two days after the treatment, in comparison to the negative control (HBSS), there were no statistically significant differences in the vascular density of CAMs treated with HBSS+45S5 or HBSS+45S5/bFGF. In contrast, both the CAMs treated with HBSS+45S5.2B and those treated with HBSS+45S5.2B/bFGF showed higher vascular density, 58% and 84%. The response observed was equivalent to that achieved by applying 10 µg/mL of basic fibroblast growth factor (HBSS+bFGF) (Fig. 3 and 4).

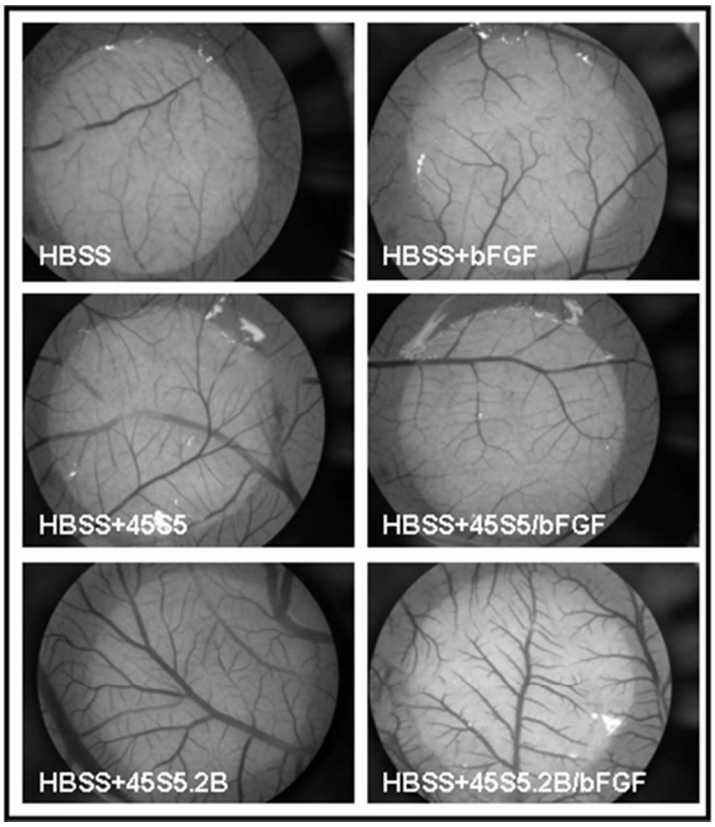


Fig. 3 Angiogenic response of CAMs at two days post-treatment. Original magnification X20.

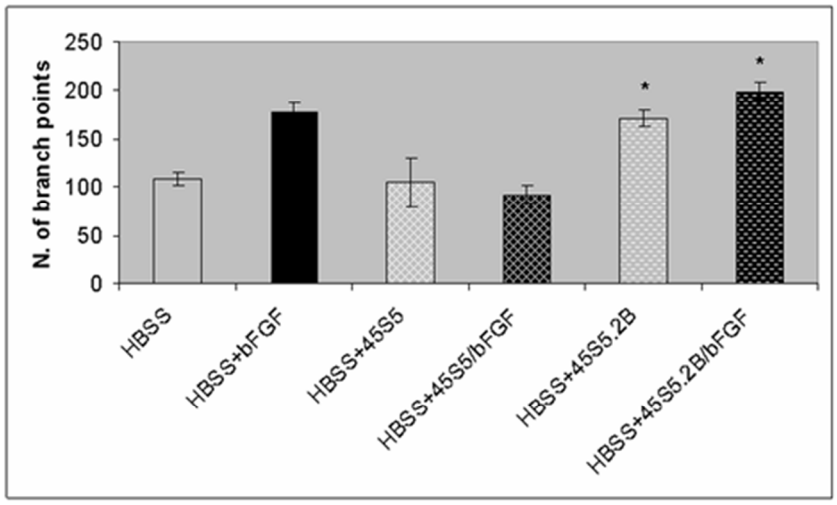


Fig. 4 Number of blood vessel branch points at two days post-treatment (*$p<0.05$ with respect to HBSS).

Five days after the treatment, in comparison to the negative control (HBSS), we found no statistically significant differences in the vascular density of CAMs treated with HBSS+45S5 or HBSS+45S5/bFGF. In contrast, both the CAMs treated with HBSS+45S5.2B and those treated with HBSS+45S5.2B/bFGF showed higher vascular density, 30% and 73%, respectively, being this response equivalent to that observed after the treatment with HBSS+bFGF (Fig. 5 and 6).

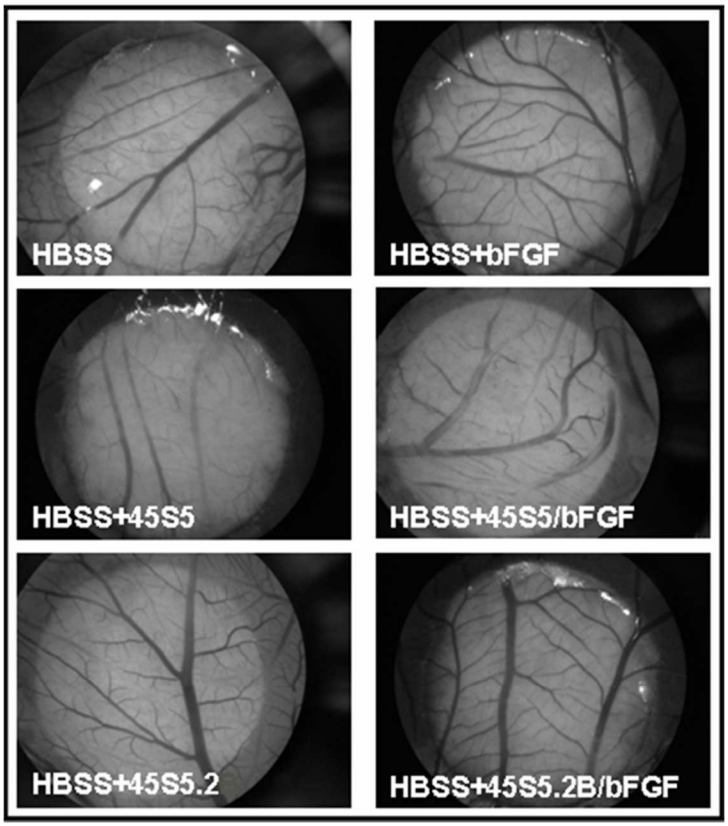


Fig. 5 Angiogenic response of CAMs at five days post-treatment. Original magnification X20.

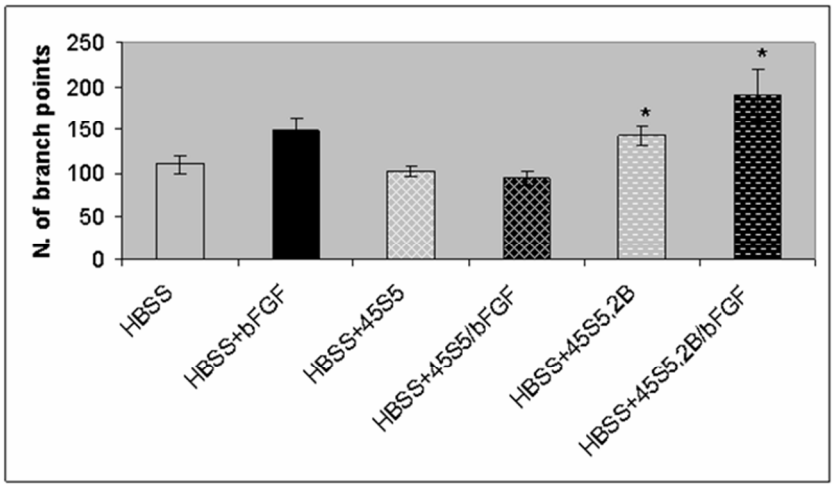
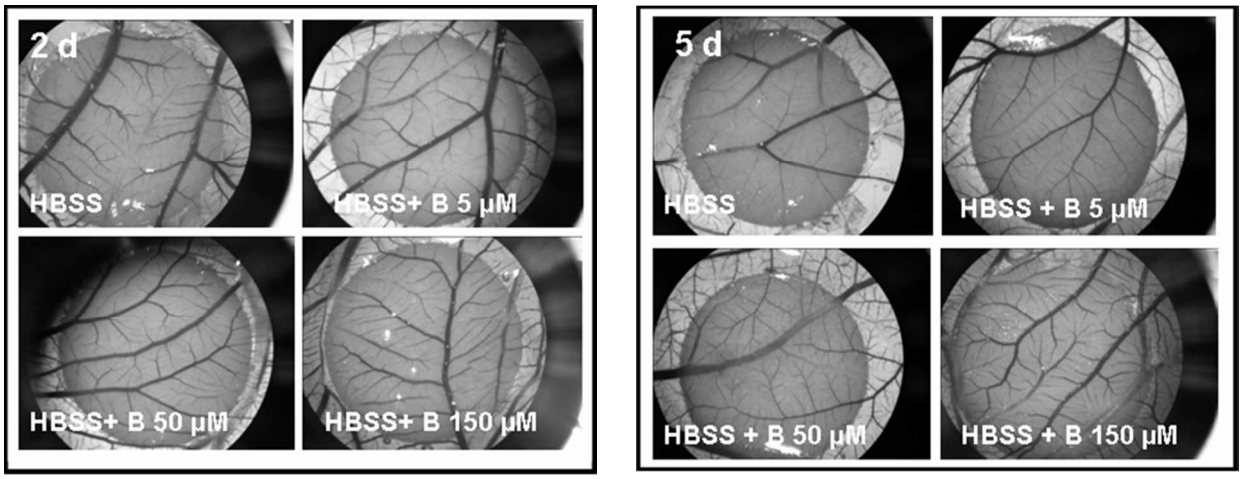


Fig. 6 Number of blood vessel branch points at five days post-treatment (*$p<0.05$ with respect to HBSS).

The effect of HBSS with different borate concentrations was evaluated to establish whether the presence of borate is responsible for the higher angiogenic activity observed in the CAMs treated with HBSS enriched with the dissolution products from the bioactive glass 45S5.2B. The results showed that, relative to the control (HBSS), all the borate concentrations used (5, 50, and 150 μM) had pro-angiogenic capacity *in vivo* (Fig. 7), evidencing greater vascular density in CAMs corresponding to 5 days post-treatment. It is important to point out that no statistically significant differences were found in the response achieved with the application of HBSS containing 50 or 150 μM borate at 2 or 5 days post-treatment (Fig. 8).



a **b**

Fig. 7 Angiogenic response of CAMs (a) at two and (b) five days post-treatment with HBSS with different borate concentrations . Original magnification X20.

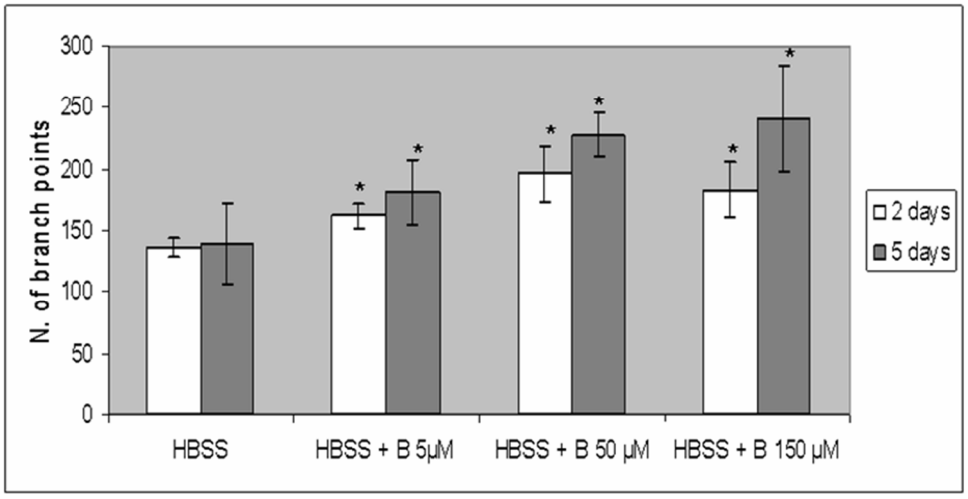


Fig. 8 Number of blood vessel branch points at two and five days post-treatment with HBSS with different borate concentrations (*$p<0.05$ with respect to HBSS).

5.Discussion

The chorioallantoic membrane (CAM) of quail embryos has been widely used as an *in vivo* experimental model to evaluate angiogenesis and anti-angiogenesis in response to different factors.[21, 24-27] Although in the present study the ionic dissolution products of the bioactive glass (BG) 45S5 showed no angiogenic capacity, they did not affect the normal development of the vasculature of embryonic quail CAMs. This observation is in line with previous studies,[28,29] which found that 45S5 Bioglass®-based scaffolds were unable to induce an angiogenic response in the chick embryo CAM. These results are probably associated with an insufficient ions release from the scaffolds due to the smaller contact area of the scaffolds to the CAM. In contrast, Arkudas *et al* [30] were able to show an increased neovascularization of a particulated sintered 45S5 Bioglass® matrix in the rat arteriovenous loop model.

Recent publications indicate that silicon (Si) ions (0.7-1.8 μg/mL) released from silicate-based ceramic materials stimulate the *in vitro* secretion of pro-angiogenic growth factors, proliferation of endothelial cells and promote angiogenesis *in vivo*.[31,32] The ionic dissolution products used in the present work have insufficient concentration of Si given that they were obtained using HBSS containing glucose (1g/L), agent known to delay the dissolution of BGs.[33] However, our preliminary degradation studies in HBSS for 72h revealed that Si concentrations leached from particles (< 5 μm) of 45S5 BG and 45S5.2B BG were 18.79 μg/mL and 17.82 μg/mL, respectively, while the amount of borate ions released from the 45S5.2B BG particles was about 20 μM. The combination of Si ions with other ions including B released from bioactive glasses might have synergistic effect and further enhance the angiogenesis.[10]

The use of ionic dissolution products released from the 45S5.2B BG showed an increase in angiogenesis, quantitatively evidenced by the greater expression of integrin $\alpha_v\beta_3$ and the greater vascular density in CAMs treated with HBSS+45S5.2B or HBSS + 45S5.2B supplemented with basic fibroblast growth factor (bFGF).

One of the major concerns regarding *in vivo* delivery of bioactive ions from biomaterials is whether the released amount of ions is within the therapeutic level to have any positive effect.[7,8,10] HBSS+45S5.2B contains 160±10 μM borate, a concentration that is below the reference values with which cytotoxic effects become apparent[34] and that represents approximately ten times the normal concentration of B in human plasma (13 ±3 μM).[34,35]

Considering also that HBSS with different borate concentrations (5, 50, and 150 μM) demonstrated pro-angiogenic ability in CAMs, it can be assumed that the presence of borate in the ionic dissolution products from 45S5.2B BG is the factor responsible for the greater angiogenic activity. It has been demonstrated that B in the form of H_3BO_3 potently activates the mitogen-activated protein kinase (MAPK) signaling pathway to markedly increase cell proliferation and growth at low concentrations and inhibits these activities at high concentrations.[34] Accordingly, we have previously shown that the ionic dissolution products from 45S5.2B BG stimulated human umbilical vein endothelial cells (HUVECs) proliferation and migration by phosphorylation of extracellular signal-related kinase (ERK) 1/2, focal adhesion kinase (FAK) and p38 protein. It was also shown that ionic dissolution products from 45S5.2B BG could enhance *in vitro* HUVECs tubule formation and secretion of interleukin 6 (IL6) and bFGF.[18] The effects observed in the present study are probably due to the fact that borate stimulates the secretion of cytokines and/or growth factors with pro-angiogenic activity and the proliferation of endothelial cells of the CAM vasculature, as evidenced by the higher expression of integrin $\alpha_v\beta_3$. The process of angiogenesis involves coordinated endothelial cell proliferation,

migration and tube formation. This process is induced by vascular growth factors in coordination with extracellular matrix interacting molecules such as integrins. Among integrins, αvβ3 heterodimer is expressed at low levels on quiescent endothelial cells *in vivo*, but is significantly up-regulated on proliferating endothelial cells during angiogenesis and vascular remodeling.[36,37] It is important to note that the response observed at 2 and 5 days post-treatment with HBSS+45S5.2B was equivalent to that after the application of 10 µg/mL of bFGF. In this context, the ionic dissolution products from 45S5.2B BG may act as inexpensive inorganic angiogenic agents providing a convenient alternative to the application of conventional angiogenic growth factors. Concerns about the toxicity of B to cells *in vivo* have been alleviated by results showing that borate ions released from bioactive glasses are non-toxic in small animals. Recently, Lin *et al*[38] showed that high amounts (up to 1120 mg/animal) of 13–93B3 borate glass microfibers containing 53 wt% of B_2O_3 implanted subcutaneously in rats can be safely used with no systemic toxicity. Overall, it can be expected that the controlled and localized release of borate ions from 45S5.2B BG could represent a promising alternative therapeutic strategy to achieve neovascularization in regenerative medicine and tissue engineering of vascularized tissues, such as bone.

6. Conclusions

The results of this study demonstrate that the ionic dissolution products released from microparticles of the bioactive glass 45S5.2B stimulate angiogenesis *in vivo*. We attribute these effects to the presence of borate in the ionic dissolution products.

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Disclosure Statement

No competing financial interests exist.

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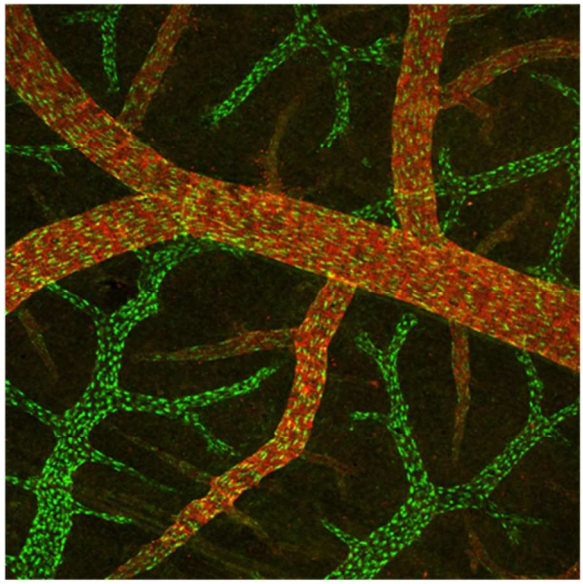

In vivo angiogenic effects of the ionic dissolution products released from microparticles of a boron-modified 45S5 bioactive glass (BG) prepared by partially replacing the SiO_2 content of 45S5 BG with 2 wt% of B_2O_3