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Angiogenic potential of boron-containing bioactive glasses: in vitro study

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ABSTRACT

Boron-containing bioactive glasses (BGs) are being extensively researched for the treatment and regeneration of bone defects because of their osteostimulatory and neovascularization potential. In this study, we report the effects of the ionic dissolution products (IDPs) of different boron-doped, borosilicate, and borate BG scaffolds on mouse bone marrow stromal cells in vitro, using an angiogenesis assay. Five different BG scaffolds of the system SiO_2–Na_2O–K_2O–MgO–CaO–P_2O_5–B_2O_3 (with varying amounts of SiO_2 and B_2O_3) were fabricated by the foam replication technique. Bone marrow stromal cells were cultivated in contact with the IDPs of the boron-containing BG scaffolds at different concentrations for 48 h. The expression and secretion of vascular endothelial growth factor (VEGF) from the cultured cells was measured quantitatively using the VEGF ELISA Kit. Cell viability and cell morphology were determined using WST-8 assay and H&E staining, respectively. The cellular response was found to be dependent on boron content and the B release profile from the glasses corresponded to the positive or negative biological activity of the BGs.

Introduction

Achieving neovascularization is a major challenge in bone tissue engineering (TE) strategies [1]. The ability to induce vascular growth during tissue repair is critical for the successful regeneration of the new tissue as the transport of oxygen and nutrients required for the survival of cells is facilitated initially by diffusion. Bone is a highly vascularized tissue which depends on the close spatial and temporal connection and interaction between blood vessels and bone cells to maintain skeletal integrity. The importance of blood vessels in the development and repair of bone tissue was reported as early as in the 1700s [2]. Trueta et al. [3] proposed that a vascular stimulating factor (VSF) is released at the fracture site of bone. There are a number of growth factors involved in the neoangiogenesis, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), various members of the

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Table 1 Composition (in wt%) of the prepared BG scaffolds with varying B_2O_3 content

Composition	Na$_2$O	K$_2$O	MgO	CaO	B$_2$O$_3$	P$_2$O$_5$	SiO$_2$
0106	5.9	12.0	5.3	22.6	0.2	4.0	50.0
0106-B1	5.9	12.0	5.5	22.6	12.5	4.0	37.5
0106-B2	5.9	12.0	5.5	22.6	25.0	4.0	25.0
13-93B3	5.5	11.1	4.6	18.5	56.6	3.7	0
13-93	6.0	12.0	5.0	20.0	0.0	4.0	53.0

transforming growth factor beta (TGFβ) family, and hypoxia related growth factors such as hypoxia-inducible factor (HIF) [2]. Among these, endogenous VEGF is important for bone formation and repair and it is expressed in a similar pattern during both processes [4, 5]. Mesenchymal stromal cells are a small subset of mesenchymal stem cells and were first reported by Friedenstein et al. [6, 7] as hematopoietic supportive cells in the bone marrow. They demonstrated that these cells were nonphagocytic, exhibiting a fibroblast-like appearance, and they could differentiate in vitro into bone, cartilage, adipose tissue, tendon, and muscle cells [8, 9].

The growth and development of a mature vascular tissue is one of the earliest events in organogenesis [10]. This requires the presence of a three-dimensional environment which, in TE strategies, is provided by the scaffolds. Thus, if the biomaterial-based scaffolds can promote vascularization, this will increase the viability of cells present within the matrix, thereby enhancing the possibility of engineering larger tissues [11]. In recent years, in vitro and in vivo studies have shown that bioactive glasses (BGs) in biomaterial-based TE applications are capable of stimulating angiogenesis and vascularization [12]. In vitro studies have demonstrated that ionic dissolution products (IDPs) of several BG compositions can induce the secretion of VEGF from exposed cells to induce vascularization [12–16]. In addition, it has been shown that the particle size and concentration of BG particles of composition (wt%) 53% SiO_2, 23% Na_2O, 20% CaO, 4% P_2O_5 (known as S53P4 composition) influences the expression of VEGF in human fibroblasts to stimulate angiogenesis [17]. Boron has been considered an important component in BGs exhibiting osteogenic and angiogenic effects [15, 18]. Durand et al. [19] for example, showed that the presence of boron in the IDPs of a 45S5 BG composition doped with 2 wt% B_2O_3 stimulated angiogenesis in vivo. Borate glass, in the

form of three-dimensional scaffolds has been shown to promote angiogenesis during bone repair in rodent calvarial defects [20–22].

In this study, we report for the first time on the interaction of stromal (ST2) cells and the IDPs of scaffolds made from a series of B-containing glasses of varying B_2O_3 and SiO_2 contents. The cell viability and VEGF secretion of ST2 cells exposed to IDPs were quantified. The influence of the presence/absence of varying amounts of B ions, with respect to cell viability, cell morphology, and release of angiogenic agents is discussed.

Materials and methods

Preparation of samples and cells for cells culture

The stimulation of VEGF secretion by BG scaffolds was assessed using ST2 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunscgweig, Germany) derived from mouse bone marrow of BC8 mice. The bone marrow stromal cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in cell culture medium (CCM) containing RPMI 1640 medium (Gibco, Germany), 10 vol% fetal bovine serum (FBS, Sigma-Aldrich, Germany), and 1 vol% penicillin/streptomycin (Gibco, Germany). Cells were grown to confluence in 75 cm^2 culture flasks (Nunc, Denmark), harvested using Trypsin/EDTA (Gibco, Germany), and counted using a hemocytometer (Roth, Germany).

The compositions of the BGs used are shown in Table 1. The fabrication of the five types of BG scaffolds was carried out as reported previously [23]. In brief, melt-derived glass frits of 0106, 0106-B1, 0106-B2, 13-93B3, and 13-93 BGs were crushed and ground to fine powders of 2–5 µm size using a Jaw Crusher (Retsch, Germany). Three-dimensional scaffolds were prepared using the foam replication technique [24] wherein, the glass powders are dispersed in de-ionized water along with a binder to form a slurry. In the case of 0106 and 13-93 glasses, polyvinylalcohol was used as the binder, and for 0106-B1, 0106-B2, and 13-93B3, ethylcellulose was used as a binder. Cubic polyurethane foams were dipped into the glass slurry, allowed to dry overnight, and then sintered according to the characteristic temperature of each glass [23]. In all cases,

Figure 1 Schematic representation of the experimental set-up for the cell biology study.
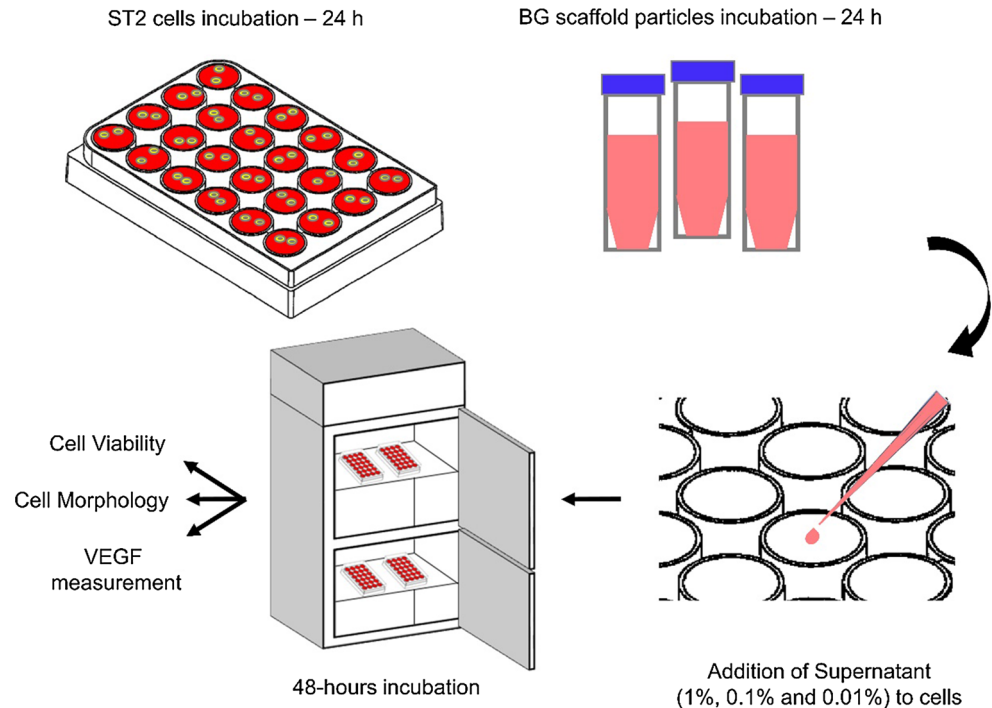
scaffolds of nominal dimensions of $7 \times 7 \times 7$ mm^3 were produced. The five types of BG scaffolds were sterilized at 160 °C for 2 h in a furnace (Naberthem, Germany). All scaffolds were pre-incubated in RPMI for 48 h. They were then removed from the solution, washed with PBS, and allowed to dry in air.

The experimental set-up for the cell biology study is schematically described in Fig. 1. To prepare cell cultures, 1,000,000 ST2 cells were seeded in 1 ml CCM in 24-well plates and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Next, the scaffolds were crushed using a mortar and pestle, and 0.1 g of the crushed scaffold powder was weighed. This 0.1 g of crushed scaffold granules was added to 10 ml of CCM to form a 1% suspension, which was incubated for 24 h in similar conditions. After 24 h, the 10 ml CCM with the IDPs of the crushed BG scaffold granules were diluted to form concentrations of 0.1 and 0.01 wt/vol% of BG in CCM. The CCM from the cells was removed, and 1 ml of 1, 0.1, and 0.01 wt/vol% of BG in CCM was added to the now-attached ST2 cells in 24-well plates and incubated in similar conditions as mentioned above. The duration of the study was 48 h, and the cell culture medium was not changed during this time. The cells seeded directly on well plates were taken as a reference. Every sample type was investigated in six replicates.

Cell viability

The viability of the cultivated cells was evaluated using a WST-8 assay (Sigma-Aldrich) after a cultivation period of 48 h. By analyzing the conversion of tetrazolium to formazan by mitochondrial enzymes, the viability of ST2 cells could be determined. A solution containing 1 vol% WST reagent and 99 vol% RPMI was prepared. The CCM was extracted into Eppendorf tubes, to be taken for VEGF release measurement studies, and 400 µl of the prepared WST solution was added to the samples and incubated for 2 h. Then, 100 µl of the solution was extracted from the samples and transferred to a 96-well plate. The supernatant was spectrometrically analyzed using a microplate reader (PHOmo, anthos Mikrosysteme GmbH, Germany) at 450 nm.

VEGF measurement

The quantity of VEGF secreted by the ST2 cells in the presence of the IDPs of the BG scaffolds was measured using RayBio Mouse VEGF ELISA (Enzyme-Linked Immunosorbent Assay) kit. This assay measures accurately the recombinant mouse VEGF in cell culture supernatants by engaging an antibody specific for mouse VEGF. The assay was carried out according to the protocol supplied by the manufacturer. Briefly, the extracted cell culture
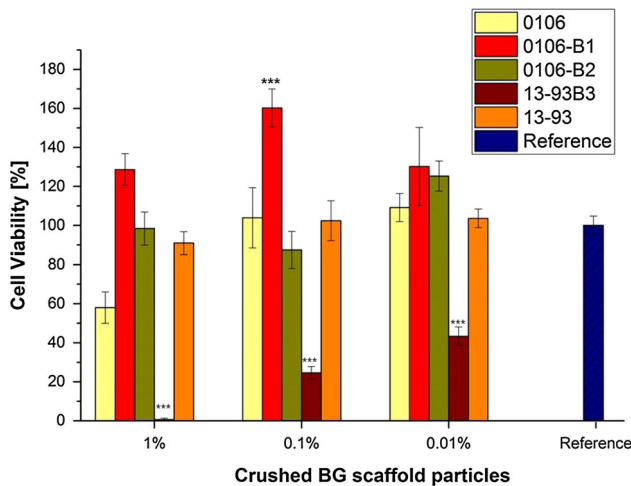
Figure 2 Relative viability of ST2 cells cultured with IDPs of BG scaffold particles of different BG composition and dilutions. *Asterisks* denote significant difference compared with the reference, ***$p < 0.001$ (Bonferroni's post hoc test was used).
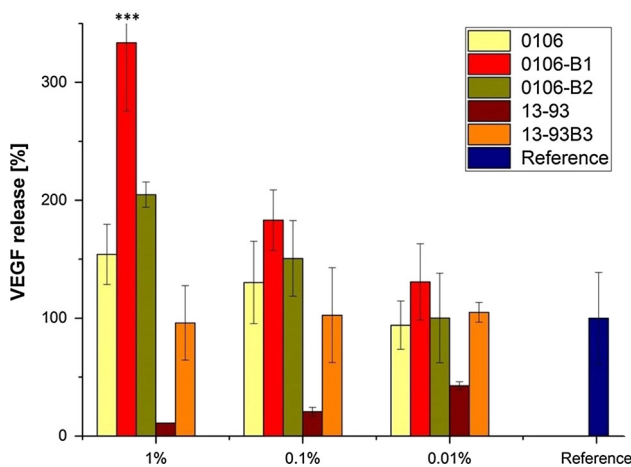
Figure 3 VEGF release from ST2 cells treated with different dilutions of IDPs of BG scaffold particles of different compositions. *Asterisks* denote significant difference compared with the reference, ***$p < 0.001$ (Bonferroni's post hoc test was used).

supernatants and standards of known VEGF concentrations were transferred to a 96-well plate, provided along with the kit, which is pre-coated with the mouse antibody, and they were allowed to complex with the bound VEGF antibody for 2.5 h. Following further reactions, a change in color from blue to yellow was detected and it was measured spectrometrically at 450 nm using a microplate reader (PHOmo, anthos Mikrosysteme GmbH, Germany). The intensity of the colored solution is directly proportional to

the concentration of VEGF secreted by the ST2 cells in the presence of the BG particles.

Cell morphology

In order to observe the morphology of the cells cultivated with different dilutions of the IDPs of the BG scaffolds, H&E (Hematoxylin & Eosin) staining was performed. Hematoxylin binds to the DNA/RNA and stain them dark blue or violet. Eosin binds to the amino acids/protein including cytoplasmic filaments and intracellular membranes and stains them red/pink. Once the WST solution was extracted for measurement, the cells attached to the well plates were washed once with PBS and were fixed using Fluoro-fix. Subsequently, samples were washed with de-ionized water and stained with Hematoxylin for 10 min. The samples were washed with tap water, followed by 'Scott's tap water' and then using de-ionized water for 1–5 min to remove all Hematoxylin. Eosin solution containing 0.1% Eosin in 90% ethanol and 5% acetic acid was prepared. The samples were then stained with the prepared Eosin solution for 1–5 min. Further washing with 95% and 100% ethanol was done, and the samples were observed under a light microscope (Primo Vert, Carl Zeiss).

Results

Cell viability

The cell viability of ST2 cells in the presence of the different BG scaffold powders in different concentrations is shown in Fig. 2. The reference, which relates to cells cultured only with cell culture medium, was normalized to 100%. This value was taken as a control, and significant differences were determined in comparison with the reference.

Quantitative assessment after 48 h of culture shows that there is an increase in cell viability with decrease in the concentration of glass particles in the cell culture medium. It can be seen that for all glasses, except 0106-B1, cell viability was the highest for the 0.01% concentration. In the case of 0106-B1, the highest cell viability was achieved by the 0.1% concentration and no difference was observed between the 1 and 0.01% concentrations. The 13-93B3 glass particles were found to be cytotoxic, considering the much lower viability detected for ST2 cells. The 0106-B1 glass of
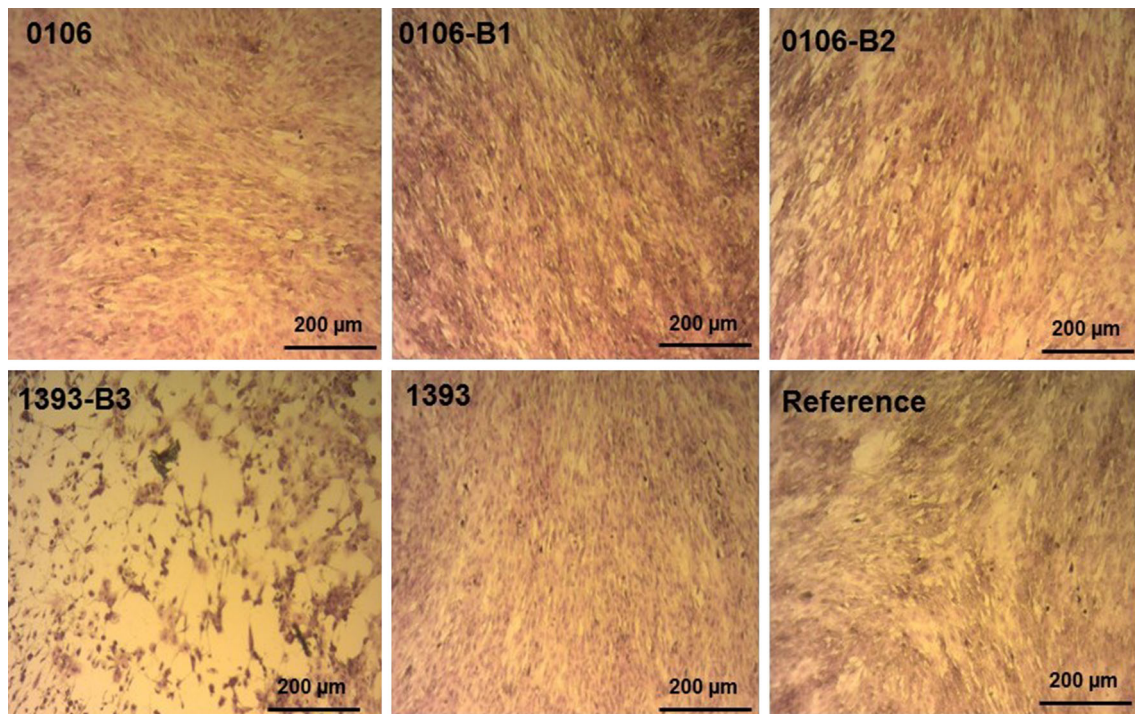
Figure 4 Light microscopy images of H&E-stained ST2 cells cultured with 0.1% dilution of BG scaffold particles of different compositions (Table 1).

0.1% dilution exhibited a significantly higher cell viability compared to the reference. In addition, the 0106-B1 glass resulted in higher cell viability for all three dilutions.

VEGF measurement

In Fig. 3, the VEGF release from ST2 cells cultured in CCM with different dilutions (1, 0.1 and 0.01%) of IDPs of boron-containing BG scaffold particles is shown.

The 0106, 0106-B1, and 0106-B2 BGs increased VEGF secretion with increasing concentration of glass particles in cell culture medium. For the 13-93 composition, VEGF concentration remained almost the same for all three dilutions. The 0106-B1 sample showed the highest release of VEGF for all three dilutions compared to the other glasses. The 13-93B3 glass showed increase in VEGF release with decrease in the concentration. These results were also in accordance with the data obtained from the cell viability study. However, the 1% dilution of 0106-B1 BG scaffold showed a significant increase in the expression and secretion of VEGF in comparison with the reference.

Cell morphology

Light microscopy images of H&E-stained ST2 cells cultured with 0.1% dilution of all five compositions of BG scaffold particles are shown in Fig. 4. From the images, it can be seen that cells exhibited their phenotypical cell morphology and also showed adhesion to the well plate. No obvious difference in the morphology and spreading of the ST2 cells could be observed. What stands out distinctly is the 13-93B3 images, where relatively poor cell proliferation and adhesion could be qualitatively noted. The 0106-B1 and 0106-B2 BGs show a higher cell density than the reference. However, this is based on visual observation only and quantitative data should be obtained in future studies. Since similar images were obtained for the 1% and 0.01% dilutions, results are not shown here.

Discussion

The role of angiogenic factors in early bone development is indispensable. The mesenchymal precursors differentiate into cartilage cells and expand to form a structure which will lay the foundation for the
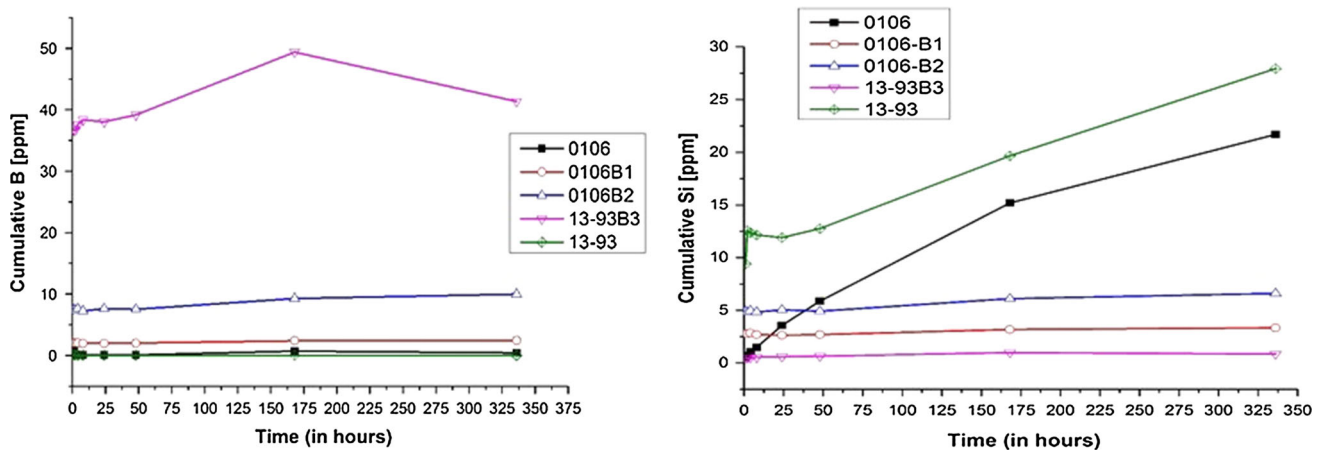
Figure 5 Release of B and Si from BG scaffolds of different compositions after immersion in RPMI for 14 days (Reproduced with permission from Ref. [23]).

bone. Chondrocytes undergo hypertrophic differentiation and lay down an extracellular matrix with different composition to that of cartilage [2]. VEGF A, a signaling molecule involved in early blood vessel development, is secreted by the hypertrophic chondrocytes and induces angiogenesis from the perichondrium resulting in the recruitment of osteoblasts, osteoclasts, and haematopoietic cells [2]. Thus chondrocyte proliferation, hypertrophy, apoptosis, and vascular invasion lead to the longitudinal growth of long bones [25]. It is well known that VEGF is an essential coordinator of chondrocyte death, chondroclast function, extracellular matrix remodeling, angiogenesis, and bone formation in the growth plate [26]. Moreover, blocking of VEGF activity results in the decrease in primary osteoblast differentiation in vitro [27] leading to an enlarged area of hypertrophic cartilage, loss of metaphyseal blood vessels, and impaired trabecular bone formation (in growing mice) [26, 28].

Low concentrations of boron released into cell culture medium have been shown to support better proliferation of MC3T3-E1 cells in vitro [29]. Boron ions (less than 100 ng/ml) have been shown to significantly improve bone-related gene expression and the formation of nodules [30]. Boron has been also reported to stimulate in vitro secretion of angiogenic growth factors [31–33], and the controlled and localized release of boron from BGs was shown to promote vascularization in vitro [31] and in vivo [19]. Boric acid is found to increase the production of RNA and translation of proteins ranging from 14 to

80 kDa, especially those encoding growth factors involved in angiogenesis and wound repair, such as VEGF and TGFβ [32].

The quantity of boron in the dissolution products of the five glasses investigated in this study has been discussed previously [23]. This dissolution profile, as shown in Fig. 5, has a direct relation to the angiogenic potential of the glasses. 0106 BG had a negligible release of boron and in turn, there is no notable enhancement in the cell viability or VEGF release from the ST2 cells in contact with the dissolution products of 0106 BG granules. Similar is the case for 13-93 BG which is a silicate glass with no boron. However, the VEGF secretion from the ST2 cells in contact with the IDPs of 0106 with 0.2 wt% B_2O_3 is higher than that for 13-93 glass (although no statistical significance was observed). The B release from 13-93B3 was quite high and correspondingly, a negative influence is found on cytocompatibility. The cell viability, VEGF secretion, and cell attachment were poor for 13-93B3. This result is in accordance with data in the literature [34], and although a boron release level as high as 50 ppm was measured for the 13-93B3 scaffolds, borate scaffolds are reported to support soft tissue infiltration and extracellular matrix formation in vivo irrespective of their cytotoxic behavior in vitro [20, 34]. A controlled release profile of boron ions is observed for 0106-B1 and 0106-B2 scaffolds in the range of 0–10 ppm, similar to the results reported by Wu et al. [35]. In addition, a moderate release of Si ions is also observed for the 0106-B1 and 0106-B2 glasses. The IDPs of 0106-B1 and 0106-B2 BG scaffolds promoted

mitochondrial activity of the ST2 cells, and a significant enhancement in the release of VEGF from the ionic release products of 0106-B1 is observed in our study. Park et al. [36] showed that boron in the form of H$_3$BO$_3$ triggers nitrogen-activated protein kinase signaling pathway which increases cell proliferation at lower concentrations and inhibits these activities when boron is present in higher concentrations. Of the presently investigated B-containing scaffolds, the composition 0106-B1 is superior with respect to cell viability and angiogenic potential. In addition, 0106-B1 BG scaffolds exhibited good compressive strength (in the range of 1.4 ± 0.3 MPa) and excellent cell adhesion and cell proliferation when cultured with ST2 cells [23]. Therefore, the developed 0106-B1 BG scaffolds are promising candidates for bone TE applications and further osteogenic differentiation studies are being carried out to understand their relevant biological capabilities as alternative to the standard 45S5 Bioglass® scaffolds [24].

Conclusion

Angiogenesis is key for bone repair. In this paper, the effect of culturing ST2 cells with different dilutions of the ionic dissolutions from 0106, 0106-B1, 0106-B2, 13-93B3, and 13-93 BG scaffolds is discussed. The dilutions of 0106-B1 gave the best results in terms of cell viability and VEGF secretion as well as formation of dense cell layer. The dissolution products from 13-93B3 were found to be cytotoxic for the cells. The influence of increase in boron content in the glass composition, in terms of angiogenic capabilities and cell viability, has been established. The results from our study show that the controlled release of ions (in particular, B and Si) is critical for VEGF secretion and cytocompatibility. It was asserted that the 0106-B1 glass has potential for stimulating angiogenesis, which could be exploited to great extent for applications in soft tissue repair, requiring vascularization, in addition to bone TE.

Acknowledgements

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