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In vitro endothelial cell response to ionic dissolution products from boron-doped bioactive glass in the SiO_2-CaO-P_2O_5-Na_2O system

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Abstract

As it has been established that boron (B) may perform functions in angiogenesis and osteogenesis, the controlled and localized release of B ions from bioactive glasses (BGs) is expected to provide a promising therapeutic alternative for regenerative medicine of vascularized tissues, such as bone. The aim of this study was to assess the *in vitro* angiogenic effects of the ionic dissolution products (IDPs) from BGs in the SiO_2-CaO-Na_2O-P_2O_5 (45S5) system and of those from 45S5 BG doped with 2 wt% B_2O_3 (45S5.2B). The results show, for the first time, that the IDPs from 45S5.2B BG stimulated human umbilical vein endothelial cells (HUVECs) proliferation and migration that were associated with phosphorylation of extracellular signal-related kinase (ERK) 1/2, focal adhesion kinase (FAK) and p38 protein. It was also shown that IDPs from 45S5.2B BG could enhance *in vitro* HUVECs tubule formation and secretion of interleukin 6 (IL6) and basic fibroblast growth factor (bFGF). The effects observed are attributed to the presence of B in the IDPs. These findings are relevant to bone tissue engineering and regeneration because the IDPs from 45S5.2B BG may act as inexpensive inorganic angiogenic agents providing a convenient alternative to the application of conventional angiogenic growth factors.

***Keywords*:** angiogenesis, bioactive glass, boron, regenerative medicine

1. Introduction

One of the main limitations in regenerative medicine of vascularized tissues is the difficulty to achieve a fast neovascularization, necessary for the transport and exchange of oxygen, nutrients, growth factors and cells involved in the process of tissue repair or regeneration.[1-7] Due to the insufficient angiogenic potential of the vast majority of natural and synthetic materials, tissue engineering procedures towards neovascularization have focused mainly on gene therapy and cell-based therapy, as well as on the addition of pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in various biomaterials.[8-16] However, no consensus has been reached about which strategy is ideal for this purpose. In this regard, and given their great therapeutic potential, there is increasing interest in developing biomaterials that have inherent capacity to modulate the angiogenic activity.[17-20] Although a growing number of publications have reported the pro-angiogenic potential of bioceramic materials, such as bioactive glasses (BGs), glass-ceramics and calcium phosphates[21-24], the specific mechanisms that accelerate neovascularization in the presence of the ions released by the dissolution of BGs of different chemical composition have not been elucidated.

Experimental evidence indicates that boron (B) stimulates *in vitro* secretion of pro-angiogenic growth factors.[25-27] However, the angiogenic effects of the B released from biomaterials have not yet been evaluated. B is a trace element that satisfies several criteria to be considered essential for animals and humans: a) it is present at comparable concentrations in healthy tissues from different animals, b) it has a homeostatic mechanism of control, and c) its deficiency results in the alteration or loss of important physiological functions associated with the metabolism of calcium and formation and remodeling of bone tissue.[28-34]

Given that it has been established that B plays roles in angiogenesis and osteogenesis, it can be expected that the controlled and localized release of B ions from BGs could represent a promising therapeutic alternative for the repair and regeneration of tissues that require a high degree of vascularization, such as bone tissue. Hench established that substitutions in the 45S5 BG formula up to 15 wt% B_2O_3 for SiO_2 has no measurable effect on the ability of the material to form a bone bond.[35] Accordingly, Brown *et al.* reported that with increasing B_2O_3 content, greater than ~1/3 of the SiO_2 in 45S5 BG, the glasses produced a greater inhibition of cell proliferation, particularly in static culture conditions.[36] However, Vrouwenvelder *et al*. showed that osteoblasts cultured on 5 wt% boron-containing 45S5 BG demonstrated lower proliferation and osteoblasts phenotype markers expression when compared with 45S5 BG.[37] The aim of this study was to assess the *in vitro* angiogenic effects of the ionic dissolution products (IDPs) from BGs in the SiO_2-CaO-Na_2O-P_2O_5 (45S5) system and of those from 45S5 BG doped with 2 wt% B_2O_3 (45S5.2B).

2. Experimental

2.1 Preparation and characterization of ionic dissolution products from bioactive glasses

We used the following BGs: 45S5 and 45S5 doped with B (45S5.2B), previously developed in our laboratory.[34] 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 $B_4Na_2O_7.10\ H_2O$. 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. Mixing was carried out in a platinum

crucible at 1350°C using a 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[38,39], in the present study, IDPs were obtained by incubating 45S5 BG or 45S5.2B BG particles (<5 μm) in M199 growing medium for 24 h on an orbital shaker at 37°C. The dose-dependency effect on dissolution and bioactivity is important for experiments that involve use of BG and BG-extracts, such as *in vitro* cell cultures. Previous work[39] has shown that as melt-derived 45S5 BG concentration increased above a value of 2 mg/mL in simulated body fluid (SBF) the concentration of active ions in solution increased but the formation rate of a biologically active hydroxycarbonate apatite layer on the surface of the glass decreased, showing a reduction in bioactivity.[39] Therefore, in the present study, IDPs were obtained by incubating 1 mg/mL of 45S5 BG or 45S5.2B BG particles in M199 culture medium. The medium was centrifuged and then filtered through a 0.22-μm filter and buffered (pH 7) to be used in the different experiments. To determine the optimum concentration of the IDPs for subsequent experiments, serial dilutions from original extracts of 45S5 BG and 45S5.2B BG were prepared using M199 medium. The preliminary results indicated that BG-extracts diluted at ratio 1/6 demonstrated non-cytotoxic effects. The soluble ions (B, Si, P, Ca, and Na) were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 3100 XL, Perkin Elmer, Wellsley, MA, USA). The ion concentrations of M199 and dilution 1/6 of M199 modified with IDPs from 45S5 BG (M199+45S5) and 45S5.2B BG (M199+45S5.2B) are detailed in Table 1.

2.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were used for the *in vitro* assays. HUVECs were cultured between passage +3 and passage +8 in T75 flasks at 37°C and 5% CO_2 atmosphere. The culture medium was M199 medium (GIBCO, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 50 μg/mL of gentamicin and supplemented with bFGF or VEGF at a final concentration of 2 ng/mL and 10 ng/mL respectively.

2.3 Proliferation assay

HUVECs were trypsinized, counted in the Neubauer chamber, and grown in 96-well plates at a density of 3,500 cells/well in 100 μL of M199 medium containing 10% FBS and 50 μg/mL gentamicin. After 2 h, 50 μL of M199 enriched in IDPs from both BGs (M199+45S5; M199+45S5.2B) or either supplemented with 2 ng/mL of bFGF (M199+45S5/bFGF; M199+45S5.2B/bFGF) or 10 ng/mL of VEGF (M199+45S5/VEGF; M199+45S5.2B/VEGF) was added. The assay was performed in quadruplicate.

We also studied the effect of M199 enriched with boron (H_3BO_3, Sigma, St. Louis, MO, USA) at the following final concentrations of B: 10 μM, 50 μM, 100 μM, 500 μM and 1 mM, either supplemented or not with bFGF (1 ng/mL) or VEGF (5 ng/mL). M199 with bFGF or VEGF was used as a positive control, whereas M199 without IDPs or growth factors was used as a negative control.

At 24 h, 25 μL/well of M199 containing methyl-[^{3}H]-thymidine to a final concentration of 2.5 μCi/mL was added and incubated for another 24 h. The reaction was stopped by the addition of 50 μL of 6 M guanidinium chloride. The cells were lysed completely through three cycles of freezing and thawing. Cellular DNA was collected on Whatman GF/C filters by using a cell harvester (*Cell Harvester 8*, Nunc, Rochester, NY, USA), fixed with ethanol 96%, and air-dried. The incorporated radioactivity was determined in the presence of 1 mL

of scintillation solution (OptiPhase HiSafe 3, Perkin-Elmer, Waltham, MA, USA) in a liquid scintillation counter (Packard 1600 TR, Canberra Company, Ramsey, MN, USA).

2.4 Endothelial wound healing assay

HUVECs were grown to confluence in 96-well plates (ETC International, Buenos Aires, Argentina), left overnight to ensure their adherence to the surface of the wells, and then a wound was created by a pipette tip. After this, the cells were washed three times with PBS to remove cell debris and detached cells, and then cultured for another 8 h in M199 medium enriched either with IDPs (M199+45S5; M199+45S5.2B) or with 2 ng/mL of bFGF (M199+bFGF). M199 medium without IDPs or bFGF was used as a negative control.

Photographs were taken immediately after the wound was made (time 0) and after 8 h. Cell migration was quantified using Image J (NIH, USA). The mean area of the wound is expressed as the percentage of recovery (% R) of five wells treated in the same way, by using the following equation: $\% R = [1-(A_t/A_0)] \times 100$, where A_0 is the area of the wound at time 0 and A_t is the wounded area after 8 h.[40]

2.5 Transwell assay

To the start of the assay, 500 µL of M199+45S5, M199+45S5.2B, M199+bFGF (as positive control), or M199 without IDPs or bFGF (as negative control) was placed in each well of Transwell® plates (Corning, New York, NY, USA), in triplicate. Then, the Transwell® inserts were placed and 20,000 HUVECs in 200 µL of M199 were seeded in the upper compartment of each insert. After 6 h of incubation, the inserts were removed and placed 10 min in Crystal Violet for fixation and staining of the cells. The cells were then washed with water and the residual cells that had not migrated through the microporous membrane were removed with a cotton swab. The analysis was performed on an inverted

optical microscope and pictures of two different areas were taken to quantify the number of cells that had transmigrated to the underside of the membranes.

2.6 Tubulogenesis on Matrigel ™

For tubulogenesis on Matrigel™, 60 µL of Matrigel™ (BD Biosciences, San José, CA, USA) was placed in each well of a 96-well plate and left to solidify for 30 min at 37°C before seeding the cells. Then, 10,000 HUVECs per well were seeded onto the surface of the Matrigel™ in 150 µL of M199+45S5, M199+45S5.2B, M199+45S5/bFGF, M199+45S5.2B/bFGF, M199+45S5/VEGF or M199+45S5.2B/VEGF.

The assay was performed in triplicate. M199 medium with VEGF and/or bFGF was used as positive control, whereas M199 without IDPs from the BGs or growth factors was used as negative control. The assay was monitored every 1 h for 6 h. At the end of the assay, cells were analyzed under an inverted optical microscope and pictures of two fields for each well were taken to quantify the number of completely closed 'tubules' present within each photographed area.

2.7 Enzyme-Linked ImmunoSorbent Assay (ELISA)

An ELISA was carried out to determine the secretion levels of pro-angiogenic cytokines such as: VEGF, bFGF, EGF, TNFα, TGF-β, IL6 and IGF1. HUVECs were grown to confluence in six-well culture plates, and then, after reaching the cell monolayer, stimulated with M199+45S5 or M199+45S5.2B. After 48 h, the supernatant was removed and the protocol continued following the manufacturer's instructions (EA-1011, Signosis, Santa Clara, CA, USA).

2.8 Evaluation of protein kinases associated with proliferation and cell migration

The activation of some protein kinases associated both with proliferation and cell migration, such as extracellular signal-related kinase (ERK) 1/2, focal adhesion kinase

(FAK) and p38 protein were examined by Western blot. HUVECs were grown to confluence in six-well culture plates. After reaching the monolayer, cells were stimulated for 30 min with IDPs from 45S5 BG and 45S5.2B BG. At 30 min post-stimulation, cells were washed three times with PBS to stop the reaction and then maintained for 24 h at -4°C. The cells were then thawed and homogenized in 200 µL of lysis buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% SDS, 10% glycerol, 50 mM DTT and 0.01% bromophenol blue. Equivalent amounts of homogenates were heated for 5 min at 100°C and 20 µL of each sample were resolved in a 10% polyacrylamide gel. The 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, Hercules, CA, USA). The 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 0.45 µm nitrocellulose membrane (Hybond, Amersham Biosciences, GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England) at 100 V for 1 h at 4°C in an electrophoretic transfer cell (Mini-Trans-Blot, Bio-Rad, Hercules, CA, USA). The transferred proteins were stained in a 0.2% Ponceau S solution in acetic acid 0.5% to visualize the total proteins and verify the effectiveness of the transfer in all the lanes. The membranes were blocked with 5% skim powdered milk prepared in TBS (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) enriched in 0.1% Tween-20 (TBS-T) for 1 h, washed three times with TBS-T and incubated overnight with the following primary antibodies: anti-phospho-p44/42 MAPK (ERK1/2) (1:1500, Cell Signaling, Danvers, MA, USA), anti-phospho-p38 MAPK (1:750, Cell Signaling, Danvers, MA, USA), anti-phospho-FAK (1:750, Cell Signaling, Danvers, MA, USA), and anti-FAK (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) all produced in rabbit, and anti-p44/42 MAPK (ERK1/2) (1:2000, Cell Signaling, Danvers,

MA, USA) and anti-p38 MAPK (1:1000, Cell Signaling, Danvers, MA, USA) produced in mouse. This incubation step was carried out in TBS-T with 5% BSA at 4°C. The membranes were then washed with TBS-T and detection was achieved by incubating anti-rabbit secondary antibodies developed in goat (1:2000, Cell Signaling, Danvers, MA, USA) or anti-mouse antibodies produced in horse (1:2000, Vector PI-2000, Burlingame, CA, USA) conjugated to peroxidase in TBS-T with 5% skim powdered milk at room temperature for 2 h. The membranes were then washed and incubated for 1 min with a chemiluminescent substrate for peroxidase (ECL™, Amersham Biosciences, GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England). The result was visualized by autoradiography of the chemiluminescent reaction.

2.9 Statistical analysis

The results were statistically analyzed assuming values of α=0.05 and β=0.01. For all the data, we calculated the mean ± standard deviation. The results were analyzed by analysis of variance (ANOVA) and Bonferroni test as a post-hoc test.

3. Results

3.1 Proliferation assay

At 48 h post-stimulation, HUVECs showed a significant increase of cell viability in the presence of the IDPs from 45S5.2B BG as compared to controls, and a higher proliferative response (44%) in M199 supplemented with bFGF (M199+45S5.2B/bFGF). In contrast, the presence of this growth factor together with IDPs from 45S5 BG showed an inhibitory effect (51%) on the proliferation of HUVECs. However, this response was not observed with the use of M199+45S5/VEGF (Figure 1).

To elucidate whether the presence of B in the culture medium enriched in IDPs from 45S5.2B BG could explain the effect observed on the proliferative capacity of HUVECs, a proliferation assay was performed using M199 with different concentrations of B either alone or supplemented with growth factors (VEGF or bFGF). Only 1 mM of B in the medium without growth factors stimulated the *in vitro* proliferation of HUVECs (Fig. 2). However, in the presence of growth factors, B determined a dose-dependent increase in the number of cells at 48 h post-stimulation. For all the concentrations of B studied, an increased proliferative response was observed using medium supplemented with bFGF, being 50 μM the lowest concentration of B with which a statistically significant increase in cell proliferation was observed, without significant differences with the response attained with 100 μM, 500 μM, or 1 mM B in the presence of bFGF (Figure 2).

3.2 Endothelial wound healing assay

A statistically significant increase was observed in the migratory response of HUVECs when stimulated with IDPs from 45S5.2B BG (M199+45S5.2B) and the wound was closed in approximately 60% of the total area at 8 h post-stimulation (Figure 3 and 4).

3.3 Transwell assay

At 6 h post-stimulation, the number of HUVECs that had migrated was significantly higher in M199 enriched in the IDPs from 45S5.2B BG (M199+45S5.2B) than in the negative control (M199) and M199 + 45S5. This response was equivalent to that found with the use of medium supplemented with bFGF (M199+bFGF) (Figure 5 and 6).

3.4 Tubulogenesis on Matrigel™

At 6 h post-stimulation in M199 enriched in IDPs from 45S5.2B BG (M199+45S5.2B), the ability of HUVECs to form endothelial tubules on Matrigel™ increased significantly, being this response similar to that found with medium supplemented with growth factors

(M199+VEGF; M199+bFGF) (Figure 7 and 8). However, no increased formation of endothelial tubules was observed with the use of M199+45S5.2B supplemented with VEGF (M199+45S5.2B/VEGF) or bFGF (M199+45S5.2B/bFGF) (Figure 7 and 8). On the other hand, the presence of VEGF or bFGF together with IDPs from 45S5 BG showed an inhibitory effect (37% and 53%, respectively) on the formation of endothelial tubules (Figure 7 and 8).

3.5 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Regarding the expression of pro-angiogenic cytokines, we observed an increase in the expression levels of IL6 and bFGF in endothelial cells treated with IDPs from 45S5.2B BG (Figure 9).

3.6 Protein kinases associated with proliferation and cell migration

We first evaluated the activation of protein kinases associated with proliferative signals in HUVECs after 30 min of incubation with M199 alone or enriched with IDPs from 45S5 BG and 45S5.2B BG, and also in the presence or absence of bFGF or VEGF. In the presence of bFGF alone, ERK 1/2 phosphorylation levels increased as compared both with the control and with M199 enriched in IDPs from 45S5 BG both in the presence and in the absence of bFGF. However, the incubation of HUVECs in M199 enriched only with IDPs from 45S5.2B BG resulted in higher phosphorylation levels than those observed for bFGF alone or added to this culture condition (Figure 10 a,b).

When the incubation was carried out in the presence of VEGF, both the addition of VEGF and IDPs from 45S5 BG and 45S5.2B BG generated a proliferative response compared to the control medium (M199 alone), without significant differences between them. A significant increase with respect to each separate condition was observed only when the cells were incubated with IDPs from 45S5.2B BG and VEGF, suggesting a synergistic

effect (Figure 11 a,b). These results were consistent with those of the proliferation assay (Figure 1).

To elucidate whether the presence of B in the medium triggers a mitogenic signal by itself, we next assessed ERK phosphorylation levels in the presence of increasing concentrations of B after 30 min of incubation (similar experimental conditions used in the proliferation assay). The response was positive for the different concentrations, with a strong dose-dependent increase in ERK 1/2 phosphorylation levels, even in the absence of growth factors exogenously added to the culture medium (Figure 12 a,b).

To establish a mechanistic link between effects of B on MAPK activation and HUVECs proliferation, we tested the effect of the specific MAPK inhibitor U0126 At 10 μM, U0126 inhibited proliferation stimulated by IDPs from 45S5.2B BG and B (Figure 13 a,b).

Next, to evaluate whether the IDPs from BGs affect signaling cascades associated with migratory responses in HUVECs, we analyzed the phosphorylation levels of two proteins closely linked to this process: FAK and p38. For this purpose, we determined the phosphorylation levels of these enzymes after 30 min of incubation in M199 (control) or M199 enriched in IDPs and/or bFGF. At 30 min post-stimulation, only the IDPs from 45S5.2B BG induced an increase in the phosphorylation levels of FAK (Figure 14 a,b) and p38 (Figure 15 a,b), either by themselves or in the presence of bFGF. Inhibition of p38 with 10 μM SB203580 inhibited cell migration stimulated by IDPs from 45S5.2B BG and 55 μM of boron (Figure 16 a,b).

Although there were no significant differences in the levels obtained in the presence of 45S5.2B BG as compared with the response obtained for bFGF alone, this result indicates that the IDPs from 45S5.2B BG stimulate cell migration even in the absence of exogenous

growth factors. These results were consistent with those observed with the migration assay (Figure 3 and 4).

4. Discussion

Previous studies have shown that 45S5 BG (Bioglass ®) stimulates the *in vitro* proliferation of human endothelial cells and secretion of growth factors with pro-angiogenic activity such as VEGF and FGF.[41-51] Leach *et al*. showed that scaffolds based on poly-lactic-co-glycolic acid (PLGA) coated with 0.5 mg of micro-particles of 45S5 Bioglass® can stimulate the *in vitro* proliferation of human microvascular endothelial cells (HMVEC).[45] Leu and Leach also described an increase in cell proliferation and VEGF production in HMVEC exposed to IDPs released from collagen sponges containing 0.12 and 1.2 mg of 45S5 Bioglass® microparticles in their structure.[46] More recently, Deb *et al.* observed an increase in the proliferation of HUVECs directly cultured on scaffolds of 45S5 Bioglass®.[47] In accordance with these studies, Handel *et al*. showed that minimal medium containing 1% of crushed 45S5 Bioglass®-based scaffolds induced a significant proliferation of HUVECs.[48] However, these results do not agree with those by Aina *et al*., who showed that proliferation of endothelial cells from bovine aorta (BAE-1) in 45S5 Bioglass® used as substrate was lower than that observed using 45S5 BG modified with 5% ZnO (w%).[49] The authors attributed the lower proliferation rate to the alkaline pH resulting from the sudden release of ions from 45S5 BG.

Also, regarding the effects of 45S5 Bioglass® on secretion of growth factors with pro-angiogenic activity, previous studies have shown an increase in the *in vitro* production of bFGF in human fibroblasts (CCD-18Co) grown on polystyrene plates coated with 0.1-2% (w/v) of microparticles (< 5 μm) of 45S5 Bioglass®.[43,50] Day *et al*.[41,43] showed a higher

concentration of VEGF in the culture medium of human (CCD-18Co) and rat fibroblasts (208F) grown on surfaces coated with colloidal suspensions (0.01 and 0.1 w/v) of particles (< 5 μm) of 45S5 Bioglass®. Similar results were observed when mouse fibroblasts (L929) were cultured on the surface of discs from a composite material based on PLGA with 0.01, 0.1, and 1 % w/v particles (< 5 μm) of 45S5 Bioglass ®.[42] On the other hand, Keshaw *et al.* quantified higher VEGF secretion by human fibroblasts (CCD-18Co) either grown in the presence of microporous spheres of PLGA or encapsulated in alginate beads with 10% (w/w) or 0.01% and 0.1% (w/v) of particles (~ 4 μm) of 45S5 Bioglass®, respectively.[44,51] These authors observed an increase in the proliferation of human dermal microvascular endothelial cells (HDMEC) treated with the culture medium from the incubation of human fibroblasts (CCD-18Co) encapsulated in alginate beads with 0.1 (w/v) of glass particles (~ 4 μm) of 45S5 Bioglass®.[44] These results are consistent with those by Day *et al.*, who described an increase of 61.5% in the number of HDMEC after exposure for 24 h to a culture medium obtained from the incubation of human fibroblasts (CCD-18Co) grown on polystyrene plates coated with 0.1% (w/v) of microparticles (< 5μm) of 45S5 Bioglass ®.[43] Recently, Gerhardt *et al*. found that human fibroblasts (CCD-18Co) grown on films of Poly-D, L-lactic acid (PDLLA) added with 5% and 20% of nanoparticles (35-40 nm) or 20% of microparticles (0.1-25 μm) of 45S5 Bioglass® secrete up to 5 times more VEGF than those grown on PDLLA films without the bioactive glass particles.[52]

In the present work, HUVECs stimulated with IDPs from 45S5 BG supplemented with VEGF did not show a higher proliferative response. On the other hand, in the presence of bFGF, the IDPs from 45S5 BG showed an inhibitory effect on the proliferation of HUVECs. The mechanism involved in the observed effects will be subject of future studies. The results achieved are probably associated with the insufficient concentration of silicon

(Si) in the IDPs from 45S5 BG and are in agreement with an increasing number of studies reporting that Si ions released from ceramic materials based on silicate compositions stimulate the secretion of pro-angiogenic growth factors, the proliferation of endothelial cells *in vitro,* and angiogenesis *in vivo*.[23,53-57]

The results of this study show that, when stimulated with IDPs from the bioactive glass 45S5.2B, HUVECs show greater proliferative and migratory response, greater capacity of *in vitro* formation of tubules and greater secretion of the pro-angiogenic cytokines IL-6 and bFGF. These positive effects would be due to the presence of 55±5 μM of boron (B) in the culture medium, a concentration that represents approximately four times the normal concentration of B in human plasma (13 ±3 μM)[29,31] and were below toxic levels (5-25 mM).[29,58,59] Concerns about the toxicity of B to cells *in vivo* have been alleviated by results showing that borosilicate and borate glasses are non-toxic in small animals. Our previous study[34] showed no systemic adverse effects at 15 and 30 days after implantation of 15 mg of 45S5.2B BG particles (300– 350 μm) in rat tibia bone marrow. Moreover, borate bioactive glass containing 54 mol% of B_2O_3 implanted in rabbit tibiae produced B concentrations in the blood far below the toxic level[60] and within the human tolerable upper intake level of 20 mg/d for B.[30] Boron released into the blood of the rabbits as a result of the degradation of the glass particles reached its highest concentration (~10 μg/mL) 24 h post implantation, and subsequently decreased to the value (~3 μg/mL) for control rabbits within 7–10 days.[60] More recently, the possible systemic cytotoxicity of dissolved borate ions released after subcutaneously implantation of 13–93B3 borate glass microfibers containing 53 wt% of B_2O_3 was investigated in a rodent model study by Lin *et al*.[61] Results

indicates that high amounts of borate glass microfibers (up to 1120 mg/animal) implanted in the rats can be safely used with no systemic toxicity.[61]

So far, the information regarding the effects of B at cellular and molecular level is very limited. Park *et al.* reported that a concentration of B between 0.1 and 1 mM stimulates the proliferation of the epithelial cell lines HEK293 and HeLa[29], whereas Fu *et al.* showed that a concentration of B ≤ 0.65 mM released from scaffolds of the bioactive glass 13-93B2 (in moles %: 18% SiO_2, 36% B_2O_3, 22% CaO, 2% P_2O_5, 8% K_2O, 6% Na_2O, 8% MgO) has *in vitro* mitogenic effects on bone marrow mesenchymal cells, as well as on the cell line of osteoblast-osteocyte lineage MLO-A5.[58] In addition, B has dose-dependent effects on pre-osteoblasts MC3T3-E1, with a positive response on cell proliferation and expression of osteogenic markers with concentrations of up to 1 mM.[36,59]

The present study provides original results of the effects of B on the *in vitro* behavior of HUVECs. Considering these results and those previously reported in the literature, B possibly acts through a borate co-transporter, analogous to that described by Park *et al.*[29], which, by phosphorylation of ERK, FAK and SAPK/p38, activates the proliferation and migration of HUVECs. The production and release into the culture medium of the pro-angiogenic cytokines IL6 and bFGF would stimulate the angiogenic response through an autocrine and paracrine mechanism with positive feedback. This is because bFGF acts by activating both cell proliferation via MAPKs, evaluated in this work through ERK phosphorylation levels, and cell migration. Also, bFGF mediates activation of p38 (which belongs to the family of cJun, JAK/JNK kinases) by enabling cell migration during wound healing and angiogenesis.[62] IL6 also acts both on this signaling cascade and on the

proliferation and regulation of the expression of growth factors that in turn activate migration via FAK phosphorylation.[63]

Both plasticity and cytokine cross-talk are significantly involved in pro- and anti-inflammatory/regenerative properties of IL-6-type cytokines.[63] The duration and strength of IL-6-type cytokine-mediated signaling is tightly regulated to avoid overshooting pro-inflammatory activities. However, also anti-inflammatory activities have to be restricted since premature termination of the inflammatory response might prevent efficient reparative/regenerative process.[63] Previous research suggests a role for IL-6 in angiogenesis. *In vitro* studies have shown that, under appropriate stimulation, endothelial cells produce and secrete IL-6.[64,65] Transient expression of IL-6 mRNA in endothelial cells was also demonstrated in two independent angiogenic processes: the formation of a vascular sheath surrounding developing ovarian follicles, and extension of blood vessels in the maternal decidua following embryonic implantation.[66] Furthermore, other groups have indicated that IL-6 acts as a pro-angiogenic regulator of coronary vasculature formation and that IL-6-loss will be detrimental to the formation of the vascular bed.[67,68] Additional studies are therefore necessary to clearly identify these different contributions of IL-6 by HUVECs transplantation, which are however beyond the scope of the present investigation at this stage.

In future studies, it will be of interest to assess the angiogenic effects of 45S5.2B BG particles of different size (nano- and micrometric) using more complex cell cultures such as the co-culture of osteoblasts with microvascular endothelial cells, so as to reproduce the interactions that occur normally among the different cell populations during bone repair.[11]

6. Conclusions

The results of the present study show, for the first time, that HUVECs possess greater proliferative and migratory response and greater ability to form *in vitro* tubules and to secrete pro-angiogenic cytokines (IL-6 and bFGF) when stimulated with the ionic dissolution products from the bioactive glass 45S5 enriched with 2% B_2O_3 (45S5.2B).

The effects observed are attributed to the presence of B in the ionic dissolution products. In this sense, these products would have potential application in different strategies of tissue engineering and regenerative medicine of tissues that require a high degree of vascularization, such as bone.

Acknowledgements

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Figure Captions

Fig. 1 Proliferative response of human umbilical vein endothelial cells (HUVECs) (mean ± SD; **p*<0.05 and ***p*<0.01 compared to its controls: M199, M199+VEGF, M199+bFGF). cpm: counts per million.

Fig. 2 Proliferative response of HUVECs in M199 medium with different concentrations of boron (B) (mean ± SD; **p*<0.05 and ***p*<0.01 compared to its controls). GF: growth factors; cpm: counts per million.

Fig. 3 Endothelial wound healing assay. Note the migratory response of HUVECs. Orig. Mag. X100.

Fig. 4 Percentage of recovery (% R) of the wounded area by migration of HUVECs (mean ± SD; **p*<0.05 with respect to its controls: M199, M199+bFGF and M199+45S5).

Fig. 5 Photographs depicting the cells that migrated in the transwell assay. Crystal Violet staining. Orig. Mag. X100.

Fig. 6 Number of HUVECs that migrated after 6 h incubation (mean ± SD; *p<0.05 compared to M199 and M199+45S5).

Fig. 7 Microphotographs showing the formation of endothelial tubules on Matrigel™. Note the disorganization in tubular structures when incubated with M199+45S5/bFGF and M199+45S5/VEGF. Orig. Mag. X200.

Fig. 8. Number of endothelial tubules after 6 h of incubation (mean ± SD; **p<0.01 compared to its controls: M199, M199+VEGF, and M199+bFGF).

Fig. 9 Expression levels of pro-angiogenic cytokines (*p<0.05 compared to M199 and M199+45S5). OD: optical density.

Fig. 10 Expression of protein kinases associated with proliferative signals: a) Western blot of HUVEC homogenates to evaluate ERK phosphorylation level as an indicator of activation of mitogenic signals. Incubation was assayed in the presence or absence of bFGF. b) Densitometry of the protein bands (ratio of OD values p-ERK/ERK) (mean ± SD; *p<0.05 compared to M199+bFGF).

Fig. 11 Expression of protein kinases associated with proliferative signals: a) Representative Western blot of HUVEC homogenates to evaluate ERK phosphorylation level in the presence or absence of VEGF. b) Densitometry of the protein bands (ratio of OD values p-ERK/ERK) (mean ± SD; *p<0.05 compared to all groups).

Fig. 12 ERK phosphorylation levels in HUVECs incubated for 30 min at increasing concentrations of boron (B). a) Representative Western blot of HUVECs homogenates. b) Densitometry of the protein bands (ratio of OD values p-ERK/ERK) (mean ± SD; *p<0.05 compared to M199 +VEGF).

Fig. 13 MAPK inhibitor U0126 inhibited proliferation stimulated by IDPs from 45S5.2B BG (a) and boron (b) (mean ± SD; *p<0.05 compared to its controls). cpm: counts per million.

Fig. 14 Expression of protein kinases associated with migration signals: Focal Adhesion Kinases (FAK). a) Representative Western blot of HUVEC homogenates incubated for 30 min in the different experimental conditions. b) Densitometry of the protein bands (ratio of OD values p-FAK/FAK) (mean ± SD; *p<0.05 compared to all groups).

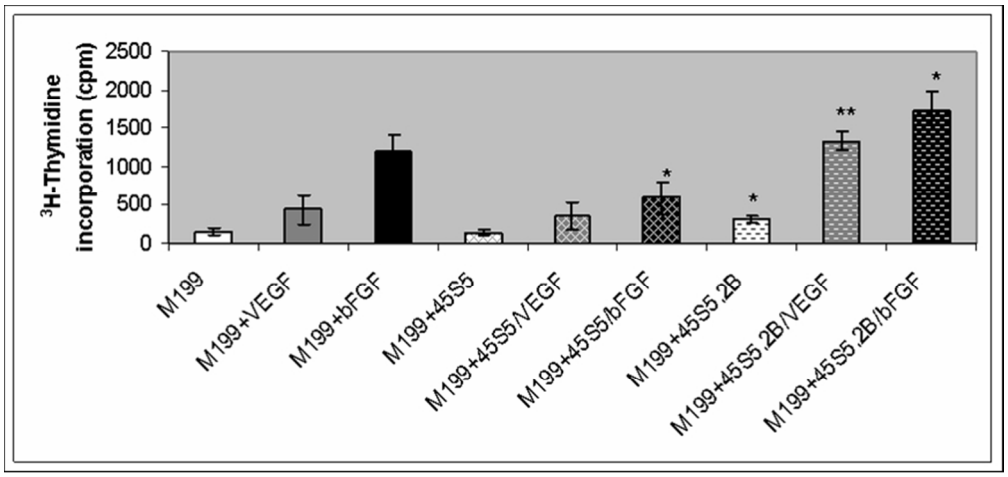
Fig. 15 Expression of protein kinases associated with cell migratory signals: p38 kinase. a) Representative Western blot of HUVEC homogenates incubated for 30 min in the different experimental conditions. b) Densitometry of the protein bands (ratio of OD values p-p38/p38) (mean ± SD; *p<0.05; **p<0.01 compared to M199 + bFGF).

Fig. 16 The p38 inhibitor SB203580 inhibited cell migration stimulated by IDPs from 45S5.2B BG and 55 μM of boron. a) Photographs depicting the cells that migrated in the transwell assay. Crystal Violet staining. Orig. Mag. X100. b) Number of HUVECs that migrated after 6 h incubation (mean ± SD; *p<0.05 compared to its controls).

Table 1. Ionic 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)**
M199	Bld	Bld	28.9 ± 1	64.8 ± 1	3345 ± 15
M199 + 45S5	Bld	Bld	28.7 ± 1	70.8 ± 1	3352 ± 10
M199 + 45S5.2B	55 ± 5	Bld	27.1 ± 2	66.2 ± 1	3340 ± 15

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



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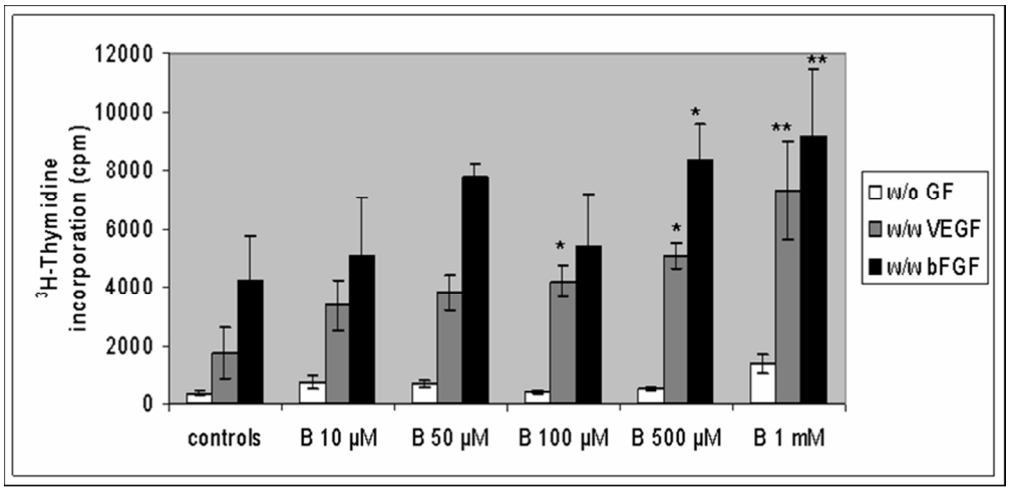

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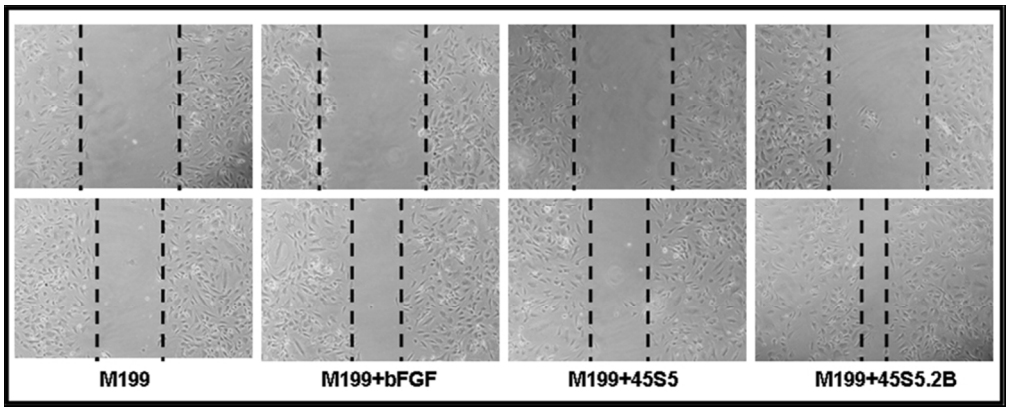

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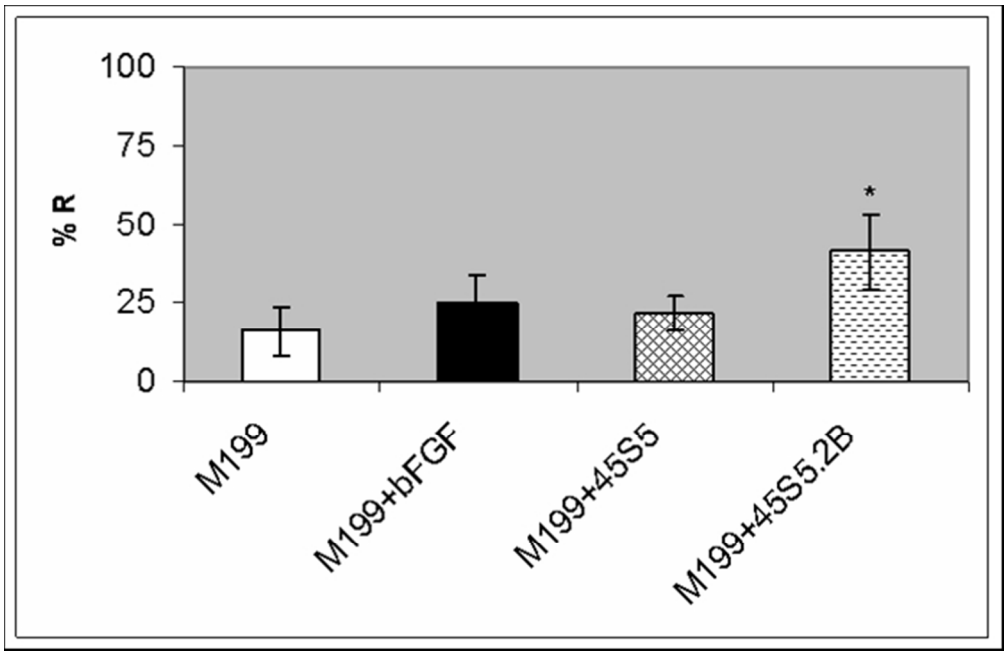

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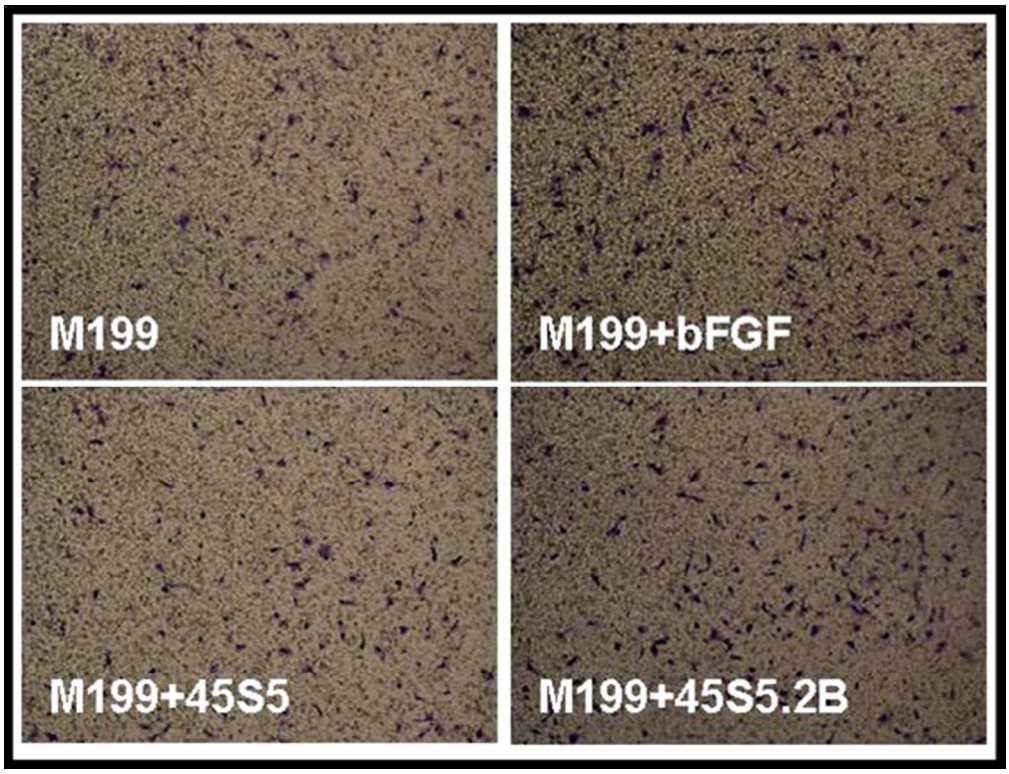

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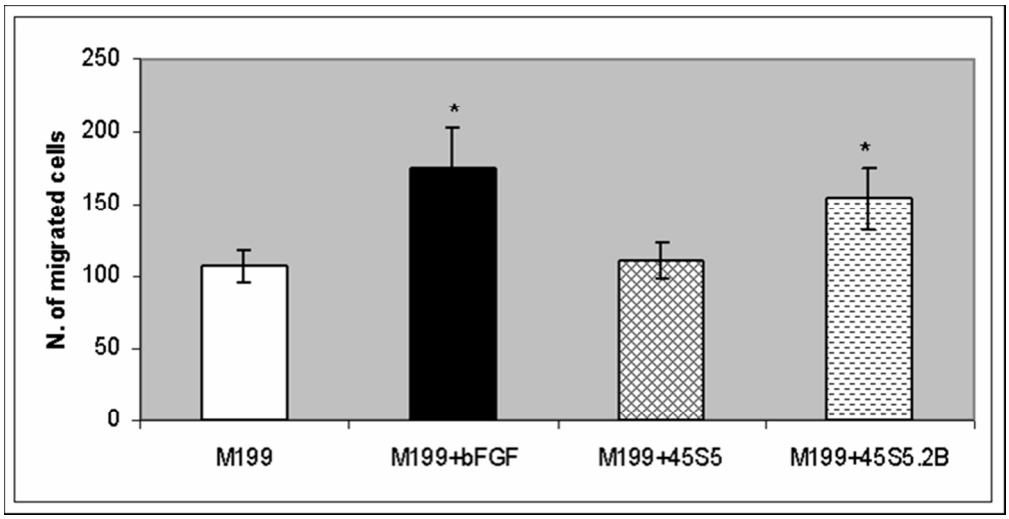

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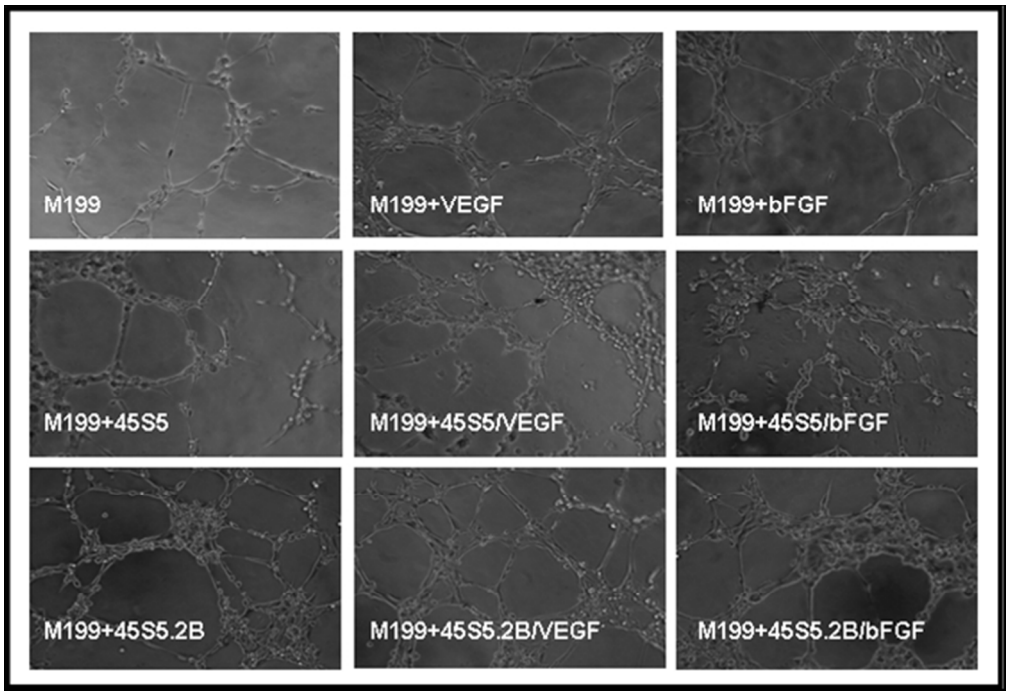

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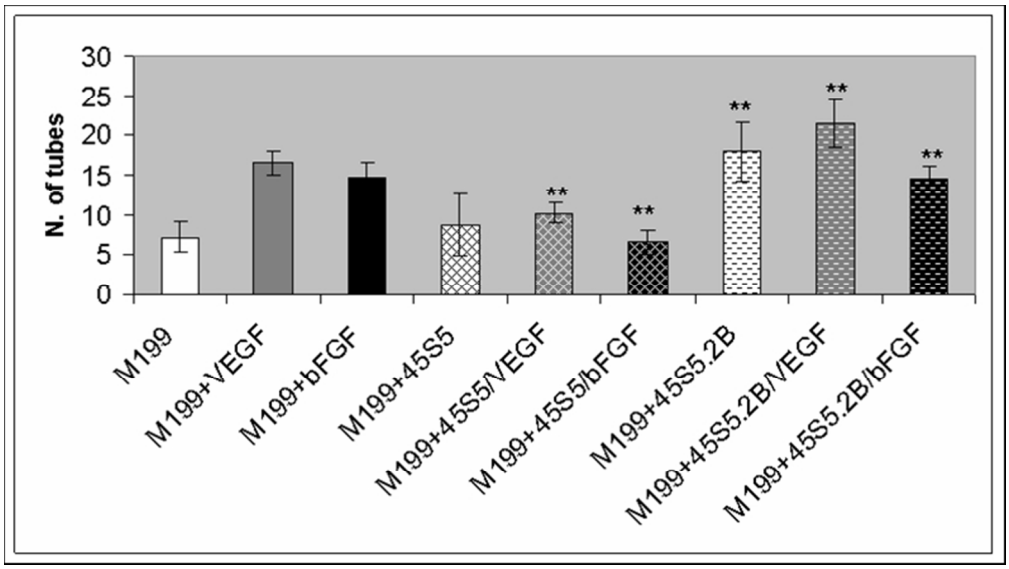

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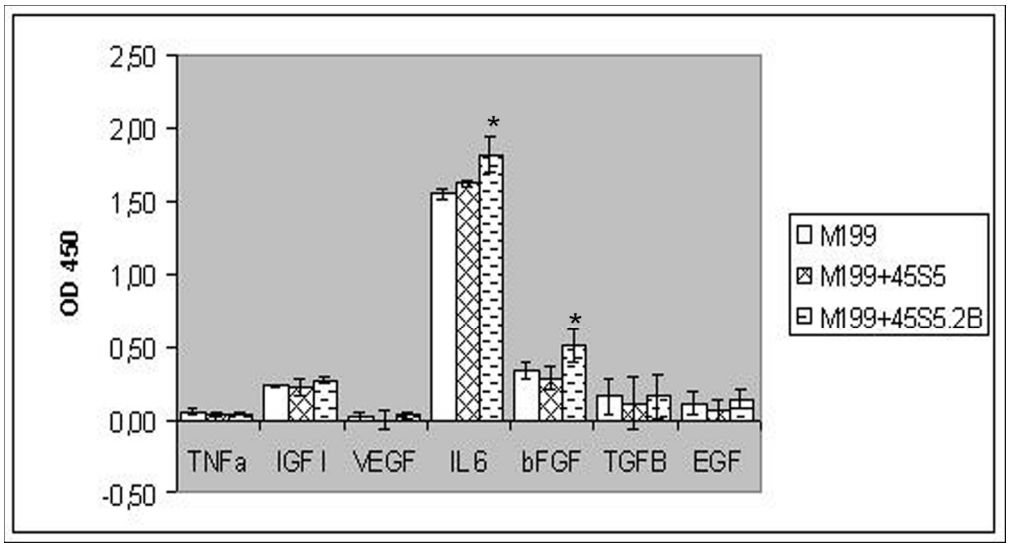

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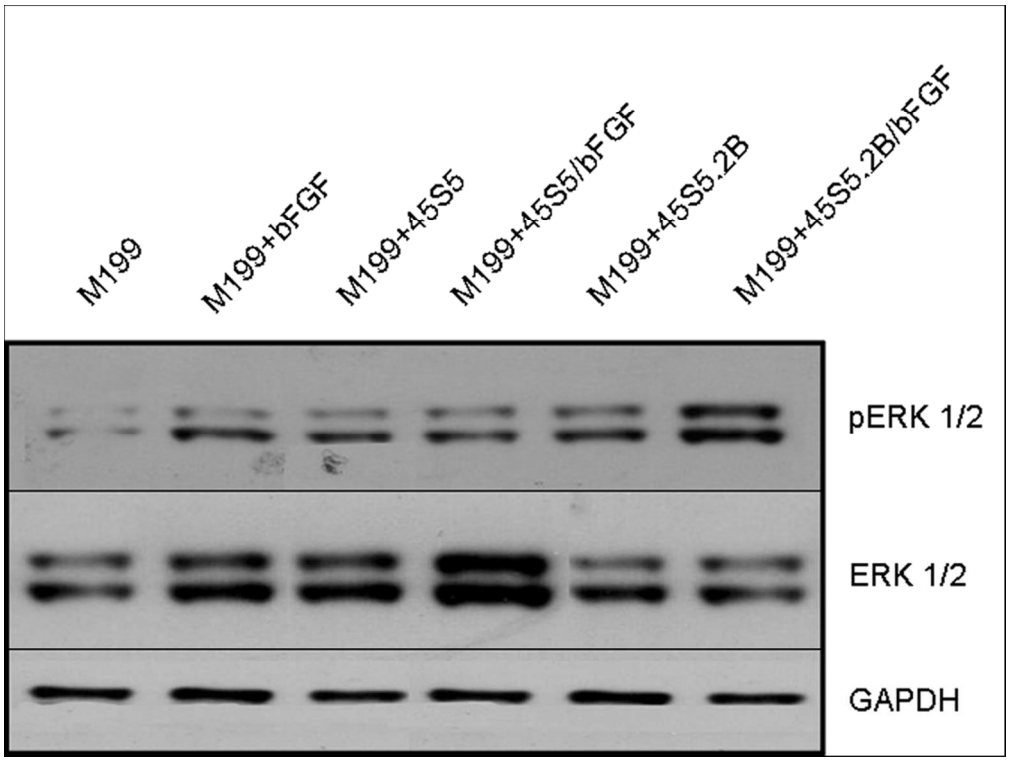

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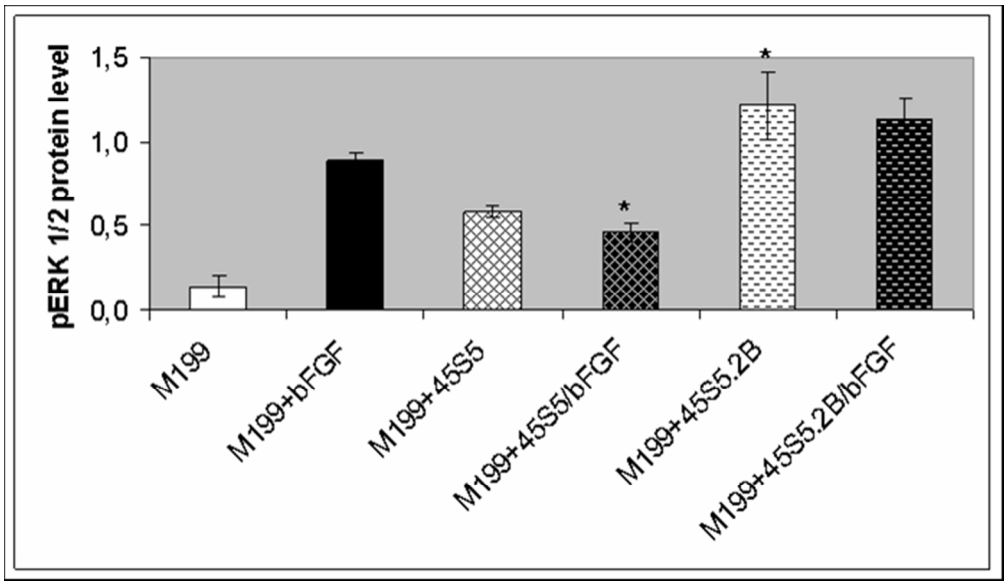

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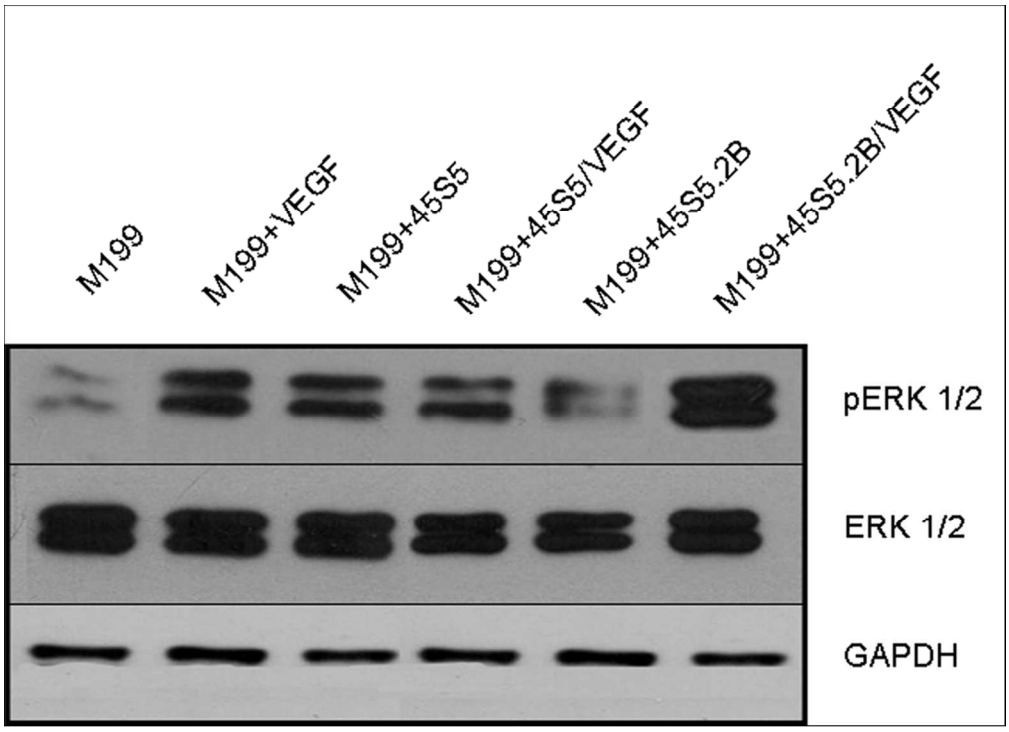

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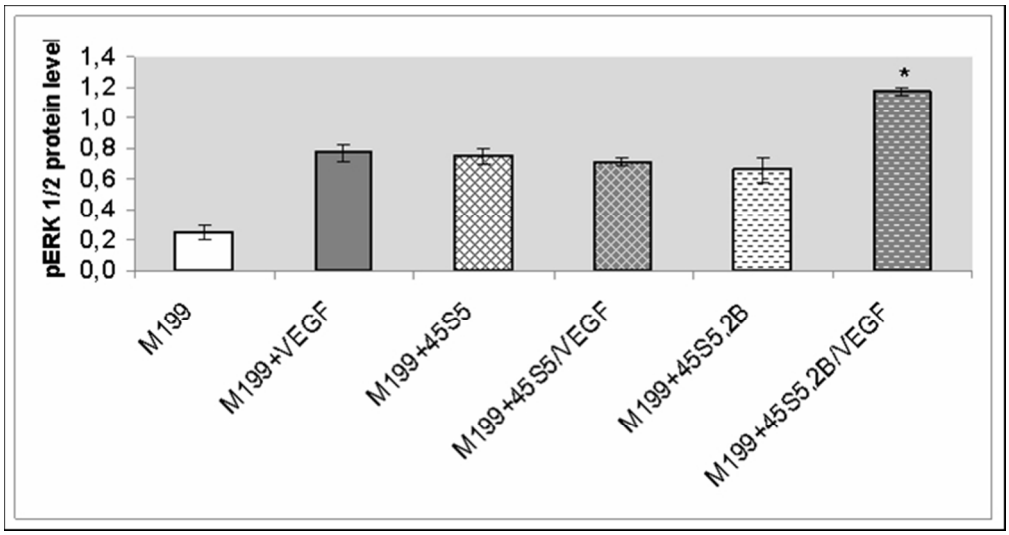

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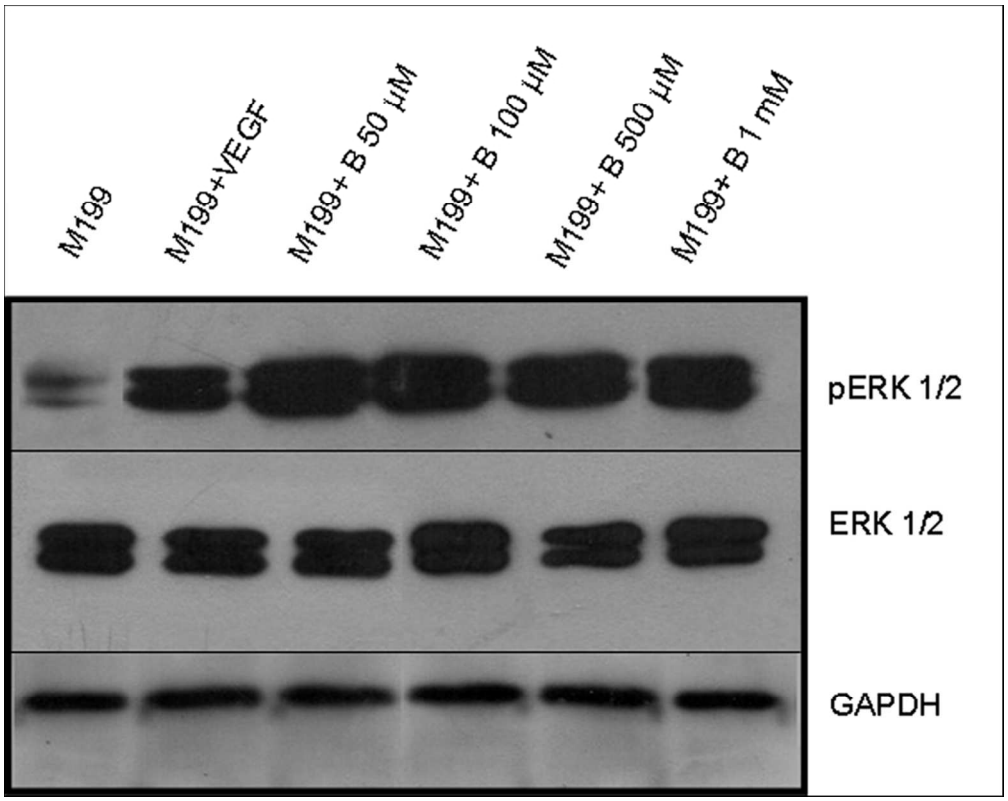

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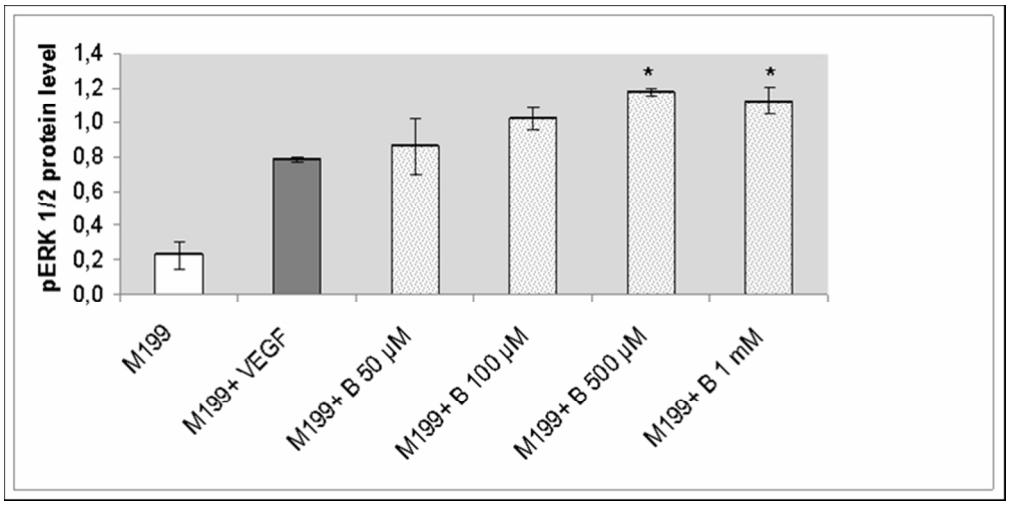

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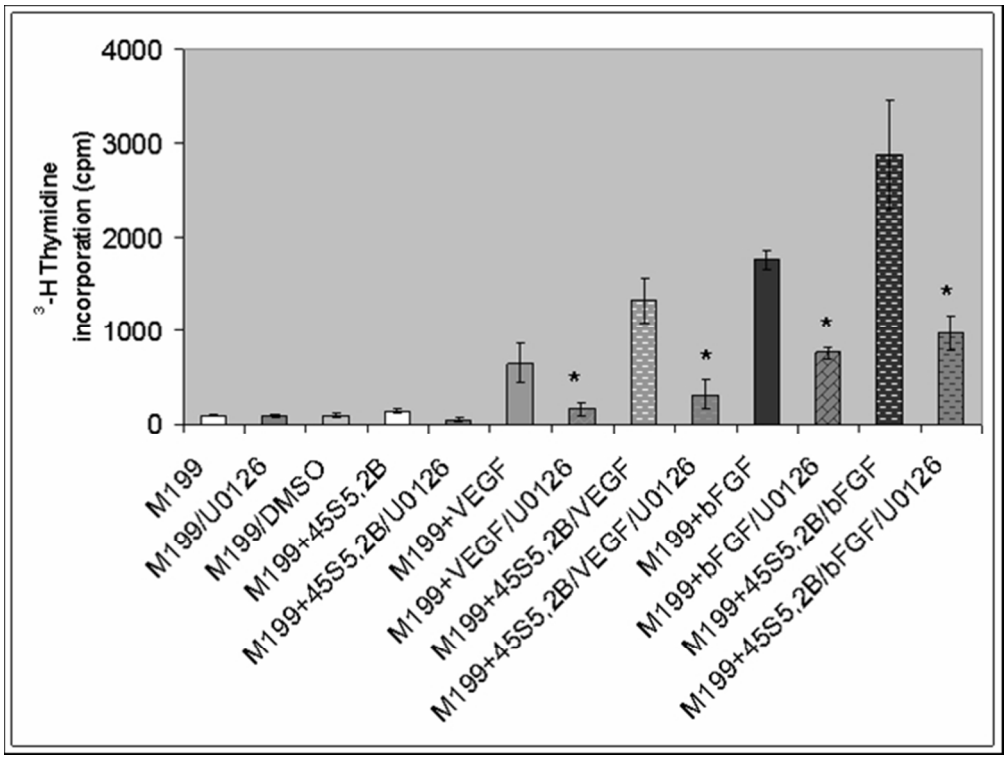

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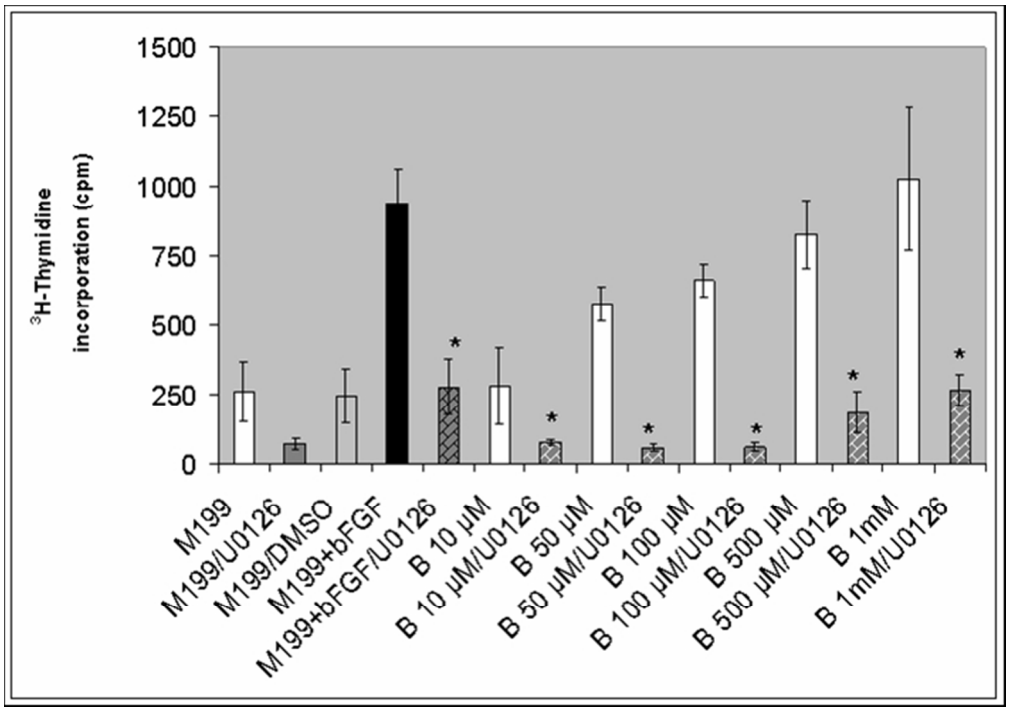

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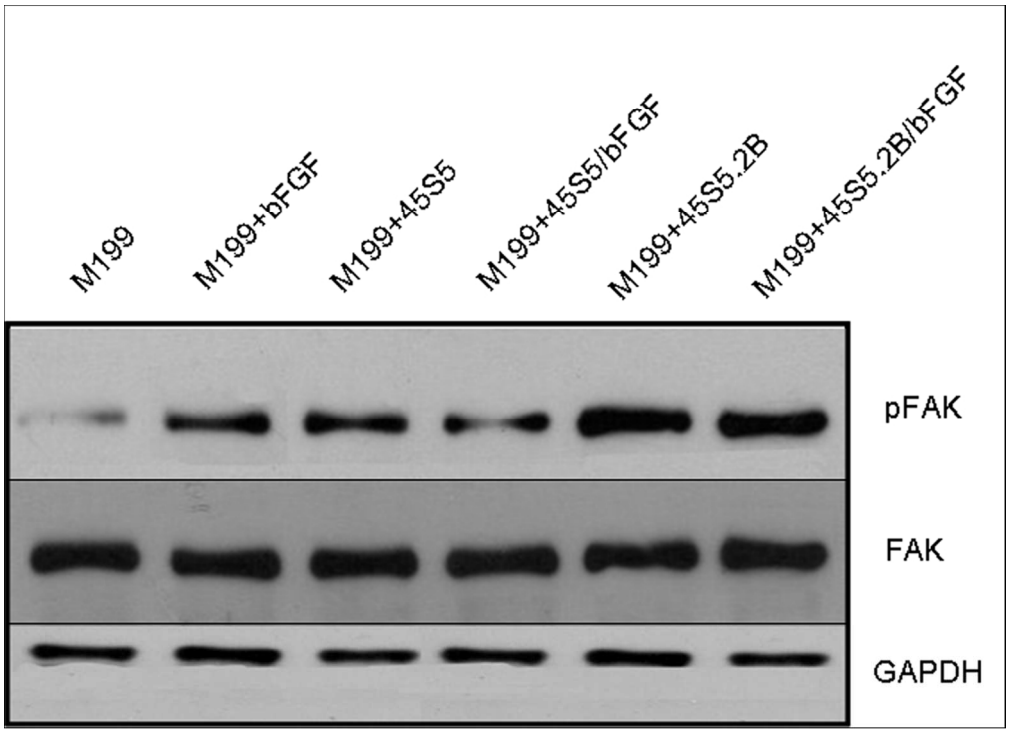

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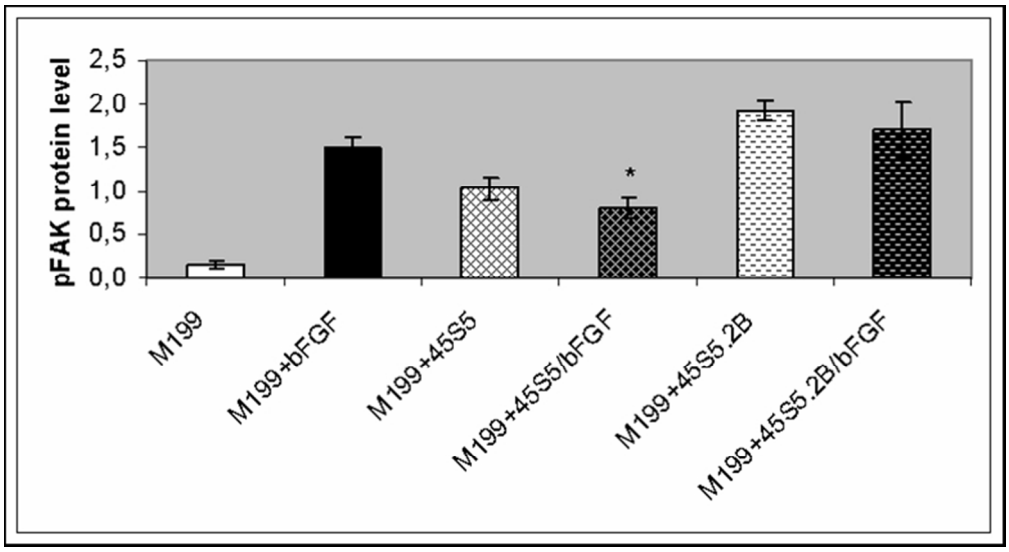

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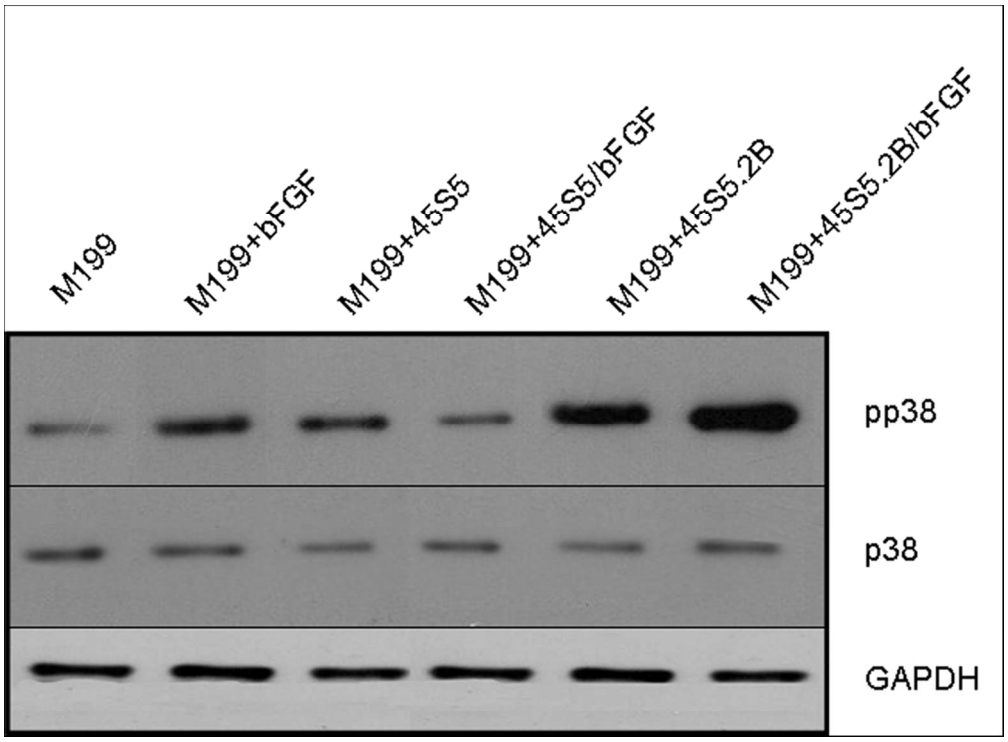
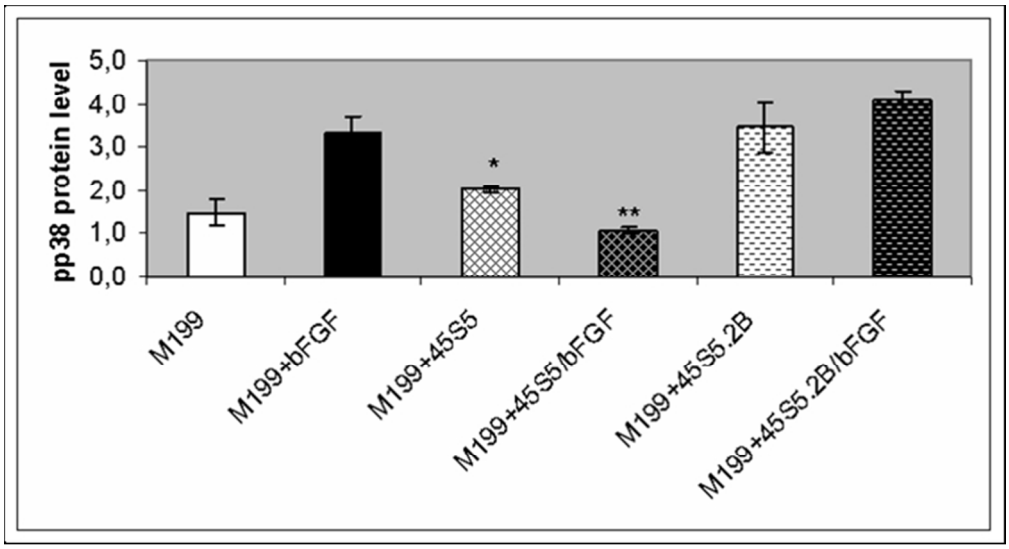
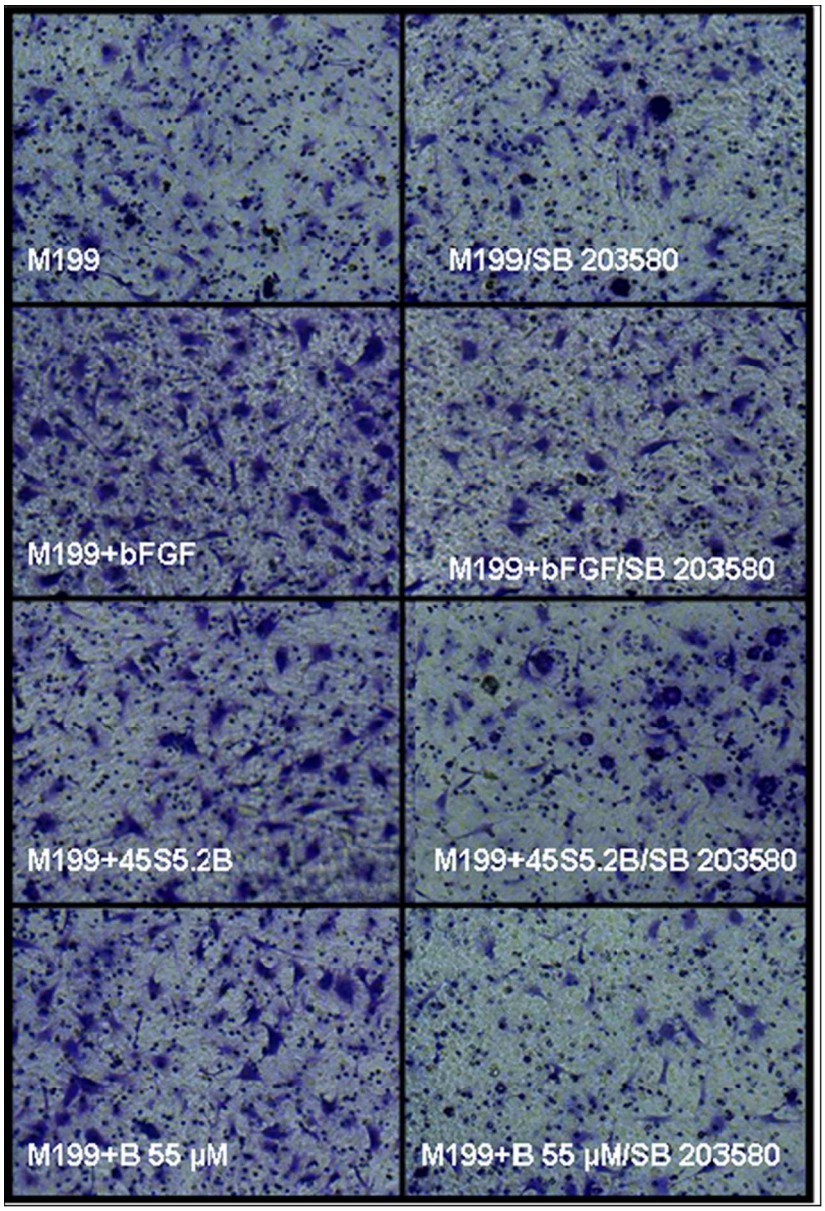


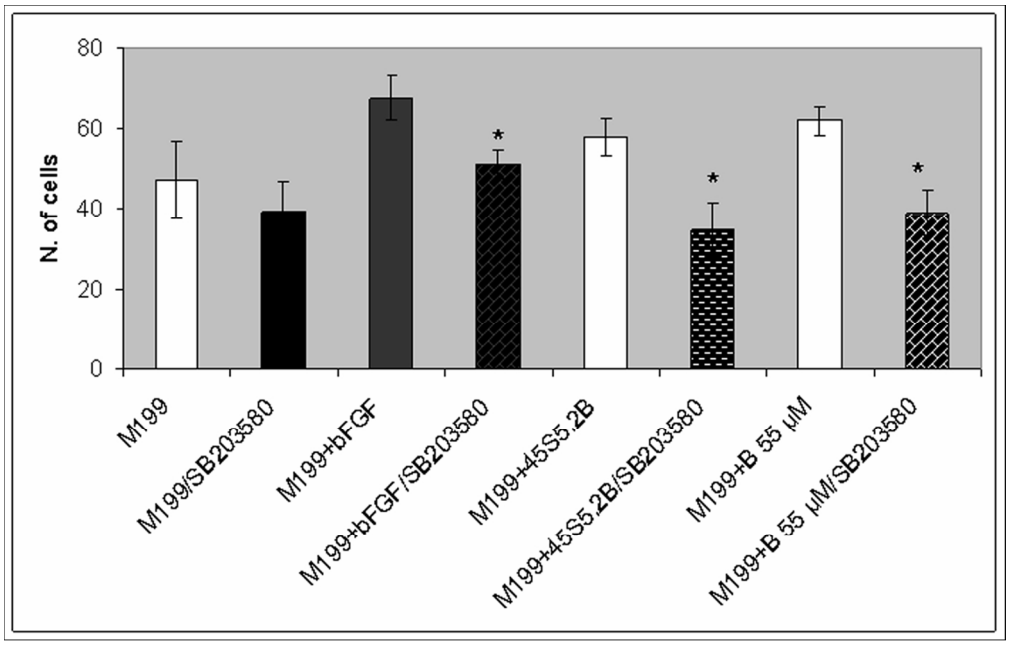
190x139mm (150 x 150 DPI)



254x139mm (76 x 75 DPI)



126x185mm (150 x 150 DPI)



235x149mm (150 x 150 DPI)