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Boron regulates mineralized tissue-associated proteins in osteoblasts (MC3T3-E1)

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ABSTRACT

The aim of this study was to determine the effects of boron (B) on the cell-survival, proliferation, mineralization and mRNA expression of mineralized tissue-associated proteins. Additionally, determination of the effects of B on the BMP-4, -6 and -7 protein levels of pre-osteoblastic cells (MC3T3-E1) was also intended. The effects of B (pH 7.0) concentrations (0, 0.1, 1, 10, 100, 1000, 2000, 4000, 8000 and 10,000 ng/ml) on the survival of the cells were evaluated at 24 and 96 hrs with MTT assay. To evaluate the proliferation in long term, MC3T3-E1 cells were treated with different concentrations of B (0, 0.1, 1, 10, 100 and 1000 ng/ml) and were counted on days 2, 5, and 14. While in short term, decreased cell survival rate was observed at 1000 ng/ml and above, at long term no statistically significant difference was detected in different B concentrations applied. Slight decreases at the proliferation of the B-treated groups were determined on days 5 and 14 but one-way analysis of variance revealed that the difference was statistically insignificant. In mineralization assay, increased mineralized nodules were apparently observed in B treatment (1 and 10 ng/ml concentrations) groups. Based on quantitative RT-PCR results, remarkable regulation in favor of osteoblastic function for Collagen type I (COL I), Osteopontin (OPN), Bone Sialoprotein (BSP), Osteocalcin (OCN) and RunX2 mRNA expressions were observed in B treatment groups in comparison with untreated control groups. Increased BMP-4, -6 and -7 protein levels were detected at 0.1, 1, 10 and 100 ng/ml B concentrations. Results of the study suggest that at the molecular level B displays important roles on bone metabolism and may find novel usages at the regenerative medicine.

Introduction

Boron (B) is an essential micronutrient of organisms and plays a crucial role in osteogenesis and the maintenance of bone [1,2]. Dietary intake is an important factor for bone health and inadequate intake of nutrients including calcium, protein, magnesium, phosphorus, vitamin D, potassium, fluoride, manganese, copper, B, iron, zinc, vitamin A, vitamin K, vitamin C, and the B vitamins increases the risk for bone loss and impairs bone remodeling. Moreover, in deficiency conditions development and regeneration of bone is negatively influenced [3–5].

B may interact with steroid hormones, and involve in the prevention of calcium loss and bone demineralization. It has been shown that B supplementation markedly reduces the urinary calcium and magnesium excretion, increases serum levels of estradiol [6] and calcium absorption [7], in peri- and postmenopausal women. B has also been related to vitamin D function by stimulating growth in vitamin D deficient animals and alleviating perturbations in mineral metabolism that are characteristic of vitamin D deficiency [8].

No recommended levels have been set for B, only upper limits of intake (20 mg/day) [9]. Because no data are available on the adverse effects of very high intakes of B in humans, animal studies were used to extrapolate the upper limit. These studies were based on the effects of high B intakes on reproductive and developmental function. The level of B intake associated with gains in bone was about 3 mg/day [10] well below this limit. Bone is strongly influenced by the intake of several nutrients. The recommended levels of many of these nutrients may not be optimal for bone health. Several recent epidemiological studies and clinical trials have reported higher intake levels of some of these nutrients for building bone mass, preventing bone loss and fractures, and decreasing bone resorption and/or increasing bone formation.

Recently, Gorustovich et al. using rat [4] and also mice [11] models investigated the deficiency of B on bone healing and periodontal alveolar bone remodeling. They found that B deficiency resulted in altered bone healing due to apparent reduction in osteogenesis and B deprivation altered periodontal alveolar bone

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modeling and remodeling by inhibiting bone formation. Nielsen and Stoecker [12] performed a study to determine whether B deprivation affects vertebra bone micro-architecture and whether any adverse affect would be modified by dietary fatty acid composition. They investigated female rats which fed diet containing 0.1 mg B/kg (B deprived) or 3 mg B/kg (B adequate) and fat sources of 75 g safflower oil/kg or 65 g fish oil/kg plus 10 g linoleic acid/kg. Their results demonstrated that B deprivation decreased bone volume fraction and increased trabecular separation and structural model index. Moreover, B deprivation decreased the maximum force needed to break the femur. Feeding fish oil instead of safflower oil decreased connectivity density in vertebrae of B deficient but not in B-adequate rats. Their results confirm that B and fish oil are beneficial to cortical bone strength and show that nutritional intake of B is beneficial for trabecular bone micro-architecture and influence the beneficial effects of fish oil on bone.

Although recent studies [4,11,12] indicated that B plays an important role in bone formation and maintenance, and histomorphometric analysis of *in vivo* studies showed that decreased osteoblast surface and increased quiescent surface percentage were observed in B deficient rats, the exact molecular mechanism of B regulation in bone remains unclear. According to our knowledge there is no study investigating the effects of B in osteoblasts in cellular/molecular plane and the possible mechanisms in transcriptional and translational level. Hence, the purpose of this study was to explore the effects of B in osteoblasts, specifically:

- the effects of B on the cell viability, proliferation and morphology
- the effects of B on the mineralization of cells
- the effects of B on the mineralized tissue-associated proteins mRNA expression
- the effects of B on the bone morphogenetic proteins-4, -6 and -7 levels on the cells

The null hypothesis tested was that B induces biomineralization of osteoblasts regulating gene expressions related with mineralized tissues.

Materials and methods

Subclone #4 of MC3T3-E1 cells were used to test the effects of B. MC3T3-E1 cells were provided kindly from Dr. Martha J. Somerman (University of Washington) and Dr. Renny Franceschi (University of Michigan) [13]. Cells were maintained in α-MEM containing 10% FBS, penicillin, streptomycin and glutamine (Biological Industries, Israel).

Preparation of boron

Boric acid (Sigma–Aldrich, MO, USA) was used to obtain B concentrations since the cells (Sigma–Aldrich, MO, USA) in human and animals use boric acid for B trafficking [14]. Boric acid was prepared as a stock solution of 10 μg/ml and pH of the solution was arranged as 7, appropriate for cell culture conditions. The pH was adjusted to 7.0 by the addition of small quantities of base (NaOH) as needed. Other concentrations were obtained from the stock solution. At pH 7.0, the predominant species is monomeric borate anion. In this study, very low concentrations of boric acid were used and at these concentrations, only trace amounts of polyborate species could possibly exist in equilibrium at pH 7.0. Such trace amounts have negligible effects on the outcome of the study [15].

MTT experiments

The 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used as previously described [16]. For viability experiments, cells were allowed to adhere for 24 hours (hrs) in α-MEM with 10% FBS, after which media were changed to α-MEM with 5% FBS containing B (0.1, 1, 10, 100, 1000, 2000, 4000, 8000, and 10,000 ng/ml). MTT experiments were repeated 3 times (6-well per each treatment).

Proliferation experiments

For long term evaluation to test B on the cell proliferation, MC3T3-E1 cells were allowed to adhere for 24 hrs in α-MEM with 10% FBS, after which media were changed to α-MEM with 5% FBS containing B (0.1, 1, 10, 100, and 1000 ng/ml). Cells were counted with Neauber hemocytometer using trypan blue on days 2, 5 and 14. Proliferation experiments were performed 2 times as triplicate.

Morphology

Images of MC3T3-E1 cells treated with different concentrations of B were examined visually using phase contrast microscope (Nikon TS100F, Japan) on days 3 and 8 after treatment.

Mineralization assay

von Kossa staining was used to detect mineralized nodules in MC3T3-E1 cells and 0.1, 1, 10, 100, and 1000 ng/ml B concentrations were used. Cells were plated at 5×10^4 cells/cm^2 in 24-well plates in α-MEM containing 10% FBS. After 24 hrs, cells were exposed to the following factors: (a) 5% FBS + mineralization media [MM = ascorbic acid (AA, 50 μg/ml) and β-glycerophosphate (BGP, 10 mM)], (b) 5% FBS + B (0.1 ng/ml) + MM, (c) 5% FBS + B (1 ng/ml) + MM, (d) 5% FBS + B (10 ng/ml) + MM, (e) 5% FBS + B (100 ng/ml) + MM, (f) 5% FBS + B (1000 ng/ml) + MM. Mineralization of extracellular matrix was determined on day 30 by von Kossa staining [20]. Briefly, cells were washed with Phosphate Buffered Saline (PBS) twice and fixed with 100% ethanol at 37 °C for 1 hr and washing was performed by different alcohol concentrations to deionized water. Cells were treated with 5% $AgNO_3$ and incubated at 37 °C in dark for 15 min and washed with deionized water. Plates were exposed to fluorescent light for 20 min and photographs were taken [17]. Mineralization experiments were repeated 2 times as triplicate.

RNA isolation

To determine gene expression, MC3T3-E1 cells were plated in 60 mm cell culture dishes (Corning, NY, USA) at 5×10^4 cells/cm^2 and treated after 24 hrs as described above. Total RNA was isolated using monophasic solution of phenol and guanidine isothiocyanate (Invitrogen, CA, USA) on days 3 and 8. RNA concentration was quantified using NanoDrop ND-1000 and RNA samples were stored at −80 °C.

cDNA synthesis and Real-Time Quantitative PCR

For Quantitative RT-PCR analysis, cDNA was synthesized from 1.0 μg total RNA with a cDNA synthesis kit (Applied Biosystems, Foster City, USA) (High Capacity RNA-to-cDNA kit) for RT-PCR. From the resulting cDNA product 1.0 μl was used per reaction in the Strategene MX3000P. Quantitative RT-PCR reactions were carried out with the Brilliant SYBR Green QPCR Master Mix kit (Strategene, TX, USA), within a total volume of 25 μl. Primers were designed by DNA-Star design software. A BLAST search of GenBank was performed on the primer sequences to ensure specificity. GAPDH served as a housekeeping/reference gene for normalization. The amplification profile for OCN, RunX2, COL I, GAPDH used on the Strategene MX3000P was: 94/3 min preheat; 94/45:54/45:72/60; (temperature (°C)/time (s)) and 35–40 cycles;

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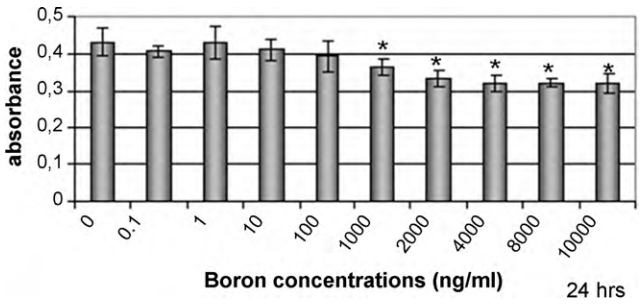

Fig. 1. The effects of different B concentration on MC3T3-E1 cell survival at 24 hrs. Each bar represents the mean ± standard deviation. * represents statistical significance, compared with control (without B) group ($p < 0.05$).

72/10 min final extention. The amplification profile for BSP, OPN, GAPDH used on the Strategene MX3000P was: 94/3 min preheat; 94/45:52/45:72/60 (temperature (°C)/time (s)) and 35–40 cycles; 72/10 min final extention. Quantitative RT-PCR experiments were repeated 3 times.

Bone morphogenetic protein-4, -6 and -7 detection

Enzyme-linked Immune Absorbent Assay (ELISA) (Raybiotech, Inc., GA, USA) was used to detect BMP-4, -6 and -7 levels. MC3T3-E1 cells were plated in 60 mm cell culture dishes (Corning, NY, USA) at 5×10^4 cells/cm^2 and treated after 24 hrs with different concentrations of B. Cells were treated every other day. On days 3 and 8, culture dishes were treated with serum-free media and the conditioned media was obtained after 24 hrs namely; on days 4 and 9. Conditioned media of the different B groups were kept in −80 °C until to ELISA experiments. ELISA experiments were repeated 2 times as triplicate.

Statistical analysis

The statistical analysis used was one-way analysis of variance (ANOVA) and Tamhane test for multiple comparison tests. The data are represented as mean ± standard deviation.

Results

Cell viability experiments (Figs. 1 and 2)

While, in short time period (at 24 hrs), decreased cell survival rate was observed at 1000 ng/ml and above this concentration, at 72 hrs there was no statistically significant difference in different B concentrations when compared to untreated control group (without B).

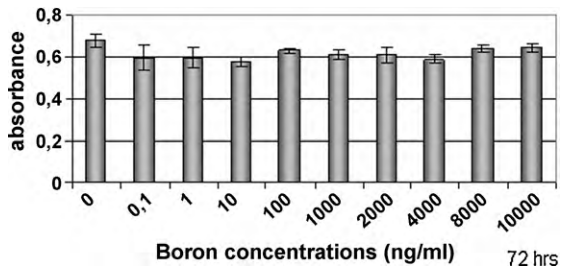

Fig. 2. Cell survival rate of MC3T3-E1 cells after 72 hrs. Each bar represents the mean ± standard deviation.

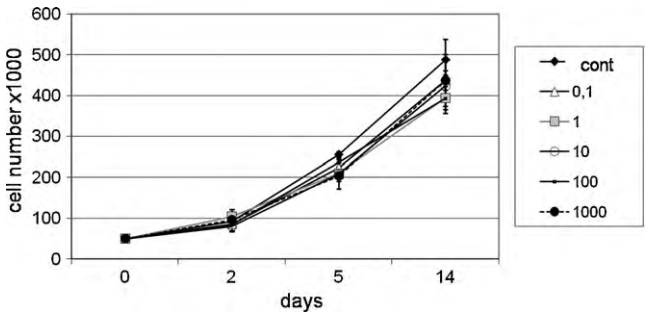

Fig. 3. Proliferation of MC3T3-E1 cells on days 2, 5 and 14. No significant difference was observed in B treatments when compared to control group (without B). The data are represented as mean ± standard deviation.

Cell proliferation experiments (Fig. 3)

A slight decrease in proliferation of cells was observed in B treatments, but the difference was not statistically significant.

Morphology of the MC3T3-E1 cells (Figs. 4 and 5)

The morphology of cells was not affected by different B concentrations in both on days 3 and 7 using inverted microscope.

Mineralization of the MC3T3-E1 cells (Fig. 6)

When compared to untreated control group, increased mineralized nodules were observed in B treatment groups. Especially, in 1 and 10 ng/ml concentrations B treatment groups, increase in mineralized nodules was apparent.

Gene expression results (Fig. 7)

COL I: On day 3, remarkable increase was observed in only 100 ng/ml B treatment, while on day 8, significant increase was noted in all B concentrations when compared to control group (without B).

OPN: Increased OPN mRNA expression was seen in the groups of 100 and 1000 ng/ml B on days 3 and 8.

BSP: On day 3, all B concentrations stimulated BSP mRNA expression in comparison with untreated control group. On day 8, only 1000 ng/ml B treatment increased significantly BSP transcript.

OCN: B treatments including 10, 100 and 1000 ng/ml up-regulated OCN mRNA expression in both days examined.

RunX2: While, statistically significant increase was noted in only 1000 ng/ml B application on day 3, on day 8 this pattern in transcript was changed and up-regulated RunX2 mRNA expression was observed in 0.1, 1, and 10 ng/ml B concentrations.

BMP-4, -6 and -7 protein results (Fig. 8)

Increased BMP-4 and BMP-6 levels were noted in 1 ng/ml B treatment both on days 4 and 9. When BMP-7 protein level was considered, a different dose-dependent tendency was evidenced. Significant stimulation in BMP-7 level was observed in 100 ng/ml B treatment on day 4 and in 1, 10 and 100 ng/ml B treatments on day 9. While, 1 and 10 ng/ml B treatments increased BMP-7 levels on day 9, 1000 ng/ml B treatment resulted in levels similar with control group (without B). Results suggest that the effects of B on BMPs of cells at differentiation phase are time- and dose-dependent.

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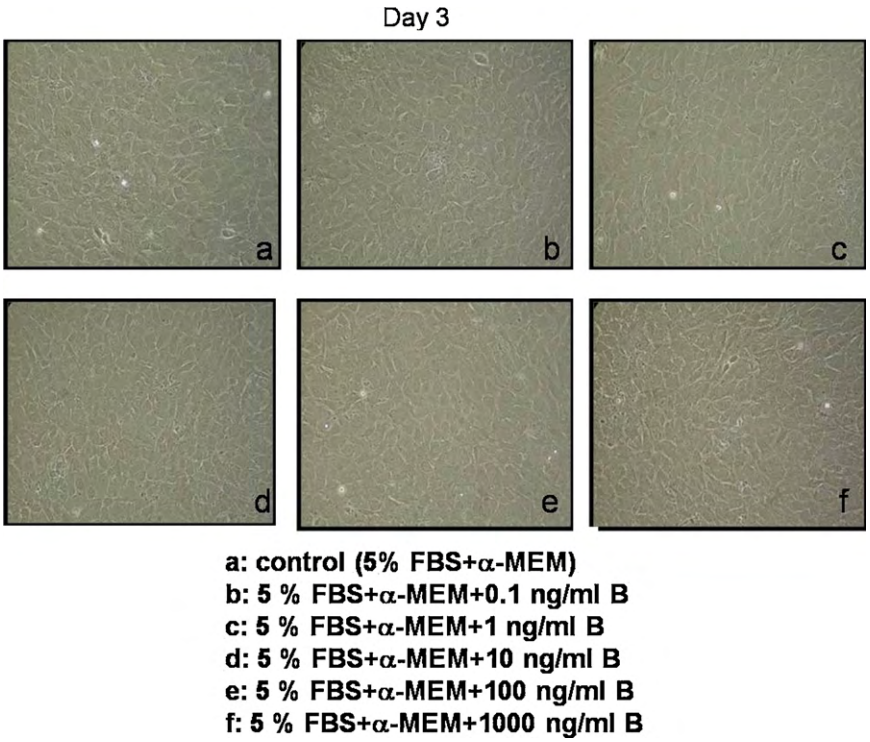

Fig. 4. Cell morphology on day 3. No differences were observed among the treatments.

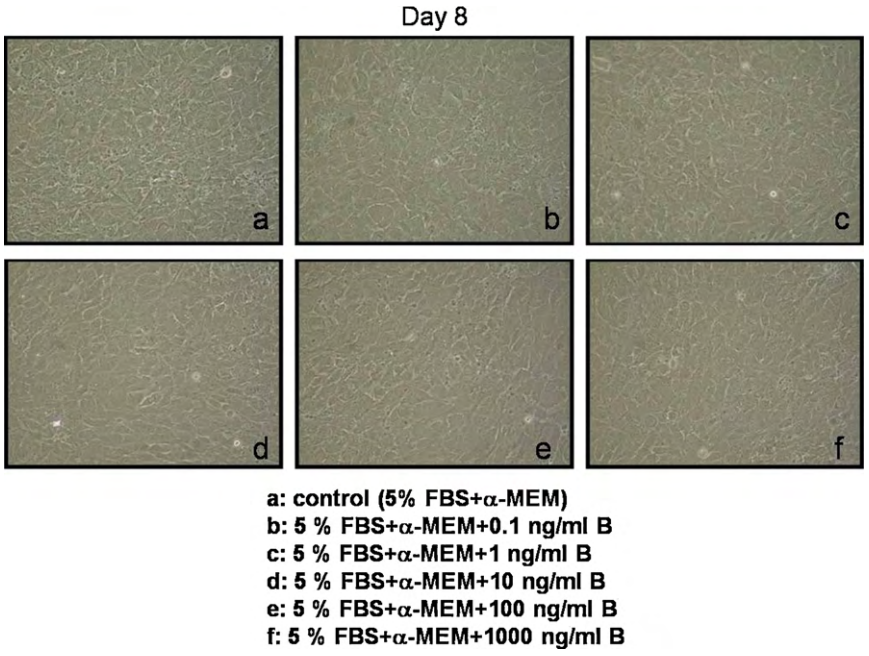

Fig. 5. Cell morphology on day 8. Healthy morphologies of cells were similar to those found on day 3.

Discussion

The mineral phase of bone is made up principally of calcium and phosphate. In the course of mineral deposition, a variety of metals are taken up that may be present in the bloodstream as the blood plasma courses over the skeletal tissue. Uptake by the bone mineral is a function of the affinity of a given metal for the bone mineral and extracellular matrix and of the metals concentration in the plasma. It is also a function of degree of mineralization of the skeleton [18].

The metal interacts with bone cells their metabolism may be affected which in turn may alter osteoblasts and osteoclasts function. B is essential element for plants and mammals. It was reported that a low B diet appears to exacerbate the effects of vitamin D deficiency in chicks, with B supplementation reducing the effects of vitamin D deficiency [19]. Nielsen et al. [6] reported that increasing the dietary intake of B from 0.25 to 3.25 mg/day in postmenopausal women increased plasma estradiol and testesterone concentration and decreased urinary calcium output. Their results demonstrated that B plays a role in postmenopausal osteoporosis. Chapin et al.

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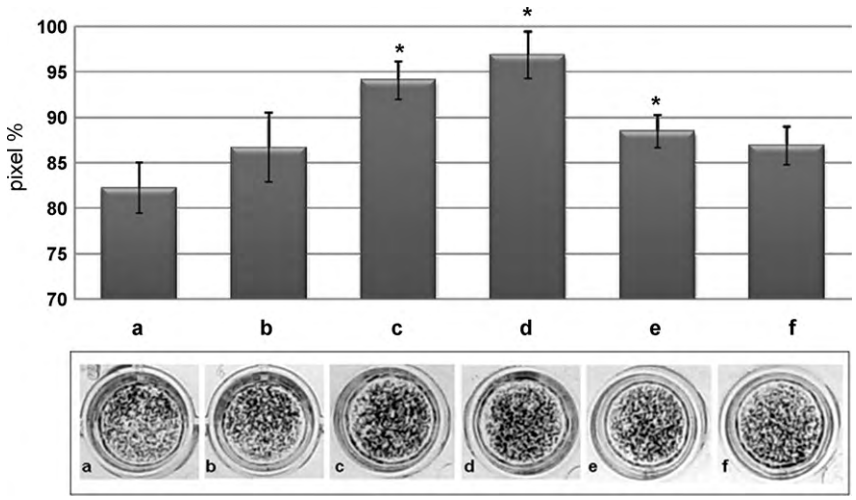

Fig. 6. Mineralization of MC3T3-E1 cells after B treatment on day 30 using von Kossa staining. Pixel values (as percentage) were calculated for comparison. Each bar represents the mean ± standard deviation. * represents statistical significance, compared with control (without B) group ($p < 0.05$). Apparent increase in mineralized nodules was noted in 1 and 10 ng/ml B treatments groups when compared to control group. There was also a slight increase in 0.1, 100 and 1000 ng/ml when compared to control group (without B). (a) 5% FBS + control (without B) + MM [MM = ascorbic acid (AA, 50 μg/ml) and β-glycerophosphate (BGP, 10 mM)], (b) 5% FBS + 0.1 ng/ml B + MM, (c) 5% FBS + 1 ng/ml B + MM, (d) 5% FBS + 10 ng/ml B + MM, (e) 5% FBS + 100 ng/ml B + MM, and (f) 5% FBS + 1000 ng/ml B + MM.

[20] studied the effect of increasing B intake from 0 to 9000 ppm boric acid for 9 weeks in young adult male rats. Investigators found a 5–10% increase in vertebral resistance to crush force.

B is a critical trace element in humans [21–23]. Significant evidence has been reported that B has beneficial effects on bone formation, composition and physical characteristics [23]. Although B can be toxic when fed in higher amounts (>20 mg/kg) like all minerals, in rats 500 mg/kg diet did not affect femur calcium and phosphorus concentration or tibia bone density. Thus, over nutritional B intakes (10–20 times nutritional intakes) may not be harmful. In our study, while at 24 hrs there was a decrease at 1000 ng/ml and above concentrations of B, at 72 hrs, there was no significant decrease in cell survival rate according to MTT assays. Based on MTT experiment, we did not use over the 1000 ng/ml concentration of B for other experiments including proliferation, mineralization, mRNA expressions and protein detection. In long term cell proliferation experiments, there was no significant difference among the groups treated with B. The higher concentration of B may reduce cell survival rate at short time period but later this effect disappears. Thus, we observed that B has no toxic effects for MC3T3-E1 cells. These concentrations should also be tried for other cell types including primary cells obtained from human bone to understand exact effects of B in human.

B clearly affects bone formation regulating hormones involved in bone growth and turnover [24]. B enhances the ability of 17-beta-estradiol (E2) but not that of PTH to improve bone quality in ovariectomized rats. The B and E2 combination increased trabecular bone surface and decreased trabecular plate separation in ovariectomized rats [25].

Nzietchueng et al. demonstrated that B modulates turnover of the extracellular matrix and increases TNF-alpha release. They investigated the direct effect of B on specific enzymes (elastase, trypsin-like enzymes, collagenase and alkaline phosphatase) implicated in extracellular matrix turnover and they found that B may regulate these enzymes in fibroblasts, *in vitro* [26]. Benderdour et al. also established that boric acid solution improved wound healing through action on the extracellular matrix, *in vitro* [27]. In our study, we also found that B regulates mRNA expression of extracellular matrix proteins including COL I, OPN, BSP and OCN which are mineralized tissue-associated proteins. In the literature, there is no study published, related to the interaction of B with pre-osteoblastic cells. The positive effects of B on the bone development and maintenance of bone in the clinical level may be explained with the results of this *in vitro* study.

Boric acid has well-defined biological effects such as stimulation of wound healing *in vivo*, release of growth factors and cytokines, and increase of the extracellular matrix turnover. Dzondo-Gadet et al. demonstrated that 10 mM boric acid greatly increased RNA synthesis using cell-free systems. They identified the factors involved in angiogenesis and, subsequently, in wound healing (VEGF and TGF-beta) by slot blot, but not FGF1 and TNF-alpha. Their results displayed that B may contribute to biological cell activities at both the transcription and translation levels [28]. In our study, we did not check the growth factors but we did examine differentiation factors including BMP-4, -6 and -7. B regulated also the protein levels of these morphogenetic proteins which are vital for bone morphogenesis.

B plays a role in bone metabolism, interacting other minerals and vitamins such as calcium, magnesium and vitamin D and hormones which are important for bone formation. Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor b (TGFb) superfamily and induce new bone formation of both cartilage and bone. BMPs also play a role in a number of non-osteogenic developmental processes. To date, around 20 BMP family members have been identified and characterized [29,30]. The roles of BMPs in embryonic development and cellular functions in postnatal and adult animals have been extensively studied. BMP-2, -4 and BMP-6, -7 induce osteoblastic differentiation in primary human mesenchymal stem cells [31]. It was reported that there is requirement of a bone morphogenetic protein for the maintenance and stimulation of an osteoblastic phenotype [32]. In the study here, the effects of B on BMP-4, -6 and -7 levels that are important in mineralization and morphogenesis of MC3T3-E1 pre-osteoblastic cells were determined with ELISA after treatment of the cells every other day with different B concentrations. In our study, it was demonstrating for the first time that B regulates BMP-4, -6 and -7 which are important proteins in the morphogenesis of bone. In the present study, B induces BMPs protein expressions under 1000 ng/ml concentration.

B can be used for modification of alloplastic materials or coating for osteoconductive biomaterials to induce bone regeneration. Gorustovich et al. [33] characterized the neoformed bone tissue around B-modified bioactive glass particles implanted in rat tibia bone marrow and they found that the thickness of osseointegrated

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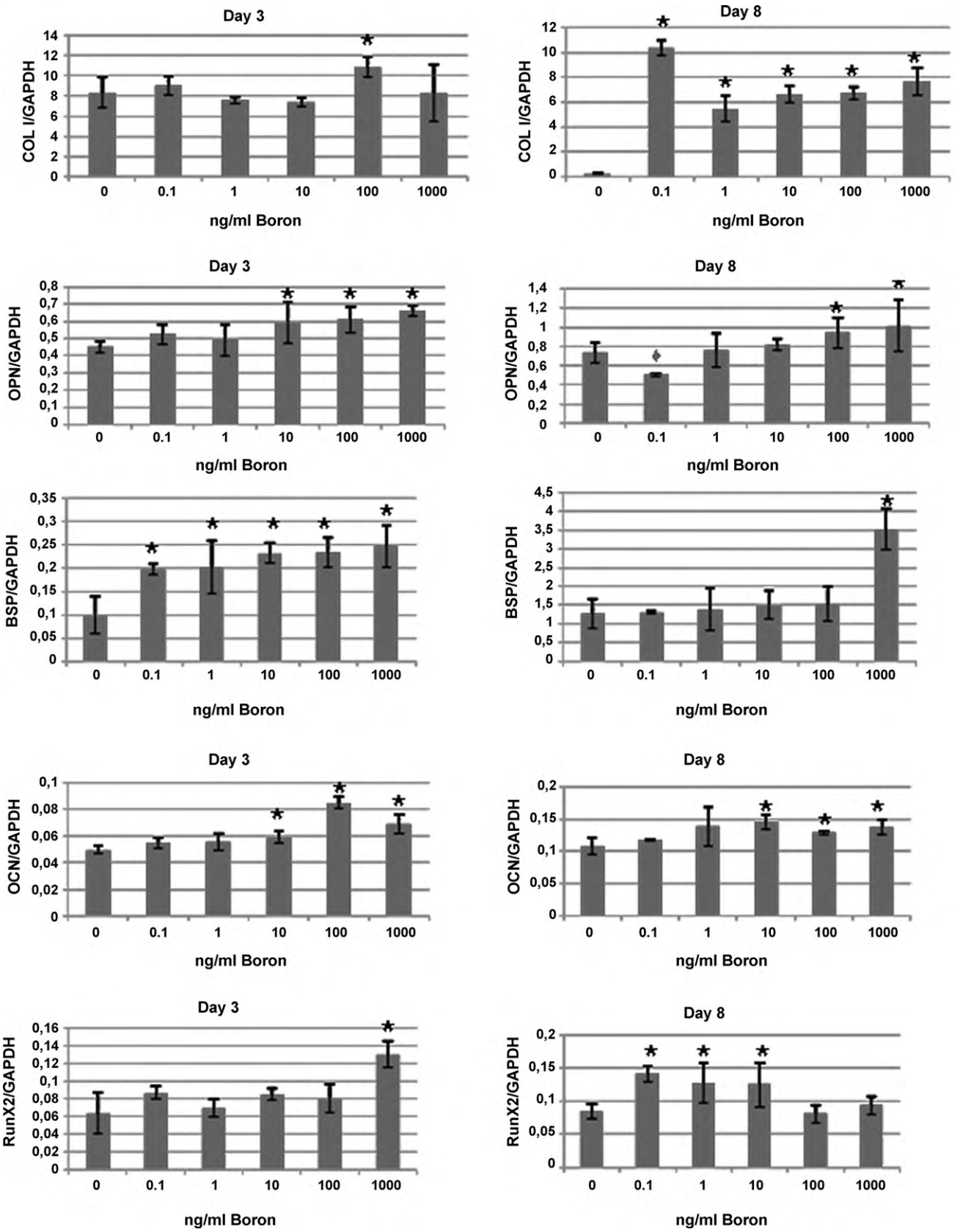

Fig. 7. Normalization graphs of mRNA expressions of mineralized tissue-associated proteins including COL I, OPN, BSP, OCN and RunX2 which is transcription factor for osteogenesis. Each bar represents the mean ± standard deviation. * represents statistically significant increase, compared with control (without B) group ($p < 0.05$). ♦ represents statistically significant decrease, compared with control (without B) group ($p < 0.05$).

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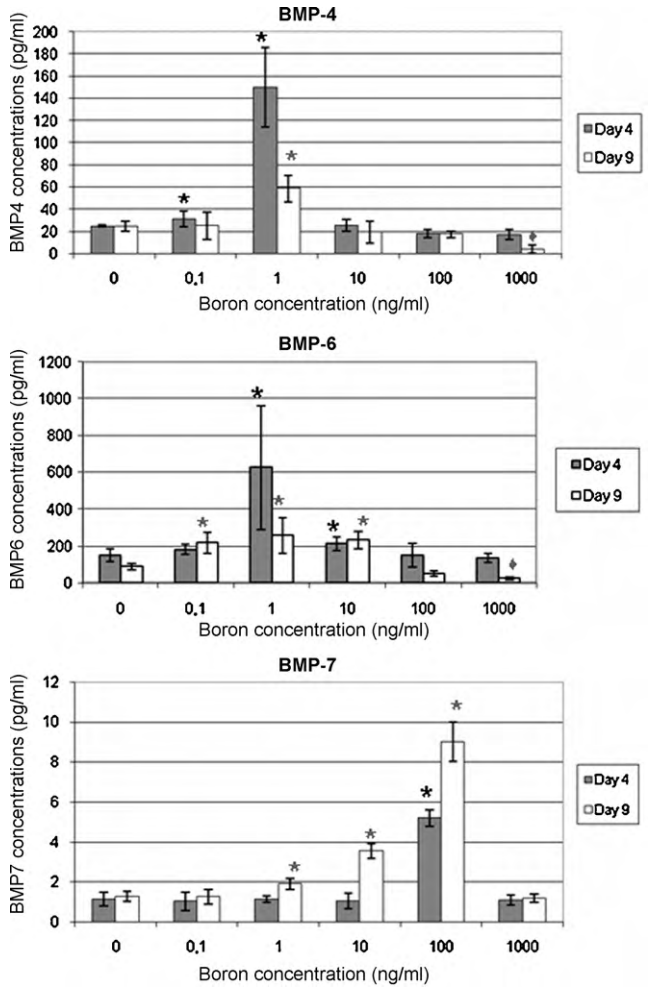

Fig. 8. BMP-4, -6 and -7 protein levels in the treatments of B on days 4 and 9. Apparent regulation was noted with B applications. Each bar represents the mean ± standard deviation. * represents statistically significant increase, compared with control (without B) group ($p < 0.05$). ♦ represents statistically significant decrease, compared with control (without B) group ($p<0.05$).

tissue and Ca:P ratio on B-modified particles was significantly greater than on the control. Moreover, Brown et al. [34] examined the effects of B_2O_3-containing glasses (borate-glass composition) on the DNA content and proliferation of osteoblastic cells (MC3T3-E1). They prepared glass-based composition with varying B_2O_3 content and they concluded that higher B_2O_3 content of the glass increased the conversion rate to hydroxyapatite, but resulted in a greater inhibition of cell proliferation under static culture conditions. When they used glasses with lower B_2O_3 content, the inhibition of cell proliferation was alleviated. Their *in vitro* evidences demonstrated that while 1B glass (low B content) had good biocompatibility and 2B glass had moderate biocompatibility, 3B glass (high B content) had little or no biocompatibility for the osteoblastic cells. In our results, we also demonstrated that higher B concentration (over 1000 ng/ml) reduced cell viability of MC3T3-E1 cells at 24 hrs but not at 72 hrs and B is a dose-dependent regulator on the osteoblastic cells. Hence, in appropriate conditions, B may be a promising component that may have positive effects on bone repair and tissue engineering.

RunX2 is a transcription factor essential for osteoblast differentiation [35,36]. It was reported that RunX2 null mice had no bone tissue, osteoblasts, or osteoclasts. RunX2 is also active in mature osteoblasts, and when active RunX2 levels have been reduced, decreased expression of the genes encoding the main bone matrix proteins including BSP, OCN, OPN and COL I were also observed [37,38]. Autocrine BMP production is necessary for the RunX2 transcription factor to be active and that BMPs and RunX2 cooperatively interact to stimulate osteoblast gene expression [39]. In our study, we observed that B regulates not only the osteoblastic transcription factor; RunX2 but also BMPs. Based on the results of our study, B is promising element to induce osteogenesis by regulating RunX2, BSP (mRNA expression level) and BMP-4, -6 and -7 (protein level).

Moreover, B affects the synthesis of the extracellular matrix and is beneficial in wound healing. Usual dietary B consumption in humans is 1–2 mg/day for adults. As B has been shown to have biological activity, research into the chemistry of boronated compounds has increased. Boronated compounds have been shown to be potent anti-osteoporotic, anti-inflammatory, hypolipemic, anti-coagulant and anti-neoplastic agents both *in vitro* and *in vivo* in animals [40]. It was also reported that B-containing compound topically reduces the formation of an inflammatory infiltrate and reduces bone loss significantly, with antibacterial and anti-inflammatory properties [41].

Evidence from numerous laboratories using a variety of experimental models [42–45], including human [46,47], shows that B is a bioactive beneficial element. According to our knowledge and literature reviews, the present study is the first study to clarify the mechanisms of B in regulation of osteoblastic behavior in MC3T3-E1 cells. Although, there are some limitations of these kind of *in vitro* studies (they always cannot mimic *in vivo* conditions), this model lead to understand some unknown mechanisms of B on the bone cells and regulation of mineralized tissue-associated mRNA expression and mineralization and BMPs. Further studies are required to realize the signaling pathway of the regulation of mineralized tissue-associated proteins of osteoblasts. Moreover, the interaction of B with some minerals (Ca, Mg) and vitamins (vitamin D) should also be investigated in osteoblasts to understand milestones in osteogenesis.

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