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The effect of boric acid on copine-7 expression and bioactivity in dental pulp stem cells

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ABSTRACT

Boron has significant impact on the mineral composition of teeth. Copine-7 (Cpne-7) is secreted by pre-ameloblasts and induces dentin formation via differentiation of mesenchymal cells of dental. The goal of this study was to see how boric acid affected the bioactivity and expression of Cpne-7, collagen type I in dental pulp stem cell. The expression of Cpne-7, COL-I were assessed in the boric acid treated pulp cells by molecular method on 3 and 8 days exposure. When comparing the different boric acid concentrations to the control group in a proliferation experiment, no significant differences were noted. At 10 ng/mL boric acid, a rise in the number of mineralized nodules was observed. Boric acid concentrations (1 ng/mL, 10 ng/mL) increased the transcripts of Cpne-7 on day 3 and 8. Additionally, 1 ng/mL boric acid concentration significantly upregulated of Cpne-7 expression compared to control group on day 3 and 8. When the 1-10 ng boric acid was compared to control group COL-I expression remarkably enhanced in cell. According to the current findings that boric acid may be a potent regulator of Cpne-7, which is a promising candidate for new dentin formation in regenerative dentistry, in dental pulp stem cells and provide osteogenic efficacy in therapies aimed at dentin formation.

1. Introduction

Copine (Cpne) is a family of calcium-associated phospholipid-binding proteins that is expressed in numerous eukaryotic organisms [1]. Copine proteins constituent structures including two C2-part (C2A and C2B) at the N-section and an A-part at the C-section [2]. The N-terminal C2 domains are calcium-associated phospholipid-binding in protein kinase C and responsible for protein–protein interaction [3]. The C-terminal A-domain is a plasma and extracellular matrix protein and play function as protein-binding domain [4]. The Cpne family of proteins consist of nine members and it was widely expressed in the organism [5]. Except for cpne-1, -2, -3, which are expressed in all normal tissues, cpne-4 has limited expression in different tissues such as prostate gland, heart and cpne-6 is specific for brain function [2, 6]. Cpne-7, which is a difussing signalling molecule, has an osteogenic diferentiation potential of mesenchymal stem cells into odontoblasts [6]. Lee et al. [7] displayed that cpne-7 stimulated odontoblast differentiation and regulated hard tissue structure. Cpne-7 stimulated odontoblast differentiation *in vitro* and regulated hard tissue structure *in vivo*, according to Lee et al. [7]. Additionally, preamelobasts stimulate differentiation for odontogenic form dental pulp stem cells (DPSCs) in media containing dental epithelium-derived factors such as Cpne-7 [8].

Boron (B) is a trace element for organisms and plays an important function in metabolism of bone formation [9,10]. Also, B may associated with steroid hormones, and prevents bone demineralization by limiting calcium loss from bone [11,12]. Our previous study demonstrated that B plays vital key function via Ca, Mg, vitamin D on bone metabolism [13]. Hakki et al. [14] confirmed that B supplementation can be beneficial for bone and teeth creation, restoration, potency *in vitro* and *in vivo*. Whereas dietary B was effective on the mineral composition and alveolar bone mineral content, it did not stimulate teeth strength and hardness [15].

DPSCs have several important potentials such as maintaining tissue homeostasis, injury repair and tissue regeneration of teeth. [16]. In the literature, it was suggested that the origin of DPSCs may be associated with neural crest-derived cells [17]. Additionally, DPSCs are highly proliferative, clonogenic and are able to differentiate into odontoblast compared to mesenchymal stem cells derived from bone marrow [18,19]. DPSCs localize at perivascular and perineural pulp region connected in the root canal to the external environments and humoral regulators such as tumor necrosis factor alpha, preameloblast-derived factor [17,20]. In this regard, DPSCs functions are crucial for pulp regeneration, dentin protect, tooth vitality and homeosta-

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sis [17,21]. Oh et al. [22] revealed that Cpne-7 induces odontoblast differentiation and dentin formation from dental-derived mesenchymal stem cell or non-dental origin. Also, Choung et al. [8] identified that Cpne-7 is a candidate signaling molecule as dental epithelium-derived factor and a key player for odontoblastic form of DPSC of mesenchymal origin.

The goal of the present experiments was to examine the influences of the boric acid (BA) on the proliferation, mineralization and level of Cpne-7, COL-I genes in DPSCs.

2. Materials and Methods

2.1. Dental Pulp Stem Cell of mesenchymal Origin

DPSCs were isolated from healthy teeth via the explant culture techniques in *in vitro* cell cultivation. To obtain dental pulp, appropriate ethical approvals should be in place. We use dental pulp tissue samples from periodontally healthy person have given their informed consent using Ethics Committee of the Faculty of Dentistry (2015/04), Selcuk University-approved consent forms.

The dental pulp tissue is rinsed with cell culture media (DMEM; GIBCO; Grand Island, NY, USA), 10% fetal bovine serum (FBS; GIBCO), L-glutamine (600 mg/ml; GIBCO;), penicillin (100 U/ml; GIBCO), streptomycin (125 mg/ml; GIBCO) to eliminate artifact and cut into small pieces mechanically. The dental pulp cell grew out from tissue pieces onto the culture dish area. Then, we observed the pulp cells can be proliferation under inverted-microscope. DPSC of mesenchymal origin characteristics were described according to International Society for Cellular Therapy [23].

To acquire a surface phenotype of DPSCs analysed with specific antibodies and immunofluorescence-based experiments, function of telomerase for mesenchymal stem cells characteristics of DPSCs [24].

2.2. Boric Acid (BA) Applications

BA (Merck Millipore, Germany) was dissolved in first in purified H_2O. The final concentrations of 0.1, 1, 10, 100, 1000, 10.000, 100.000 ng/mL BA in culture medium for various assays were made from 100 µg/mL stock solution.

2.3. Real-Time xCELLigence Impedance Analysis of Boric Acid on Proliferation of Dental Pulp Stem Cells

In this study, cell proliferation device (xCELLigence system, ACEA Biosciences, Inc., San Diego, CA, USA) was utilized to assess and BA on proliferation of DPSCs. Cell suspensions (200 µL, 10×10³ DPSMCs/well) were transferred to cell culture plate for 24 h. After 24 hours, different BA (Merck) dosages was added to each well (n=12), and the proliferative capacity (compared with untreated control cells) of the cells was assess during 170 h.

2.4. Mineralization Assay

The cells were plated at 5×10⁴ cells/cm² in 24-well plates in cell culture media for 24 h and added to the following factors: [Mineralization Media (MM)= ascorbic acid (AA, 50 µg/mL) and β-glycerophosphate (BGP, 10 mM)]; a) negative control (cell culture media), b) positive control (cell culture media + MM), c) cell culture media + MM +1 ng BA, d) cell culture media + MM +10 ng BA, e) cell culture media + MM + 50 ng BA, f) cell culture media + MM + 100 ng BA. Mineralization potential of extracellular matrix was determined on day 14 by von Kossa-based assay. For this purpose, cells were fixed in descending alcohol concentrations to ddH_2O. Then suitable silver nitrate concentration was applied and the photos were acquired.

2.5. Real-Time Polymerase chain Reaction (RT-PCR)

Copine-7 and COL-I mRNA expressions were assessed by RT-PCR. Total RNA was isolated from DPSCs treated with BA dosages (0, 1, 10 ng/mL) on 3, 8 and 16 days utilizing a RNA isolation kit (Invitrogen, Camarillo, CA, USA). The quantitative properties of the total RNA were measured by specific spectrophotometer device. Complementary DNA was performed with 1 µg RNA using synthesis kit (Thermo Scientific, Waltham, MA, USA). For gene expression analysis, mix with SYBR Green dye, forward and reverse primers were supplement to the cDNA for PCR reaction mixture (25 µL) as recommended by the supplier. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was utilized as an housekeeping gene. The following genes were assessed Copine-7 (Cpne-7), COL-I and GAPDH. Their primer sequences are as follows: Cpne-7-(human) 5'-TGGAGCTCTACAGGGTCAA -3' and 5'- CCGGGTTCAGGTTGTTCTT-3'; COL-I-(human)-5'-GCAACATTGGATTCCCTGGACC-3' and GTTCACCCTTTTCTCCCTTGCC; GAPDH- (human)-5'- ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'.

2.6. Statistical Analysis

A one-way analysis of variance (ANOVA) was utilized for proliferation assay and expression data. Subsequently, the results are presented as mean ± standard deviation. P values <0.05 were regarded statistically significant.

3. Results and Discussion

3.1. Boric Acid Did Not Affect Cell Proliferation of DPSCs

The *in vitro* proliferation activity of BA in DPSCs were

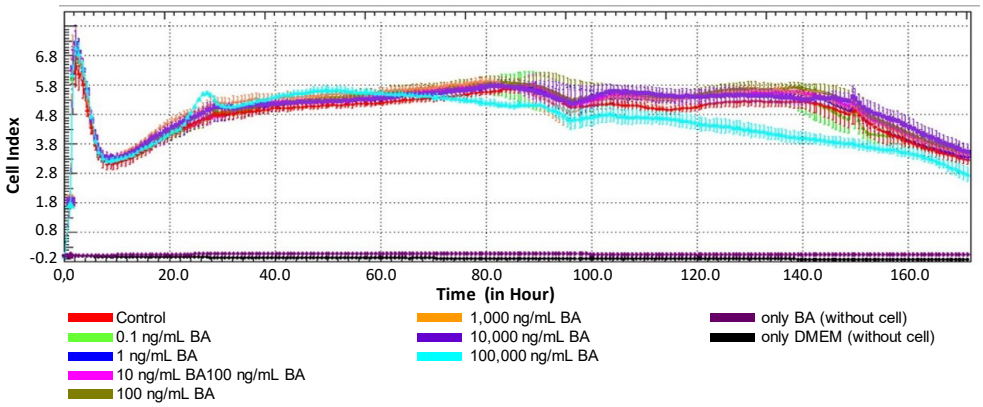

Figure 1. The effect of BA on the proliferation in DPSCs utilizing xCELLigence. The DPSCs were treated with BA dosages (0.1, 1, 10, 100, 1000, 10.000, 100.000 ng/mL). Untreated cells were used as a control (red curve) and only BA applied wells was shown as a purple curve (without cell). All cell proliferation of the DPSCs were monitored in real time for 170 hrs.

determined by utilizing real-time cell analyzer. DPSCs were treated with different BA solutions (0, 0.1, 1, 10, 100, 1000, 10.000 and 100.000 ng/mL) and cell proliferation was measured in real time for 170 h (Figure 1). When cells were exposed to 0.1-10.000 ng/mL BA, no changes in cell index were observed in real time. The control group and the six BA-treated groups had no important variations ($p>0.05$). However, 100.000 ng/mL BA concentration decreased proliferation of DPSCs compared to control group at 80 hours ($p<0.05$) (Figure 1).

3.2. Mineralization Capacity of BA on Dental Pulp Stem Cells In Vitro

The effect of different BA dosages (0, 1, 10, 50, 100 ng/mL) on mineralization potential of DPSCs was analyzed utilizing von Kossa method on day 14. Biomineralization enhancement was more significant in group applied with 10 ng/mL BA than in the untreated group (Figure 2).

3.3. Boric Acid Increased Expression Levels of Copine-7 and COL-I

Results of RT-PCR assays displayed that there were significant differences in Cpne-7 and COL-I mRNA expressions of DPSCs between the control and BA-treated groups (Figure 3 and Figure 4). To identify the effects of BA on Cpne-7 and COL-I mRNA expression levels of DPSCs were treated with BA concentrations (1 ng/mL, 10 ng/mL) and total RNA was isolated from DPSCs on days 3 and 8 with or without BA concentrations. Both 1 ng/mL BA and 10 ng/mL BA increased Cpne-7 levels on day 3 ($p<0.001$) (Figure.3a). Additionally, all BA solutions induced Cpne-7 mRNA expression on day 8 ($p<0.001$) (Figure 3b). Whereas 1 ng/mL BA concentration displayed the same tendency mRNA expression of Cpne-7 on day and 8 ($p<0.01$), 10 ng/mL BA concentrations more expression levels showed on day 3 than on day 8 ($p<0.01$).

Figure 4 shows that BA application, the expression levels of collagen type I (COL-I) in the DPSCs were significantly upregulated in a time- and dose-dependent manner when compared to the control group on 3 and 8 days ($p<0.01$) (Figure 4). Additionally, 10 ng/mL BA concentration significantly increased COL-I mRNA expression as compared with 1 ng/mL BA concentration in DPSCs on day 3 and 8 ($p<0.01$) (Figure 4a) (Figure 4b). These results indicate that 1 ng/mL BA concen-

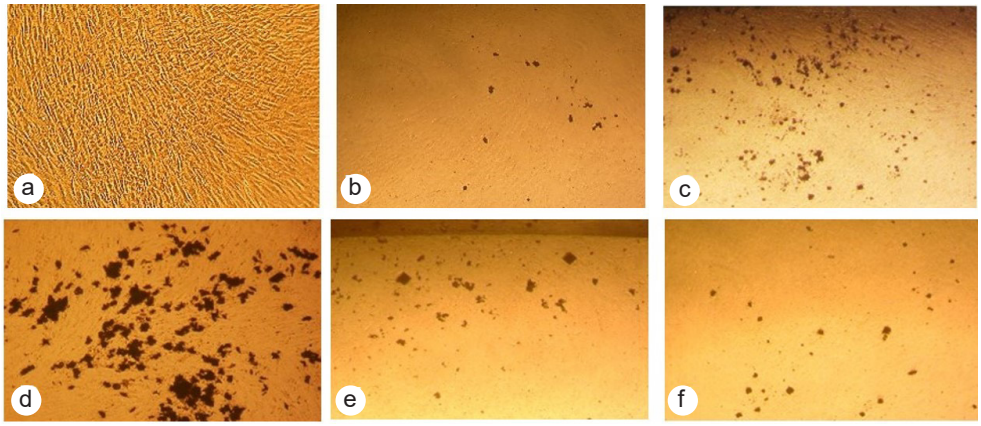

Figure 2. Biomineralization of DPSCs. Osteogenic differentiation was indicated by the formation of calcified nodules with von Kossa staining on day 14. (a) negative control (without mineralization media), (b) positive control [with mineralization media: ascorbic acid (AA, 50 µg/mL) and β-glycerophosphate (BGP, 10 mM)], (c) 1 ng/mL, (d) 10 ng/mL, (e) 50 ng/mL, (f) 100 ng/mL.

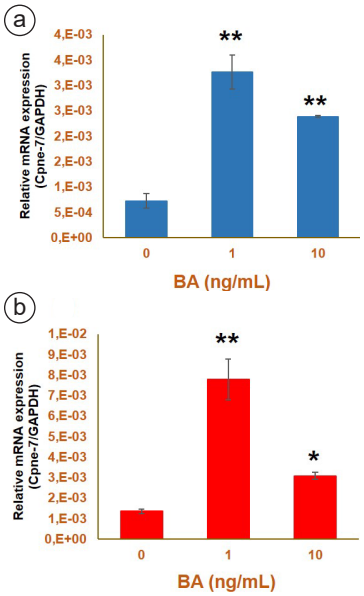

Figure 3. The expression of Cpne-7 gene in DPSCs from each group detected by quantitative RT-PCR (target genes were normalized to housekeeping genes) The cells were treated on 3 day (a) and 8 day (b) with different BA concentrations (1, 10 ng/mL). Graphical units were reflected as ($x10^{-4}$). *p<0.05, **p<0.001, compared with the control.

tration more induces Cpne-7, while 10 ng/mL BA concentrations more stimulates COL-I levels as compared to control group in DPSCs *in vitro*. BA is transformed of boron and an essential micronutrient for the human diet. In literature, there are a lot of results associated with the effect of boric acid on health outcomes *in vitro* and *in vivo*. Argument from researches have displayed that boron is a bioactive trace element for bone cells and mineralized tissue-associated genes of teeth [13-15]. Additionally, boron does not accumulate in soft tissue and is kept at a certain level in plasma, liver and brain due to homeostatic control mechanisms [25]. Cpne7 is a Ca^{2+}-dependent phospholipid associated protein that is found in the cytoplasm. Furthermore, Cpne7 is a soluble released agent produced by epithelial cells and its expression increased during tooth development. Especially, Cpne7 characterizes in predentin and odontoblasts [26]. Based on the knowledge that epithelial-mesenchymal interactions play important roles in tooth development [27]. Also, Cpne-7 both induces odontoblast differentiation and promotes dentin formation [1,22]. Cpne7 is a promising candidate for new dentin formation in regenerative dentistry as it induces the transformation of non-dental mesenchymal stem cells into odontoblasts [6]. At the developmental stage of the tooth, Cpne-7 is synthesized by enamel epithelial cells and then observed in pre-dentin and odontoblasts and pre-dentin. In literature, the level of Cpne-7 at the early form of crown structure has been displayed but its level in the mesenchymal dental pulp tissue has not yet been documented [26]. Our RT-PCR results displayed that 1 ng/mL and 10 ng/mL BA concentrations importantly upregulated Cpne-7 mRNA expressions compared to control group (0 ng/mL BA) (p<0.001). Also, both concentrations of BA (1 ng/mL, 10 ng/mL) importantly enhanced COL-I level in DPSCs a time- and dose-dependent manner (p<0.001).

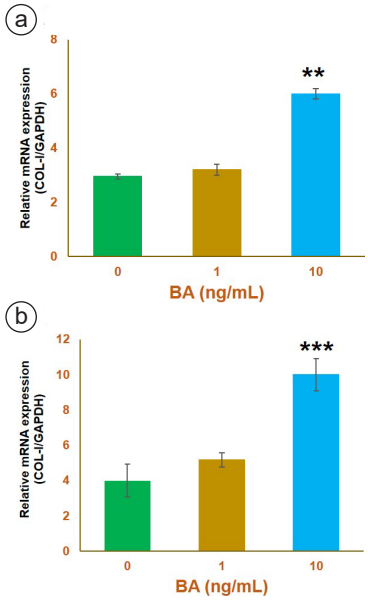

Figure 4. The expression of COL-I gene in DPSCs from each group detected by quantitative RT-PCR (target genes were normalized to housekeeping genes) The cells were treated on 3 day (a) and 8 day (b) with different BA concentrations (1, 10 ng/mL). *p < 0.05, **p < 0.001, compared with the control.

Human DPSCs are rich sources of adult stem cells situated within the tooth. DPSCs have been employed in tissue engineering and regenerative medicine because of their strong proliferative, self-renewal, and multi-lineage differentiation ability [28].

Oh et al. [22] demonstrated that Cpne7 was expressed in preameloblasts and secreted extracellularly during ameloblast development and controls the development of dental and non-dental mesenchymal cells into odontoblasts. In the present study, 1 ng/mL and 10 ng/mL BA concentrations induced Cpne-7 mRNA expression of DPSCs at different time points. This promoting effect was dose- and time-dependent. These results are in agrement with those of Seo et al. [6] who reported that Cpne-7 binding to its receptor, nucleolin, appears to play a crucial role in Cpne7 internalization into preodontoblasts in human dental pulp cells. Literature showed that conditioned medium for preameloblast such as Cpne-7 induce odontogenic transformation of human DPSCs and induce dentin structure [20].

CPNE7, a member of the Copine family comprising nine members, is found in all mammalian tissues and it has a mineralizing potential in odontoblasts as well as a high calcium ion-binding affinity *in vitro* [8]. Human dental pulp cells (hDPCs) have the ability to develop into a variety of cell types, including odontoblasts, adipocytes, chondrocytes, and osteoblasts. CPNE7

increases odontoblastic transformation of hDPCs and stimulates some structure of tooth including dentin, root via a paracrine activity utilizing nucleolin as its receptor [1]. Hakki et al. [14] displayed that boron treatments enhanced the dentin mineral density of maxillary incisor teeth *in vivo*. Our results reflected that 10 ng/mL BA concentration significantly increased COL-I mRNA expression in DPSCs ($p<0.001$). Additionally, 10 ng/mL BA concentrations up-regulated both mineralized nodules and mRNA expressions of Cpne-7 and COL-I compared to control group ($p<0.001$). Lee et al. [7] demonstrated that CDP4, which is CPNE7-derived peptide, stimulate bone and dentin formation via osteogenesis-associated genes and proteins in dental pulp cells. In a previous study, at 0.1, 1, 10, and 100 ng/ml boric acid concentrations, we found that boron as boric acid improved bone morphogenetic protein (BMP), BMP-4, -6, and -7 protein levels [13]. When compared to the control group, Cpne-7 and COL-I modulation appears to be more obvious in this experiment. This study also discovered that boric acid concentrations above 1 ng/ml have qualitative effects.

4. Conclusions

In conclusion, the upregulation of Cpne-7 mRNA in DPSCs adds to the growing body of evidence that boron, in either nutritional or physiological amounts, is required for the transformation of odontoblast-like cells from mesenchymal cells of dental source. This effect occurs at the molecular level, and as a result, it can have a variety of beneficial phenotypic effects, including regulating the differentiation of mesenchymal stem cells into odontoblasts. Since only Cpne-7 and COL-I were examined in this study, these findings should be revealed in odontoblast differentiation markers such as Dspp, nestin, and ALP to confirm that Cpne-7 expression is regulated by BA and its relevance on the dental pulp tissue are confirmed.

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