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The histopathological and morphometric investigation of the effects of systemically administered boric acid on alveolar bone loss in ligature-induced periodontitis in diabetic rats

HATICE BALCI YUCE[1], HULYA TOKER[2] & FAHRETTIN GOZE[3]

[1]*Department of Periodontology, Faculty of Dentistry, Gaziosmanpaşa University, Tokat, Turkey,* [2]*Department of Periodontology, Faculty of Dentistry, and* [3]*Department of Pathology, Faculty of Medicine, Cumhuriyet University, Sivas, Turkey*

Abstract

Objective. The purpose of this study was to evaluate the effects of systemically administered boric acid on alveolar bone loss, histopathological changes and oxidant/antioxidant status in ligature-induced periodontitis in diabetic rats. ***Materials and methods.*** Forty-four Wistar rats were divided into six experimental groups: (1) non-ligated (NL, $n = 6$) group, (2) ligature only (LO, $n = 6$) group, (3) Streptozotocin only (STZ, $n = 8$) group, (4) STZ and ligature (STZ+LO, $n = 8$) group, (5) STZ, ligature and systemic administration of 15 mg/kg/day boric acid for 15 days (BA15, $n = 8$) group and (6) STZ, ligature and systemic administration of 30 mg/kg/day boric acid for 15 days (BA30, $n = 8$) group. Diabetes mellitus was induced by 60 mg/kg streptozotocin. Silk ligatures were placed at the gingival margin of lower first molars of the mandibular quadrant. The study duration was 15 days after diabetes induction and the animals were sacrificed at the end of this period. Changes in alveolar bone levels were clinically measured and tissues were histopathologically examined. Serum total antioxidant status (TAS), total oxidant status (TOS), calcium (Ca) and magnesium (Mg) levels and oxidative stress index (OSI) were evaluated. Primary outcome was alveolar bone loss. Seconder outcome (osteoblast number) was also measured. ***Results.*** At the end of 15 days, the alveolar bone loss was significantly higher in the STZ+LO group compared to the other groups ($p < 0.05$). There was no significant difference in alveolar bone loss between the STZ+LO 15 mg/kg boric acid and STZ+LO 30 mg/kg boric acid groups ($p > 0.05$). Systemically administered boric acid significantly decreased alveolar bone loss compared to the STZ+LO group ($p < 0.05$). The osteoblast number in the BA30 group was significantly higher than those of the NL, STZ and STZ+LO groups ($p < 0.05$). Inflammatory cell infiltration was significantly higher in the STZ+LO group the other groups ($p < 0.05$). Serum TAS levels were significantly higher in the NL and LO groups than the other groups ($p < 0.05$). The differences in TOS levels were not found to be significant among all the groups ($p > 0.05$). The OSI values of the BA30 group were significantly lower than the STZ+LO group ($p < 0.05$). Also, the differences in serum calcium and magnesium levels were insignificant among the all groups ($p > 0.05$). ***Conclusion.*** Within the limits of this study, it can be suggested that BA, when administered systemically, may reduce alveolar bone loss in the diabetic rat model.

Key Words: *experimental diabetes mellitus, periodontitis, boric acid*

Introduction

Diabetes mellitus is a clinically and genetically heterogeneous group of metabolic disorders characterized by abnormally high levels of glucose in the blood, altered glucose tolerance and impaired lipid and carbohydrate metabolism [1,2]. It is associated with a number of complications directly resulting from hyperglycemia [3]. Most of these complications are vascular in nature such that macrovascular changes in diabetes lead to increased risk of myocardial infarction and stroke as a result of atherosclerosis [4]. Diabetic microvascular pathology includes retinopathy, end-stage renal disease, a variety of debilitating neuropathies, poor wound healing, enhanced risk of infection and periodontal disease [3,5–7].

A reciprocal relationship exists between diabetes mellitus and periodontal disease [1,8]. Periodontal infections have a significant impact on diabetic

Correspondence: Hatice Balci Yuce, PhD, Department of Periodontology, Gaziosmanpaşa University Faculty of Dentistry, Tokat 60100, Turkey. Tel: +90356 2124222. Fax: +90356 2124225. E-mail: htbalci@gmail.com

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control. Conversely, diabetes mellitus is a significant risk factor for the development of periodontal disease and aggravates the severity of periodontal infections [1,8]. There is considerable evidence that both diabetes mellitus and periodontal diseases can modulate the host innate immune system, such as up-regulation of inflammatory cell phenotype, elevation of pro-inflammatory cytokines and initiation of tissue damage [2,3]. The innate immune system is thought to play a major role in the progression of periodontal disease, in part through the production of pro-inflammatory cytokines [9].

Oxidative stress is defined as the disturbance of the oxidant–antioxidant balance [10] and, in addition to the periodontitis, is considered to be an important etiopathogenic factor in many other inflammatory diseases including diabetes mellitus [11,12]. As a result of oxidative stress, unbalanced radical and non-radical reactive oxygen species can damage cells by a variety of mechanisms, including peroxidation of lipid membranes, protein inactivation, induction of DNA damage and stimulating specific signaling pathways that lead to cytokine-induced tissue damage [3,13]. Reactive oxygen species are reported to be capable of inducing periodontal tissue destruction and are associated with osteoclastic bone resorption. Also, an increase in reactive oxygen species production plays a very important role in the complications arising in diabetes patients [3]. Taken together, scavenging or detoxification of reactive oxygen species is crucial for the maintenance of homeostasis in normal tissue and organ systems [14].

Boron is the fifth element in the periodic table; it contains characteristics of both metals and non-metals. Boron is abundant in nature as boric acid and borate and can be obtained in the diet through the consumption of fruits, vegetables and legumes [15].

Boron accumulates in bone in concentrations that depend on the amount of that element consumed [16] and it also has antioxidant properties [17]. It prevents oxidative damage by increasing glutathione peroxidase and its analog and by promoting other neutralizing agents of ROS [18]. Besides, boron is known to influence a variety of metabolic actions. It interacts with calcium, vitamin D and magnesium, which are all important in bone metabolism [17]. Hakkı et al. [19] showed that boron has induced osteogenesis by regulating RunX2, bone sialoprotein (mRNA expression level) and bone morphogenetic protein -4, -6 and -7 (protein level) in osteoblastic cells *in vitro*.

Additionally, boron may affect immune responses [20,21]. Supplementation of 2 mg/kg boron to a low boron diet (0.1 mg/kg boron) decreased paw swelling in rats with adjuvant-induced arthritis, suggesting boron has an effect on the inflammatory response [20]. Also a recent report has shown that systemically administered boric acid diminishes alveolar bone loss, decreases inflammatory cell infiltrate and increases osteoblastic activity in experimental periodontitis in rats [22].

Based on these favorable aspects of boric acid, we have hypothesized that boric acid is a potent suppressor of periodontal inflammation and alveolar bone loss in diabetic rats. Furthermore, there is no study investigating the effects of boric acid on alveolar bone loss and oxidant/antioxidant status in diabetic rats. Therefore, we aimed to investigate the effects of systemically administered boric acid on serum oxidant/antioxidant status and alveolar bone loss in ligature-induced periodontal disease in a diabetic rat model.

Materials and methods

Animals and experimental periodontitis model

The study protocol and experimental design were approved by the Animal Ethics Committee of Cumhuriyet University School of Medicine (no. 213/2012). In total, 44 Wistar male rats were used in the experiment. Their body weight ranged from 270–320 g at the beginning of the experiment. The animals were randomly divided into six groups as follows:

- Non-ligated (NL) group (n = 6);
- Ligature-only (LO) group (n = 6);
- STZ-only (STZ) group (n = 8);
- STZ and ligature (STZ+LO) group (n = 8);
- STZ and ligature plus 15 mg/kg/day boric acid (for 15 days) (BA15) group (n = 8); and
- STZ and ligature plus 30 mg/kg/day boric acid (for 15 days) (BA30) group (n = 8).

Induction of diabetes

Diabetes was induced by a single injection of 60 mg/kg body weight streptozotocin (Sigma-Aldrich, St Louis, MO] (STZ) dissolved in citrate buffer (0.01 M, pH 4.5) into the jugular vein. Blood glucose levels were measured with a glucometer (IME-DC, Oberkotzau, Germany) before the procedure and at the 3 days after diabetes induction (Table I). The glucose level greater than 300 mg/dl confirmed the presence of diabetes. Also blood glucose levels were measured at days 7 and 15.

Induction of experimental periodontitis

One day after diabetes confirmation the rats from the STZ+LO, BA15 and BA30 groups and LO group received ligature placement performed under general anesthesia using ketamine (Eczacibasi Ilac Sanayi, Istanbul, Turkey) (40 mg/kg). A 4-0 silk suture (Dogsan Ilac Sanayi, Istanbul, Turkey) was sub-marginally placed around the first molars of the right mandibular

Table I. Histopathological and biochemical results in study groups.

	NL	LO	STZ	STZ+LO	BA15	BA30
Osteoblast numbers	17.5 ± 8.8★	67.3 ± 26.5†	47.5 ± 17.3†	64.5 ± 23.3†	99.6 ± 16.4	108.2 ± 6.1
Osteoclast numbers	4.0 ± 1.5§	15.0 ± 10.8	17.8 ± 30	23.2 ± 28.7‡	7.8 ± 3.6	5.0 ± 2.5
Serum Ca	9.81 ± 0.25	9.65 ± 0.40	9.90 ± 0.55	9.36 ± 0.68	9.60 ± 0.55	9.05 ± 0.81
Serum Mg	2.10 ± 0.25	2.36 ± 0.28	2.17 ± 0.20	2.28 ± 0.23	2.16 ± 0.16	1.96 ± 0.14

Data are given as mean ± standard deviation.
★$p < 0.05$ vs the LO, STZ+LO, BA15 and BA30; †$p < 0.05$ vs the BA15 and BA30 groups; §$p < 0.05$ vs the LO and STZ+LO groups; ‡$p < 0.05$ vs the BA30 group.

quadrants. The sutures were checked after application and lost or loose sutures were replaced. All ligatures were placed by the same operator (H.B.Y.). The animals were kept in individual cages and received water and food *ad libitum*. BA was prepared as 15 and 30 mg/kg for 0.5 ml distilled water and systemically administered by gastric feeding at a rate of 0.5 ml daily for 15 days. On day 15, the animals were sacrificed and the blood samples were taken by cardiac puncture.

Measurement of alveolar bone loss

After the decapitation of rats, the mandibles were carefully removed and the gingival tissues around the molar teeth were excised. Then mandibles were stained with aqueous methylene blue (Merck & Co., Inc., Whitehouse Station, NJ) (1%) to identify the cemento-enamel junction (CEJ). The alveolar bone height was measured under a stereomicroscope (Stemi DV4, Carl Zeiss, Jena, Germany) (×25 magnification) by recording the distance from the CEJ to the alveolar bone crest. Measurements were taken at three points on both the buccal and lingual sides to quantify the alveolar bone level. A mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner (H.T) who was unaware of the identity of samples.

Histopathological evaluation

Histological analysis was performed by a single examiner (F.G.) who was also blinded to the identity of samples. The mandible samples were fixed in 10% formalin and demineralized in 10% formic acid. The specimens were then dehydrated, embedded in paraffin and sectioned along the molars in a mesio-distal plane for hematoxylin-eosin and Masson's trichrome staining. Sections of 6 μm thickness, corresponding to the area between the first and second molars where ligatures had been placed, were evaluated by light microscopy (Nikon Eclipse, E 600, Tokyo, Japan).

Inflammatory cell infiltration (ICI) scoring was based on the inflammatory cell accumulation around the first molars. ICI was determined by a semi-quantitative scoring [23] as no visible ICI (0), slightly visible ICI (1), moderately visible ICI (2) and the dense ICI (3). Multi-nucleated giant cells with existing resorption lacunae, ruffled border and an eosinophilic view were counted as osteoclasts. Osteoblast cell counting was performed in the visible active bone formation surfaces, which were limited by osteoid and cuboidal osteoblasts.

Biochemical analyses of blood

After the blood samples were taken by cardiac puncture, serum TAS, TOS, Ca and Mg levels were measured in the Cumhuriyet University School of Medicine Department of Biochemistry.

All laboratory analyses were performed on the same day. On the day of the assay TOS and TAS levels were measured using commercially available kits (Rel Assay, Mega Tıp, Gaziantep, Turkey) by Erel's colorimetric method [24,25] at an absorbance of 520 nm. For determination of TOS, the method utilizing oxidation of the ferrous ion-o-dianisidine complex to ferric ion by oxidants present in the sample was used. The oxidation reaction was enhanced by glycerol that was present in abundance in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micro molar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L).

Determination of TAS is based on bleaching of the characteristic color of a more stable 2, 2 ¢-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation by antioxidants. The assay has precision values of <3%. The results were expressed as mmol Trolox equivalent/L.

The ratio of TOS to TAS was accepted as OSI. For calculation the resulting TAS unit was converted to μmol/L and the OSI value was calculated using the following formula; OSI (arbitrary unit) = TOS

(μmol H_2O_2 equivalent/L)/TAS (μmol Trolox equivalent/L) [26].

Statistical analysis

Data were presented as mean ± SD or percentage as appropriate. Osteoclast and osteoblast numbers, alveolar bone loss, gingival TNF levels, serum total antioxidant status (TAS), total oxidative stress (TOS), Ca and Mg concentrations were analyzed with ANOVA followed by Tukey test for pair-wise comparisons. Presence of ICI was analyzed by chi-square test. *P*-values less than 0.05 were considered statistically significant. An alpha of 0.05 was selected for calculation. The required sample size was eight in diabetic-periodontitis groups, giving a statistical power of 85%. The alveolar bone loss and osteoblast number was determined as the expected primary and secondary outcomes of the study, respectively.

Results

Morphometric measurements

The presence of the silk ligature around the first molar induced an inflammatory reaction in the periodontal tissue. Measurement of alveolar bone loss in the mandibular molar tooth revealed significantly higher bone loss values in the STZ+LO group compared to the other groups ($p < 0.05$) (Figures 1 and 2). Diabetes increased the severity of alveolar bone loss in the STZ+LO group as the maximum destruction was 1.84 mm. Administration of both doses of boric acid normalized the negative effects of diabetes on periodontal destruction (Figure 1). The lowest alveolar bone loss was 0.57 mm in the BA15 group. Also there was no significant difference in alveolar bone loss between the BA15 and BA30 groups ($p > 0.05$). The difference between the NL and BA15 groups in alveolar bone loss was not significant either ($p > 0.05$).

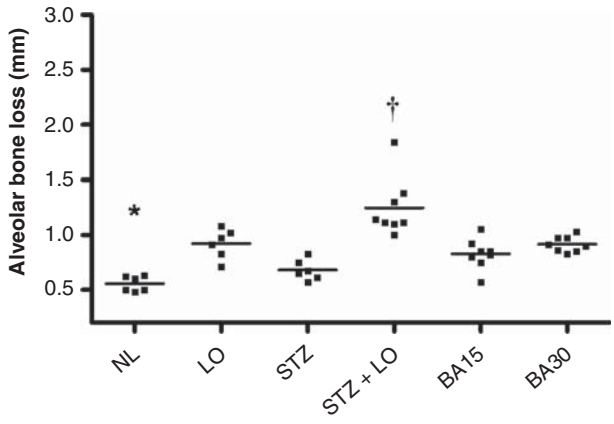

Figure 2. Mean alveolar bone loss in the study groups. *$p < 0.05$ vs LO, STZ+LO and BA30; †$p < 0.05$ vs LO, STZ, BA15 and BA30.

Histopathological analyses

A typical histological view for each group is shown in Figures 3A–F. ICI in the NL and STZ groups were significantly lower than those of the other groups ($p < 0.05$) and there was no significant difference in ICI between the NL and STZ groups ($p > 0.05$). The density of ICI in the STZ+LO group was significantly higher than those of the LO, BA15 and BA30 groups ($p < 0.05$). Regarding ICI the difference between BA15 and BA30 groups did not reach statistical significance ($p > 0.05$).

The osteoclast numbers of the study groups are shown in Table I. The osteoclast number in the STZ +LO group was significantly higher than those of the NL and BA30 groups ($p < 0.05$) (Figure 3). There

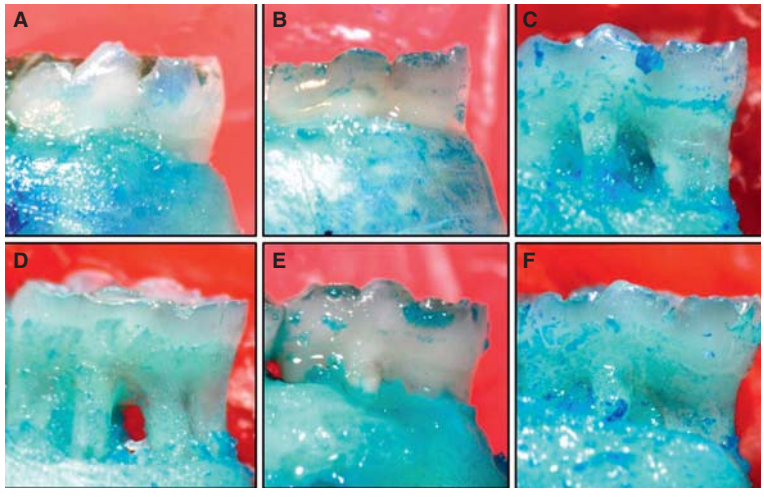

Figure 1. Representative images of the alveolar bone loss in mandibular first molars in all groups. (A) NL group; (B) STZ group; (C) LO group; (D) STZ+LO group, € BA15 group; (F) BA30 group. BA application prevents the bone loss induced by ligatures around mandibular first molars in rats.

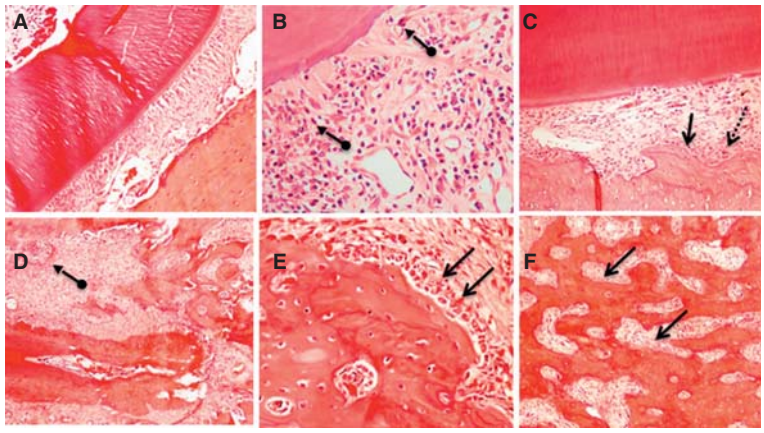

Figure 3. (A) Dentin, periodontal ligament and alveolar bone in the NL group (H&E, original magnification ×200). (B) Numerous inflammatory cells in the LO group (round-nosed black arrows indicate polimorphonuclear leucocytes) (H&E, original magnification ×200). (C) Dentin, periodontal ligament and alveolar bone in the STZ group are shown (straight black arrow points osteoblast cell and dotted black arrow points osteoclast cell) (H&E, original magnification ×400). (D) Alveolar bone loss around mandibular first molar tooth in the STZ+LO group (round-nosed black arrows indicate polimorphonuclear leucocytes) (H&E, original magnification ×40). (E) Alveolar bone in the BA15 group (straight black arrows point osteoblast cells) (H&E, original magnification ×200). (F) Alveolar bone in the BA30 group (straight black arrows point osteoblast cells) (H&E, original magnification ×100).

were no significant difference among the NL, BA15 and BA30 groups regarding osteoclast numbers ($p > 0.05$).

There was no significant difference in osteoblast numbers between the NL and STZ groups ($p > 0.05$). Also, the osteoblast numbers in the BA15 and BA30 groups was significantly higher than those of the NL, STZ and STZ+LO groups ($p < 0.05$) (Table I). There was no significant difference in osteoblast cell counts between the BA15 and BA30 groups ($p > 0.05$) (Figure 3).

Biochemical analyses

There was no significant difference in serum Ca and Mg concentrations among groups (Table I). Table II presents data relating to TOS and TAS levels and OSI in serum. TAS level was significantly higher in the LO group than those of the other groups ($p < 0.05$). There were no significant difference in TOS levels among all groups ($p > 0.05$). OSI level of the BA30 group was close to the level of the NL group and both groups were lower than those of the other groups ($p < 0.05$).

Discussion

Our study found that boric acid administration decreased alveolar bone resorption in STZ-induced diabetic rats with periodontitis. Further characterization of the bone-related changes has suggested that the suppression of the bone loss was due to reduced osteoclastic activity, OSI values and increased osteoblastic activity. Therefore, the data supports the notion that boric acid may be an effective element in prevention of streptozotocin and periodontitis-induced alveolar bone loss.

In this study streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-d-glucopyranose), a naturally produced antibiotic from *Streptomyces achromonogenes* [27], was used as a diabetogenic agent that causes selective destruction of pancreatic β cells in order to induce experimentally decreased secretion of insulin. STZ-induced hyperglycemia is a widely used experimental model [7,27–31]. Also, our results showed that plasma glucose levels increased in STZ-treated rats (Table I).

Ligature methods have been accepted as useful experimental models of periodontitis with alveolar

Table II. Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) in study groups.

	NL	LO	STZ+LO	BA15	BA30
TAS	1.5 ± 0.1	1.5 ± 0.2	0.9 ± 0.2†*	0.9 ± 0.1†*	0.9 ± 0.1†*
TOS	6.1 ± 2.1	9.0 ± 2.6	8.7 ± 3.5	9.0 ± 2.1	5.7 ± 2.1
OSI	0.4 ± 0.1	0.6 ± 0.2	0.9 ± 0.3†	0.9 ± 0.1†	0.6 ± 0.2

Data are given as mean ± standard deviation.
$^{\star}p < 0.05$ vs the LO group; $^{\dagger}p < 0.05$ vs the NL group.

bone resorption [7,31]. This ligature favored the formation of bacterial plaque and induced an inflammatory response, reproducing human periodontal disease [32,33]. In the present study, ligature placement on the first molar tooth caused a significant amount of bone loss and, also, the amount of bone loss was the highest in STZ-induced rats. However, for every animal model of a human disease, there are inherent limitations. Molars in rats are similar in anatomic configuration and structure to humans, but the molars of rats are smaller, so it was difficult to perform any sort of periodontal treatment. A further limitation to the experimental model used is that the induced periodontitis follows an acute course, during which tissue trauma and adjacent microbial accumulation accelerate the destructive process. Such pathways of acute inflammation are likely to differ from chronic periodontitis [34].

Boron can be toxic when fed in higher amount like all minerals. Boron, boric acid and boron oxide are primarily irritants under exposure conditions. Biochemical symptoms of toxicity include riboflavinuria and riboflavin deficiency, along with the inhibition of the dehydrogenase enzymes [35]. Toxic ingestions of boron may cause nausea, vomiting and diarrhea [17]; 17.48% of boric acid is boron and the fatal doses of boron for humans and rats are 640 mg/kg and 2660 mg/kg, respectively [36].

Alveolar bone resorption is an inevitable result of periodontitis and it has also been demonstrated in diabetic conditions [31,32]. Osteoclasts, in addition to phagocytes, have been shown to produce reactive oxygen species and it has been suggested that these species are involved in the process of bone resorption [37]. Boron is known to inhibit the activities of specific enzymes involved in extracellular matrix turnover and metabolism. It is shown that boron directly inhibited elastase and alkaline phosphatase activities and had no effect on trypsin-like and collagenase activities [38]. In this study systemically administered boric acid reduced osteoclastic activity and osteoclast numbers and also diminished tissue destruction in diabetic rats.

Supplemental boron as boric acid has been shown to increase bone strength measures in rats [16]. Besides, positive effects of boron also have been shown on early bone regeneration in rabbits after expansion of midpalatal suture [39]. Hakkı et al. [19] demonstrated that boron increased the mRNA expression of collagen type I, osteopontin, bone sialoprotein, osteocalcin and RunX2 and protein levels of bone morphogenetic protein -4, -6, -7 *in vitro*. In accordance with these findings, both doses of boric acid induced osteoblastic activity in this study.

Previous reports have demonstrated that dietary boron is a physiological regulator of the normal inflammatory response [40–42]. It was reported that dietary boron supplementation resulted in a significant decrease in TNF-α levels and a remarkable decrease in high sensitive C-reactive protein and interleukin-6 levels [41]. Also, Cao et al. [43] reported that boric acid has a moderate inhibitory effect on LPS-induced TNF-a production *in vitro*. They also suggested that treatment with boric acid could limit inflammatory injury without a significant impairment of defense mechanisms. In addition, Demirer et al. [22] showed that systemically administered boric acid reduced ICI and osteoclast numbers in ligature-induced periodontitis in rats. Supporting this result, the present study demonstrated that boric acid histopathologically reduced inflammatory cell infiltration in periodontal tissues in diabetic rats.

Enhanced oxidative stress was observed in patients with diabetes, as indicated by increased free radical production, lipid peroxidation and diminished antioxidant status [3,10]. There are several reports regarding the effects of periodontal status on TAS and TOS [44,45]. It has been reported that periodontitis was associated with lower TAS levels [46,47]. Another study found no relationship between chronic periodontitis and serum TAS and TOS values [48]. Moreover, although TAS and TOS values influence patients' oxidative status, an imbalance between oxidant and antioxidant status must be considered to recognize patients' exact oxidative status. The OSI gives an opportunity for evaluating this process. In a study that was investigated the relationship between these two chronic inflammatory diseases (rheumatoid arthritis and chronic periodontitis) with regard to antioxidant and oxidant status, it was found that local OSI values in groups with patients with chronic periodontitis were higher, whereas systemic OSI values showed no difference among the groups [45]. Also, it has been reported that boron pre-treatment (4 mg/kg borax) could replenish the depleted level of glutathione, with a concomitant decrease in lipid peroxidation in rats with fulminant hepatic failure [49]. Furthermore, Turkez et al. [50] reported that boron compounds increased superoxide dismutase and catalase activities at low doses (15 mg/L), while at high doses (500 mg/L) they decreased both superoxide dismutase and catalase activities in erythrocytes versus control. So it can be concluded that boric acid has a dose-dependent effect on antioxidant systems. Similarly, in this study 30 mg/kg boric acid reduced OSI values to the control levels in diabetic rats.

In conclusion, our results revealed that STZ-induced diabetes may lead to enhanced alveolar bone loss in experimental periodontitis. Furthermore, this study represents, within the inherent limitations between experimental animal and human disease interventions, the evidence that systemic administration of boric acid decreases alveolar bone loss in experimental periodontitis in the diabetic rat model. Although we are unable to make definitive

conclusions regarding the effects of BA on a diabetic animal model with periodontitis, the present data appear to be meaningful with regard to the beneficial effects of boric acid in diabetes mellitus. Also our results suggested that the inhibition of inflammation and alveolar bone loss with boric acid administration may also be a novel strategy for treating diabetes-induced periodontitis.

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