Original Article

Boric acid inhibits alveolar bone loss in rat experimental periodontitis through diminished bone resorption and enhanced osteoblast formation

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Abstract *Background/purpose:* Inhibition of bone resorption is essential for periodontal treatment. Recently, it has been suggested that boric acid suppresses periodontitis, but the mechanism of this inhibition is still not well understood. Therefore, to analyze the cellular response to boric acid administration, we histologically evaluated alveolar bone in experimental periodontitis of rats administered boric acid.

Materials and methods: 5-0 silk ligatures were placed around the cervix of the second maxillary molars of 4 week-old rats treated with or without boric acid. Five and 14 days after ligature placement, the periodontal tissues between first and second molars were investigated histologically and immunohistochemically using antibodies to CD68, cathepsin K, and α-smooth muscle actin (SMA).

Results: Five days after the beginning of the experiment, many CD68-positive cells appeared in the periodontal tissues with ligature placement without boric acid administration. Also, the number of cathepsin K-positive osteoclasts had increased on the surface of alveolar bone. However, boric acid administration prevented severe bone resorption and reduced the number of cells positive for CD68 and cathepsin K. At day 14 post treatment, cells positive for α-SMA were seen in the periodontal tissues after boric acid administration, whereas no such cells were found around the alveolar bone without the administration of boric acid.

Conclusion: Boric acid inhibited the inflammation of ligature-induced periodontitis. This agent might reduce bone resorption by inhibiting osteoclastogenesis and also could accelerate osteoblastogenesis.

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Introduction

Inhibition of bone resorption in periodontitis is a key objective of dental treatment, because periodontal diseases are a major cause of tooth loss. Since the major etiological factor of periodontitis is thought to be a microbial infection following inflammation, scaling and root planning (SRP) is performed as a standard therapy to reduce bacterial volume. In addition to SRP, severe periodontal lesions are treated using surgical techniques including guided tissue regeneration, bone grafting, advance flap surgery, and the use of Emdogain, but their success is not predictable. Thus, the development of new periodontal therapies using less invasive techniques is highly desirable.

Boric acid (H_3BO_3), or orthoboric acid, is a weak acid of boron and produces anti-microbial activity by inhibiting cell growth and cell wall synthesis.[1–3] Boric acid exerts anti-inflammatory effects by regulating the levels of oxidants and antioxidants levels in tissues.[4,5] Therefore, in the treatment of periodontitis, it has been suggested that boric acid may reduce some clinical measurements such as alveolar bone loss and bleeding on probing.[6–9] However, the mechanisms of these improvements in periodontal tissues have not yet been investigated in detail.

Bone volume is regulated by bone cells including osteoclasts and osteoblasts. Osteoclasts are multinucleated cells whose function is bone resorption. The precursors to osteoclasts originate from monocyte-macrophages which express CD68. After osteoclasts attach to the bone matrix, they secrete acid and proteinases, including cathepsin K and matrix metalloproteinase-9, and degrade collagen and other bone matrix proteins.[10] Osteoblasts produce mineralized bone matrix and are derived from undifferentiated mesenchymal cells. Previous reports have described that α-smooth muscle actin (SMA) is localized in the pericytes of blood vessels as well as some stem or progenitor cells.[11,12] In the process of osteoblast differentiation, this protein is produced in osteoblast progenitor cells during tissue repair and regeneration.[13,14] Thereafter, runt-related transcription factor 2 (Runx2) and Osterix, essential transcription factors for osteoblast differentiation, are expressed in osteogenic cells and induce the expression of bone matrix proteins.[10,15]

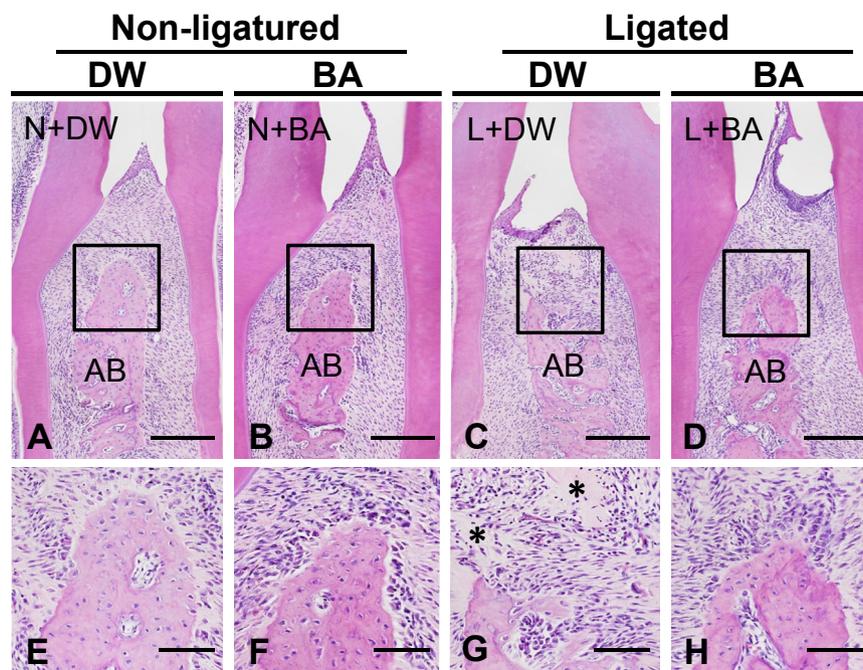In the present study, we histologically and morphometrically evaluated the structure of alveolar bone in

Figure 1 Histological observations of non-ligatured (A–B, E–F) and ligated (C–D, G–F) periodontal tissues at day 5 stained by hematoxylin and eosin. Higher magnifications of the boxed regions in "A", "B", "C" and "D" are shown in "E", "F", "G", and "H", respectively. (A–B, E–F) No inflammation is visible around the alveolar bone (AB). (C, G) There are many inflammatory cells and necrotic tissues (asterisks) beneath the ligature in the absence of boric acid. Bone resorption is observed at the crest of alveolar bone. (D, H) Boric acid administration shows the inhibitory effects of ligature-induced inflammation. Scale bars: 100 μm (A–D), 30 μm (E–H).

experimentally-induced periodontitis after the administration of boric acid. The immunohistochemical observations undertaken involved the use of CD68-, cathepsin K-, and α-SMA-specific antibodies.

Material and methods

Experimental procedures

All experiments were approved and performed according to guidelines set forth by the Animal Ethics Committee of the Health Sciences University of Hokkaido. Eighty male Sprague Dawley (SD) rats, 4 weeks of age and weighing 70—80 g, were used in this experiment. They were housed in cages and maintained on a 12 h light/12 h dark cycle at a constant temperature of 25°C. The animals were divided into four groups: non-ligatured rats with distilled water (DW) administration (N + DW), non-ligatured rats with boric acid administration (N + BA), ligature-placement rats with DW administration (L + DW), and ligature-placement rats with boric acid administration (L + BA).

In the L + DW and L + BA groups, after the animals had been anesthetized by an intraperitoneal injection of 0.3 mg/kg medetomidine hydrochloride (Domitor®, Orion Pharma Co. Ltd, Espoo, Finland), 4 mg/kg midazolam (Dormicum®, Astellas Pharma Inc., Tokyo, Japan), and 5 mg/kg butorphanol (Vetorphale®, Meiji Seika Pharma Co., Ltd, Tokyo, Japan), 5-0 silk ligatures (Mani Inc.,

Tochigi, Japan) were placed around the cervix of the right and left second maxillary molars. Either boric acid solution Sigma—Aldrich, St. Louise, MO, USA) or DW was applied daily at a dosage of 3 mg/kg via a feeding tube.

Tissue preparation

At 5 and 14 days after the beginning of the experiment, the animals were sacrificed under anesthesia. The maxillae were excised and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h, and then decalcified with ethylenediamine tetraacetic acid (EDTA, pH 7.4) at 4°C for 2 weeks. After dehydration in ethanol, the samples were embedded in paraffin and sectioned sagittally at a thickness of 5 μm. Some sections were deparaffinized with xylene (Wako Pure Chemical Industries, Ltd. Osaka, Japan), rehydrated with decreasing concentrations of ethanol in DW, and stained with hematoxylin and eosin (H-E). The periodontal tissues between the first and second molars were observed using light microscopy (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Immunohistochemical analysis

Following deparaffinization and rehydration, the paraffin sections were pretreated with 0.3% hydrogen peroxide (Wako Pure Chemical Industries, Ltd. Osaka, Japan) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 30 min
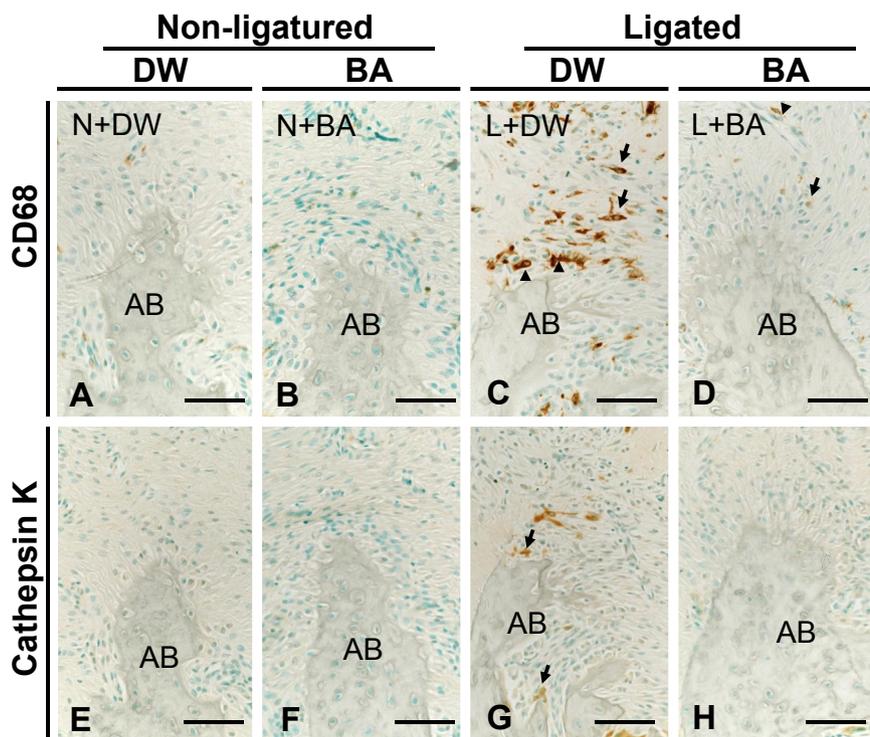

Figure 2 Immunohistochemical localizations of CD68 (A—D) and cathepsin K (E—H) in non-ligatured (A—B, E—F) and ligated (C—D, G—H) periodontal tissues at day 5. (A—B, E—F) CD68-and cathepsin K-positive cells were rarely observed in non-ligatured periodontal tissues in the absence of presence of boric acid administration. (C, G) A lot of these positive cells (arrows) were seen in ligatured periodontal tissues without boric acid administration. (D, H) Boric acid administration inhibits the formation of CD68-and cathepsin K-positive cells. AB, alveolar bone. Scale bars: 40 μm (A—H).

at room temperature. Samples were then treated with 3% bovine serum albumin (Sigma—Aldrich, St. Louise, MO, USA) in PBS for 30 min at room temperature, and incubated with mouse monoclonal antibodies against human CD68 (MCA341R, Bio-Rad, Hercules, CA, USA), human proliferating cell nuclear antigen (PCNA, MS-106-R7, Neo Markers, CA, USA), human α-SMA (M0851, Dakocytomation, Glostrup, Denmark) or rabbit polyclonal antibody against human cathepsin K (ab19027; Abcam, Cambridge, England). The antibodies against CD68, PCNA, and cathepsin K were diluted to 1:100; and α-SMA antibody was diluted to 1:500. The sections were reacted with Histofine Simple Stain rat MAX-PO (MULTI; NICHIREI Co., Tokyo, Japan) for 1 h at room temperature. Immune complexes were visualized using diaminobenzidine (Envision kit, DAKO, CA). The immunostained sections were then counter-stained with methyl green. In place of the primary antibodies, non-immune mouse or rabbit sera were diluted to the same strength and used as negative controls. The staining controls did not show any specific immunoreactivity.

Histomorphometric and quantitative analyses

The degree of alveolar bone loss was quantified by measuring the distance from the cement—enamel junction of the second molar to the tip of the alveolar bone between the first and second molars.

To compare the number of positive cells in the immunohistochemical analyses, 5 sample slides were randomly chosen for each staining. The cell numbers per 1 mm^2 of area surrounding the alveolar bone were observed by using light microscopy.

The experiments were repeated at least 3 times, and the data were expressed as the mean ± SD. Statistical significance between groups was evaluated by performing Student's t test. Values of $P < 0.05$ were considered statistically significant.

Results

Experimental day 5

Histological observations

At day 5 after the beginning of the experiment, a thick alveolar bone was observed between the first and second molars in the N + DW and N + BA groups. In these groups, osteocytes were visible in all of the bone lacunae (Fig. 1A, B, E—F). There were few inflammatory cells around the alveolar bone. The interproximal gingiva showed a triangular shape and was covered with stratified squamous epithelium (Fig. 1A, B).

Ligature placement induced periodontitis, as evidenced by the infiltration of inflammatory cells and the formation of eosinophilic matrix around the alveolar bone, in the L + DW and L + BA groups (Fig. 1C—D, G—H). Epithelial tissue defects were observed in the interproximal gingiva. Bone resorption occurred at the crest of the alveolar bone. In the alveolar bone some bone lacunae appeared empty,
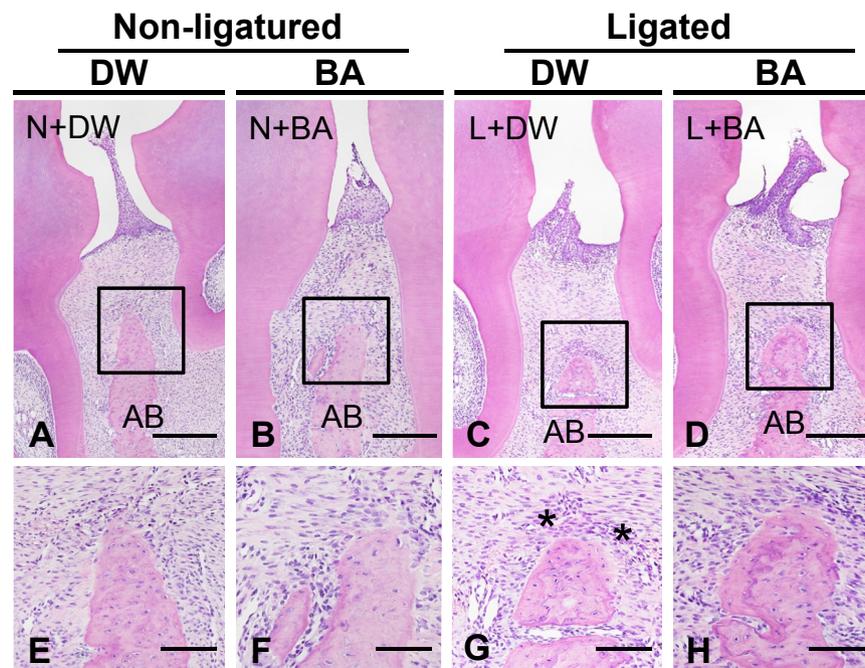
Figure 3 Histological observations of non-ligatured (A—B, E—F) and ligated (C—D, G—F) periodontal tissues at day 14 stained using hematoxylin and eosin. Higher magnifications of the boxed regions in "A", "B", "C" and "D" are shown in "E", "F", "G", and "H", respectively. (A—B, E—F) Thick alveolar bone (AB) is observed between the molars. (C, G) After ligatures were placed, the height of alveolar bone was decreased and some inflammatory cells (asterisks) were visible. (D, H) Boric acid administration maintains a bone height similar to that of non-ligatured teeth. Scale bars: 100 μm (A—D), 30 μm (E—H).

indicating that the ligature caused acute inflammatory reactions in the periodontal tissues (Fig. 1C, G). Although boric acid had been administered for 5 days, only a small number of inflammatory cells were seen in the periodontal tissues of the L + BA group (Fig. 1D, H), compared with those of the L + DW group (Fig. 1C, G). There was no eosinophilic matrix in the connective tissue beneath the ligature. The alveolar bone contained osteocytes within most bone lacunae and did not show any evidence of significant bone resorption (Fig. 1H).

The distances between the cement—enamel junction of the second molars and the crests of alveolar bone in the N + DW and N + BA groups, were 463 ± 11 and 435 ± 14 μm, respectively. In contrast, in the L + DW and L + BA groups these distances were 656 ± 13 and 604 ± 11 μm, respectively.

Immunohistochemical observations

In the N + DW and N + BA groups, immunoreactivity for CD68, which indicates the presence of macrophages, was low in the periodontal tissues (Fig. 2A, B). However, in the L + DW group, there were numerous CD68-positive cells around the crest of the alveolar bone (Fig. 2C). The prevalence of these positive cells was significantly decreased by boric acid administration in the L + BA group (Fig. 2D, Fig. 6A).

The number of cathepsin K-positive osteoclasts showed a similar pattern of immunoreactivity as CD68 (Fig. 6B).

However, cathepsin K-positive cells were localized to the surfaces of the alveolar bone. These osteoclasts seemed to have high resorption activities with respect to their cell size (Fig. 2E—H).

Experimental day 14

Histological observations

In the N + DW and N + BA groups, the morphology of the periodontal tissues appeared to be similar to those at day 5. The alveolar bone was thick, and there were no signs of inflammatory bone resorption (Fig. 3A—B, E—F).

In the L + DW and L + BA groups, fewer inflammatory cells were observed around the alveolar bone, compared with the same groups at day 5. However, no pathological bone resorption was evident in the alveolar bone. Osteocytes resided inside bone lacunae (Fig. 3C—D, G—H).

The non-ligatured teeth of the N + DW and N + BA groups had interspaces of 481 ± 13 and 495 ± 13 μm between the cement—enamel junction of second molar and the tip of alveolar bone, and this became 927 ± 16 μm in the L + DW group. This observation indicates that significant bone resorption had occurred in the experimental periodontitis generated using ligature placement. However, this bone resorption was inhibited by systemic boric acid application, as evidenced by the fact that the distance was 705 ± 14 μm in the L + BA group.
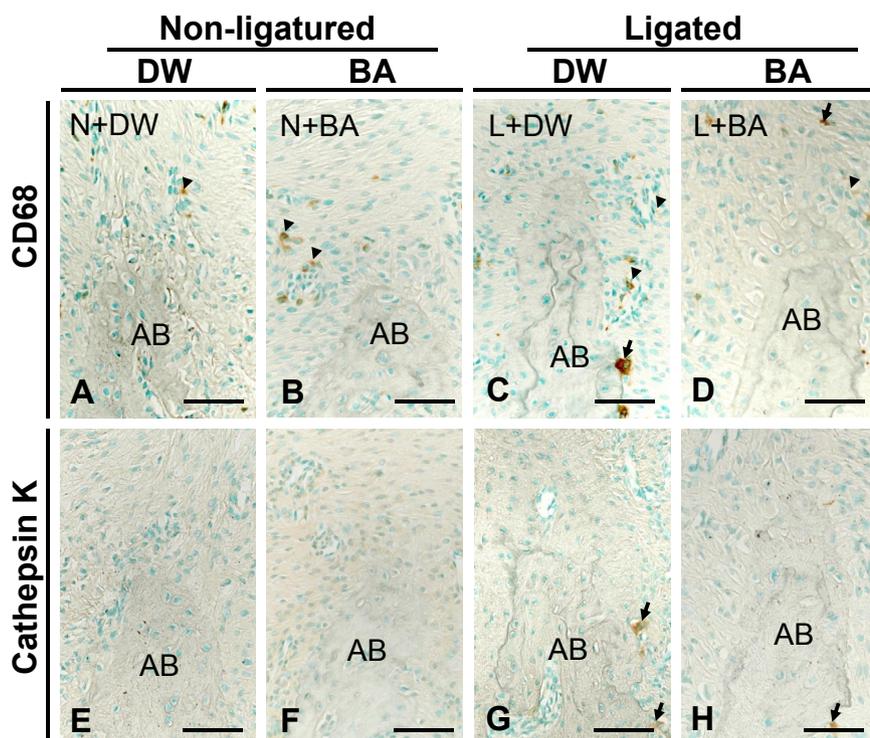

Figure 4 Immunohistochemical localizations of CD68 (A—D) and cathepsin K (E—H) in non-ligatured (A—B, E—F) and ligated (C—D, G—H) periodontal tissues at day 14. (A—B, E—F) CD68- (arrowheads) and cathepsin K-positive (arrows) cells are rarely seen around the alveolar bone. (C, G) Some of the positive cells appear in periodontal tissues after placement of a ligature without boric acid administration. (D, H) Boric acid administration reduces the number of cells positive for CD68 and cathepsin K compared with the L + DW group. AB, alveolar bone. Scale bars: 40 μm (A—H).
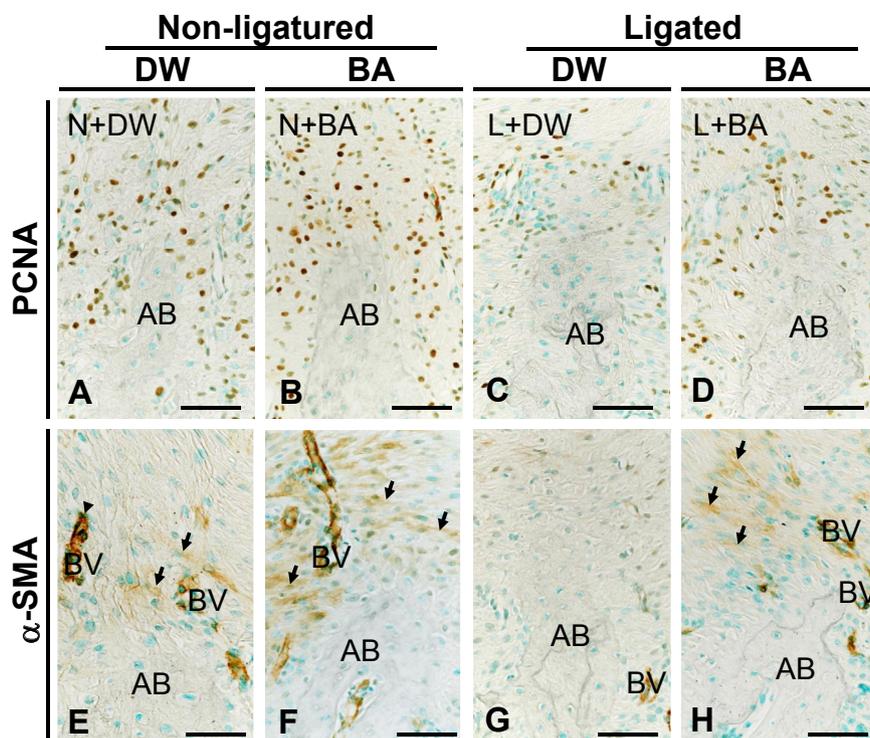
Figure 5 Immunohistochemical localizations of PCNA (A—D) and α-smooth muscle actin (E—H) in non-ligatured (A—B, E—F) and ligated (C—D, G—H) periodontal tissues at day 14. (A—B, D) There are many positive cells of PCNA, indicating cell proliferation. (C) A small number of PCNA-positive cells was observed in the periodontal tissues. (E—F, H) α-SMA localized in some cells (arrows) around the alveolar bone (AB). (G) There are few expressions of α-SMA in the periodontal tissues, except for α-SMA immunoreactivity in the vascular endothelial cells. BV, blood vessel. Scale bars: 40 μm (A—H).
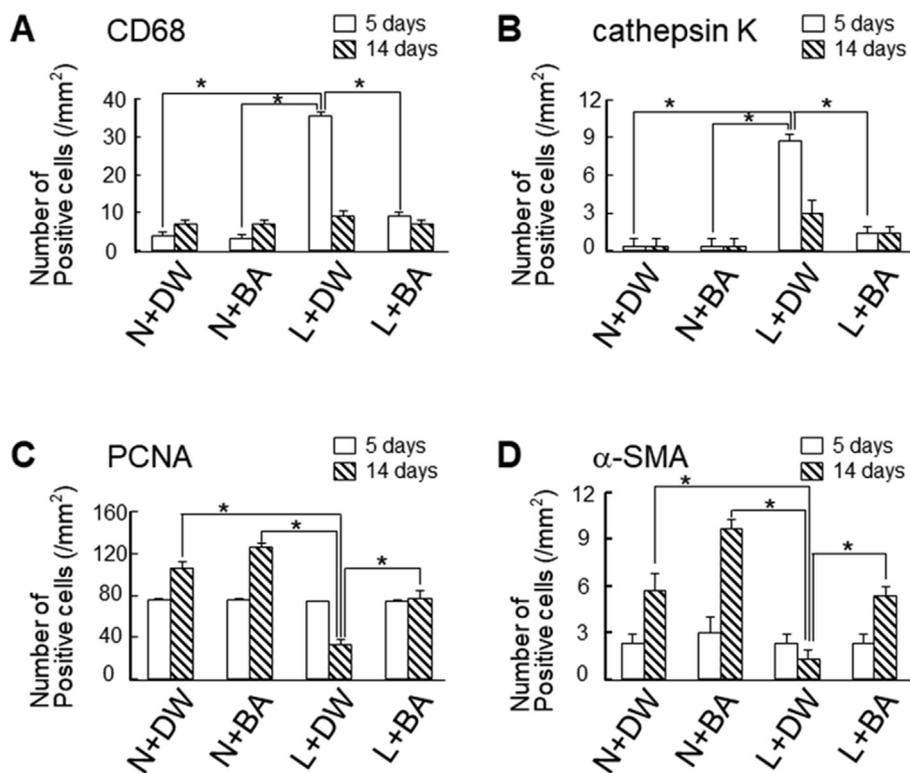

Figure 6 The quantitative analyses of the immunohistochemical observations for CD68 (A), cathepsin K (B), PCNA (C), and a-SMA (D).

Immunohistochemical observations

Regardless of the presence or absence of ligatures, there were few CD68-positive cells in the periodontal tissues around the crest of the alveolar bone (Fig. 4A−D, Fig. 6A). Osteoclasts expressing cathepsin K were also scarce on the surface of alveolar bone (Fig. 4E−H, Fig. 6B).

To detect proliferating cells in periodontal tissues, we applied an antibody against PCNA. Many PCNA-positive cells were found in the tissues of all of the experimental groups (Fig. 5A−D), while the L + DW group showed a small number of positive cells (Fig. 5C, Fig. 6C).

Pericytes and smooth muscle cells from blood vessels around the alveolar bone displayed immunoreactivity to α-SMA in all groups (Fig. 5E−H). In addition to these re-actions, the administration of boric acid induced the pro-duction of many α-SMA-positive cells around the crest of alveolar bone in the L + BA group (Fig. 5H), compared with the L + DW group (Fig. 5G). The N + DW and N + BA groups also showed some cells positive for α-SMA apart from blood vessels (Fig. 5E - F, Fig 6D).

Discussion

In this study, the effects of boric acid on periodontitis were analyzed using a ligature-induced rat model. Although several models of periodontitis have been established, we chose this model since it reproduces several pathological conditions observed in human periodontitis, such as alve-olar bone resorption and loss of gingival attachment, as well as bacterial infection.[16,17] These conditions depend on bacteria-induced host responses, as evidenced by reduced loss of bone treated with antibacterial agents and antibi-otics.[18,19] Ligature placement around the molars of germ-free rats produces little evidence of periodontitis,[20] indi-cating that the major cause of ligature-induced experi-mental periodontitis is a bacterial infection of the periodontal tissues. Therefore, this model is valuable for assessing cellar responses in periodontal tissues.

In the non-ligatured groups, no inflammation was observed in the periodontal tissues of the mesial second molar root at day 5. However, a lot of CD68-positive mac-rophages, which are the precursors of osteoclasts,[21] appeared in these tissues after ligature placement without boric acid administration. A number of cathepsin K-positive osteoclasts also appeared on the surface of alve-olar bones. Boric acid administration to the L + BA group produced a reduction in the number of cells positive for CD68 and cathepsin K. These results suggest that boric acid could reduce bone resorption in ligature-induced peri-odontitis, at least in part by inhibiting osteoclastogenesis.

Boric acid has been shown to increase bone volume after sinus floor augmentation and expansion of sutures.[22,23] MC3T3-E1, an osteoblast-like cell line, formed mineralized nodules and expressed osteoblast differentiation marker genes in the presence of boric acid *in vitro*.[24] In the present study, the number of PCNA- and α-SMA-positive cells was rarely different among all groups at day 5 (Fig. 6C−D). However, at day 14 when the inflammation has subsided as evidenced by few localizations of CD68-and cathepsin K-positive cells in all groups, many PCNA-positive cells were

present in the L + BA group, compared with the L + DW group. According to the increased number of proliferating cells, α-SMA localization was apparent in many surrounding cells of the alveolar bones in the L + BA group, but not in the L + DW group. A previous study showed that MC3T3-E1 synthesizes α-SMA during the undifferentiated stage of osteoblast differentiation,[25] suggesting that α-SMA positive cells induced by boric acid administration differentiate into osteoblasts and might be related to the inhibition of alve-olar bone loss in experimental periodontitis.

Since boric acid is highly toxic to insects, which cannot eliminate it by renal excretion,[26] it is widely used as an insecticide. However, the LD_{50} of boric acid in humans is about 5 g/kg, similar to that of dietary salt. Human studies have demonstrated that dietary boron can be beneficial to the maintenance of bone mineral density when adminis-trated at appropriate doses.[27,28] Our study indicated that boric acid can act at sites of local inflammation to prevent bone loss at day 14. This finding is in keeping with previous reports reporting on the use of boric acid for the treatment of periodontitis.[6−9] Boric acid picolinate esters have been synthesized and shown to have broad spectrum antibacte-rial activity at minimum inhibitory concentrations.[2] These compounds also have anti-inflammatory activity because of the inhibition of LPS-induced TNF-α release from human monocytes.[1] Therefore, if further research indicates an antibacterial effect of boric acid on oral flora, their use in periodontal treatment might be successful.

In conclusion, boric acid inhibited inflammation in rat experimental periodontitis. Our results suggest that boric acid induced cell proliferation in the periodontal tissues and might accelerate the genesis of osteoblasts. This agent also could reduce bone resorption in ligature-induced periodontitis, at least in part, by inhibiting osteoclastogenesis.

Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgement

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