Glyburide inhibits the bone resorption induced by traumatic occlusion in rats

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Running title: **Glyburide inhibits bone resorption by traumatic occlusion**

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Abstract

Objective: **To examine whether Glyburide inhibits bone destruction caused by traumatic occlusion in a rat occlusal trauma model.**

Background: Excessive mechanical stress, such as traumatic occlusion, induces expression of IL-1β and may be involved in bone resorption. NLRP3 inflammasomes have been linked to IL-1β expression, but it is currently unclear whether **glyburide, the inhibiter of NLRP3 inflammasome, suppressed** occlusal trauma **in rats**.

Methods: Male SD rats aged 7 weeks were used. In the trauma group, the occlusal surface of the maxillary first right molar was raised by attaching a metal wire to apply occlusal trauma to the mandibular first right molar. In the trauma + glyburide group, the NLRP3 inhibitor glyburide was administered orally every 24 h from 1 day before induction of occlusal trauma. Rats were euthanized after 5 or 10 days, and the maxillary first molars were harvested with the adjacent tissues for histopathological investigation. Immunohistochemical expression of IL-1β, NLRP3, and RANKL was also assessed.

Results: On day 5, bone resorption was significantly greater in the trauma group compared with the control group or the trauma + glyburide group, and there were significantly higher numbers of osteoclasts and cells positive for IL-1β, NLRP3, and RANKL in the trauma group.

Conclusion: **In this study, glyburide inhibits bone resorption by traumatic occlusion in rats. It** suggest**s** that the NLRP3/IL-1β pathway **might be associated with** bone

resorption induced by traumatic occlusion.

Introduction

Normal occlusal **force** is a mechanical stimulus that is required for maintenance of bone homeostasis by modulating the balance between bone formation and bone resorption.[1–3] In contrast, excessive mechanical stress such as traumatic occlusion results in loss of the lamina dura, as well as widening of the periodontal ligament (PDL) space and resorption of alveolar bone and cementum.[4–6] RANKL expression in the PDL is closely associated with an increase of osteoclasts in response to traumatic occlusion.[4] RANKL is involved in osteoclastogenesis and bone resorption, and is a member of the TNF superfamily produced by various cells, including osteoblasts, osteocytes, PDL cells, endothelial cells, B cells, and T cells.[7,8] RANKL binds to RANK, after which the RANKL/RANK signaling pathway regulates the differentiation of mature osteoclasts from precursors, as well as regulating activation and viability of these cells.[9,10]

IL-1β, a proinflammatory cytokine, is a member of the IL-1 family and is mainly produced by activated monocytes and macrophages.[11] It plays an important role in infection, injury, and immune responses, as well as in the processes of acute and chronic inflammation.[12] Patients with severe periodontitis have significantly higher levels of IL-1β in periodontal tissue, gingival crevicular fluid, and saliva than healthy individuals.[13,14] It was reported that IL-1β has a role in bone resorption associated with various diseases, including rheumatoid arthritis, osteoporosis, and periodontal disease.[13,15,16] Orthodontic treatment with excessive force increases the production of

IL-1β by PDL cells.[17,18] IL-1β induces expression of RANKL by cultured human PDL cells, as well as increasing RANKL production and activity, and is involved in osteoclastogenesis and promotion of bone resorption.[16,19–21]

The inflammasome is a multimeric protein complex and mediator of innate immune responses. It is involved in recognition and targeting of pathogen-associated molecular patterns, such as lipopolysaccharide and Flagellin, and damage-associated molecular patterns (DAMPs) like ATP and uric acid crystals.[22] IL-1β is produced as a precursor molecule that is activated through cleavage by the proteolytic enzyme caspase-1.[23] Thus, inflammasomes activate caspase-1, which in turn activates the IL-1β precursor.[24] Inflammasomes are classified according to their constituent proteins (as NLRP1, NLRP3, NLRC4, AIM2, etc.), and are activated by various stimuli such as bacteria, viruses, and extracellular ATP.[24–27] The NLRP3 inflammasome has been examined in the most detail so far. It is known to be activated by various bacterial pathogens and endogenous stimuli, but activation by excessive mechanical stress has not been reported.

Glyburide, a selective ATP-sensitive potassium (K_{ATP}) channel blocker, is widely used sulfonylurea drug for the treatment of type 2 diabetes mellitus (DM).[28] The mechanism of glyburide in the treatment of DM is due to its inhibition of K_{ATP} channel in pancreatic β islet cells, and triggers insulin release from β cells.[29] Besides its insulin promoting effect, glyburide recently has been shown to play role

in inflammation regulation. Glyburide reduces adverse neuroinflammation and behavioral outcomes in central nervous system injury.[30] Glyburide displays a protective role in inflammation-induced injury in various systems, including respiration[31], urology[32], cardiology.[33] Glyburide inhibited inflammasome activation in macrophages lacking K_{ATP} channel subunits as well. Although the exact mechanisms remain unknown, glyburide could inhibit NLRP3 inflammasome activation via upstream inhibition of the inflammasome and downstream blockade of the P2X7 receptor.[34] However, little is known about the effect of glyburide on bone destruction by excessive mechanical stress. Thus, the aim of this study was to investigate whether **glyburide inhibits** bone resorption due to **traumatic occlusion** using a rat occlusal trauma model.

Materials and Methods

Animals

Male SD rats (7 weeks old, weight 250-270 g) were used, which were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) and housed under specific pathogen-free conditions at the Animal Center of Fukuoka Dental College (Fukuoka, Japan). Animal care and experimental methods were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the Animal Care and Use Committee of Fukuoka Dental College (permit number: 18020).

Experimental schedule

To apply excessive occlusion to the mandibular first right molar, a 1.0 mm diameter cobalt chrome wire was attached to the occlusal surface of the maxillary first right molar with resin cement (SuperBond C & B®; Sun Medical, Shiga, Japan) under inhalational anesthesia with isoflurane (Wako, Osaka, Japan). To inhibit NLRP3 inflammasome function, rats were orally administered the NLRP3 inhibitor glyburide (Sigma Aldrich, St. Louis, MO, USA)[34,35] at a dose of 20 mg/kg every 24 h.

We divided 45 rats into the following three groups: a group without traumatic occlusion or glyburide administration (control group**, 9 rats**); a group with traumatic occlusion and without glyburide administration (T group**, 18 rats**); and a group with

both traumatic occlusion and glyburide administration (T+G group**, 18 rats**). The experimental period was 5 or 10 days of excessive occlusal loading (Fig. 1).

Preparation of tissues

Rats were euthanized by an overdose of isoflurane. The right mandible was rapidly resected and fixed for 10 h at 4 °C using 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Then the specimens were decalcified for 3 weeks at 4 °C with 10% EDTA-2Na in 0.01 M PBS and embedded in paraffin by the AMeX method.[36] Serial buccolingual sections (4 μm thick) were cut at the level of the central roots of the mandibular first molar.

Histopathological and histometric analyses

We cut fifty serial sections from every specimen and selected 5 sections each for staining by different methods. Sections stained with hematoxylin and eosin (H&E) were used for histological observation, and histometric analyses were also performed on the same sections with Image J software (National Institutes of Health, Bethesda, MD, USA). To assess resorption of alveolar bone, the distance from the furcation fornix of the root to the alveolar crest (fornix-alveolar bone crest distance) was measured (Fig. 2).

Staining with tartrate-resistant acid phosphatase (TRAP) was done to identify osteoclasts, as reported previously[37], followed by counterstaining with Mayer's

hematoxylin. TRAP-positive cells in a 300-μm square area of the apical region of the interradicular septum were counted as osteoclasts (Fig. 2).

Immunohistochemistry for IL-1β, NLRP3, and RANKL

To detect IL-1β, NLRP3, and RANKL protein expression, we performed immunostaining with a VECTASTAIN Elite ABC-PO kit® (Vector Laboratories, Burlingame, California, USA) according to the manufacturer's instructions. Briefly, sections were deparaffinized, treated in PBS with 0.3% Triton X-100 (PBST) for 20 min at room temperature, and immersed in 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity. Then the sections were treated with 2% normal goat serum in PBST for 30 min, followed by incubation in PBS for 1 h at room temperature with the following primary antibodies: rabbit polyclonal anti-IL-1β antibody (1:200, ab9787, Abcam, Cambridge, UK), rabbit polyclonal anti-NLRP3 antibody (1:250, NBP2-12446, Novus Biological, Cambridge, UK), and rabbit polyclonal anti-RANKL antibody (1:200, Proteintech, Wuhan, China). **Negative controls for each lacked a primary antibody but underwent all other steps in the same manner.** Subsequently, the sections were treated for 30 min with a biotinylated anti-rabbit antibody and ABC solution, incubated with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (ImmPACT™ DAB Peroxidase Substrate kit, Vector Laboratories), and counterstained with Mayer's hematoxylin. Cells positive for NLRP3, IL-1β, or RANKL were counted in a 300-μm

square region of the soft tissue of the apical interradicular septum (Fig. 2).

Statistical analysis

Results are reported as the mean ± standard deviation (SD). Analyses were performed by one-factor ANOVA and the Tukey-Kramer test, with $P < 0.05$ being accepted as indicating significance.

Results

Histopathological findings

In the control group, the width of the PDL space was constant, and there were few TRAP-positive cells and no hyaline degeneration at the interradicular septum (Fig. 3A, F). In the T group, hyaline degeneration was observed in the furcation area on day 5 and the bone surface was irregular with numerous TRAP-positive cells (Fig. 3B, G). The bone surface on the PDL side was still irregular on day 10, but hyaline degeneration was no longer seen. A few TRAP-positive cells were seen on the bone surface (Fig. 3C, H). In the T+G group, the PDL space was larger than that of the control group on day 5, while fewer TRAP-positive cells was observed compared with the T group (Fig. 3D, I). No marked changes from day 5 were noted by day 10 (Fig. 3E, J).

Histometric analysis of alveolar bone loss and TRAP-positive cells

On days 5 and 10, the fornix-alveolar bone crest distance was significantly longer in the T group compared with the control group, but there was no significant difference between the T+G group and the control group. On day 5, the fornix-alveolar bone crest distance was shorter in the T+G group compared with the T group (Fig. 4A).

On day 5, the T group had more TRAP-positive cells adjacent to bone than the control group, and significantly more TRAP-positive cells were observed in the T

group on day 5 versus day 10. There were significantly fewer TRAP-positive cells in the T+G group than the T group on day 5 (Fig. 4B).

Immunohistochemistry for IL-1β, NLRP3, and RANKL

While cells positive for IL-1β, NLRP3, and RANKL were found in the T group on day 5, few cells were seen in the control group on day 5 or in the T group on day 10 (Fig. 5A, B, C, F, G, H, K, L, M). Also, only a few cells positive for IL-1β, NLRP3, and RANKL were seen in the T+G group on days 5 and 10 (Fig. 5D, E, I, J, N, O).

On day 5, significantly more cells positive for IL-1β, NLRP3, and RANKL were seen in the T group compared with the control group (Fig. 6A, B, C). The T group had significantly more cells positive for IL-1β, NLRP3, and RANKL on day 5 than on day 10, and also had significantly more positive cells than the T+G group on day 5 (Fig. 6A, B, C). In addition, there were significantly more RANKL-positive cells in the T+G group than the control group on day 5 (Fig. 6C).

Discussion

To investigate **whether glyburide inhibits** bone resorption due to excessive mechanical stress such as traumatic occlusion, we employed a previously reported rat model of occlusal trauma.[4,38] This model has been widely used in investigations on periodontal breakdown, even though the traumatic force gradually declines over time. Bone loss and accumulation of osteoclasts were seen in the furcation region on day 5 after attachment of a wire to the occlusal surface, while the osteoclasts were no longer detected on day 10. Thus, the experimental period was limited to a term of 10 days, during which excessive traumatic force persisted. However, this model was still found to be useful in the present study because traumatic force rapidly induced bone resorption. We found that the T group showed hyaline degeneration of the PDL and there was bone resorption with accumulation of osteoclasts in the furcation area, consistent with our previous study.[4]

Glyburide has been shown to have therapeutic effect in many diseases, including ovalbumin-induced asthma[39], acute pancreatitis[40] and chronic kidney disease[41] etc. in animal model. However, there are no reports to investigate the effects of glyburide on bone destruction by excessive mechanical strain such as traumatic occlusion. In this study on day 5, TRAP-positive cells and bone resorption in the T+G group decrease compared with the T group. Therefore, it is shown that glyburide inhibits bone loss by traumatic occlusion in rats.

Glyburide is an $\mathbf{K_{ATP}}$ channel inhibitor that has been reported to suppress

activation of NLRP3 inflammasomes and is frequently used as an NLRP3 inflammasome inhibitor.[42,43] Glyburide suppresses NLRP3-dependent caspase-1 activation and IL-1β expression in mouse bone marrow-derived macrophages after stimulation with LPS and ATP.[34] Activation of NLRP3 inflammasomes following mechanical stimulation has been observed in cultured human PDL cells.[44] Although we found numerous IL-1β-positive cells in the PDL of the T group on day 5, there were significantly fewer positive cells in the T+G group. These results suggest that excessive mechanical force activated NLRP3 inflammasomes and induced IL-1β expression in the PDL.

NLRP3 belongs to the NOD-like family of intracellular receptors. It responds to intracellular molecules known as DAMPs (ATP, etc.) released by cellular damage, and forms the inflammasome, a multimeric complex that activates caspase-1.[45] Extracellular ATP stimulates potassium efflux by binding with the ATP/P2X7 receptor, a ligand-gated cation channel, while potassium efflux triggers activation of NLRP3 inflammasomes. Mechanical stress has been reported to induce significant ATP release by PDL cells.[46] A previous study showed that mechanical strain induced IL-1β expression in human PDL cells via activation of the P2X7 receptor.[47] Occlusal trauma has been found to promote thrombus formation and occlusion of PDL capillaries, followed by revascularization and prominent alveolar bone resorption.[48,49] In the present study, hyaline degeneration was observed on day 5 in the T group. Therefore, it seems

that damage to periodontal tissues due to traumatic occlusion induced the release of ATP, which activated NLRP3 in PDL cells. However, further studies will be required to elucidate the mechanisms of NLRP3 activation by occlusal trauma.

NLRP3 expression is stimulated by priming with microbial molecules such as TLR ligands, or by endogenous inflammatory cytokines, including IL-1β and tumor necrosis factor, via activation of NF-κB.[50] In this study, we found fewer NLRP3-positive cells in the T+G group than in the T group on day 5, which was consistent with downregulation of IL-1β expression. This suggests that NLRP3 expression may be induced by IL-1β via activation of NF-κB.

Immunohistochemical staining revealed many cells positive for IL-1β and RANKL among PDL cells in the T group on day 5, while there were significantly fewer positive cells in the T+G group on day 5. It has been reported that IL-1β expression is induced by mechanical stimulation of human PDL cells *in vitro*,[47] while RANKL mRNA or protein expression is increased in the PDL by orthodontic force *in vivo*.[51] Furthermore, IL-1β has been shown to induce RANKL production by PDL cells.[20] The present results suggested that RANKL expression was reduced by inhibition of the NLRP3/IL-1β pathway. However, RANKL expression **in the T+G group on day 5** was **higher than that in the control group**. According to a previous study, RANKL expression was upregulated via increased prostaglandin E_2 synthesis after application of compression force to PDL cells.[52] Hypoxia of the compressed PDL during orthodontic

tooth movement may be partially due to increased production of RANKL, and leads to upregulation of hypoxia inducible factor-1α expression by PDL cells.[53] The present results also indicated that RANKL expression was induced via an IL-1β-independent mechanism, such as prostaglandin E_2 or hypoxia inducible factor-1α.

RANKL is required for osteoclast precursors to undergo differentiation[54,55] and for bone resorption and viability of mature osteoclasts.[56,57] *In vivo* studies have demonstrated an increase of osteoclasts together with enhanced RANKL expression at sites of inflammatory bone resorption and periapical lesions.[58–60] The present study showed that there were fewer osteoclasts and less bone resorption on day 5 in the T+G group compared with the T group. These results were consistent with the changes of RANKL expression, suggesting that RANKL is closely related to osteoclast formation and bone resorption in response to occlusal trauma.

Glyburide has been reported to decrease the lipopolysaccharide-induced release of TNF-α and the corresponding mRNA in monocytes.[61] Also, glyburide has been significantly shown to reduce the expressions of TNF-α and IL-6, and the number of osteoclasts in a diabetic-induced fracture mouse model.[62] *In vivo* excessive occlusion increased the mRNA expression levels of IL-6 in periodontal tissue. *In vitro* mechanical stress induced the mRNA expression levels of TNF-α in PDL cells.[63] It has been reported that traumatic occlusion induced the release of TNF-α in rat model[64]. In this study, glyburide might reduce bone resorption by

suppressing the expression of TNF-α and IL-6.

In this experiment, NRLP inflammasome and IL-1β positive cells were analyzed immunohistologically in the rat occlusal trauma model to evaluate the suppression of the inflammatory response by orally administered glyburide. Quantitative PCR and western blotting for detecting the mRNA and NRLP inflammasome may be required to fully prove our findings in this particular site. Because the area of our interest was just below the furcation of the molar which was surrounded by hard tissue, it was hard to employ those molecular biologic approaches. Some technique such as laser dissection may be needed for compensating the limitation of this experimental model in a future study.

In conclusion, this study demonstrated that **glyburide inhibits** bone resorption and osteoclastogenesis stimulated by traumatic occlusion **in rat model. In our immunohistological study, glyburide, an NLRP3 inflammasome inhibiter,** suppressed IL-1β and RANKL expression, suggesting that a possible mechanism of bone resorption secondary to traumatic occlusion is upregulation of RANKL expression via the NLRP3/IL-1β pathway.

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Figures

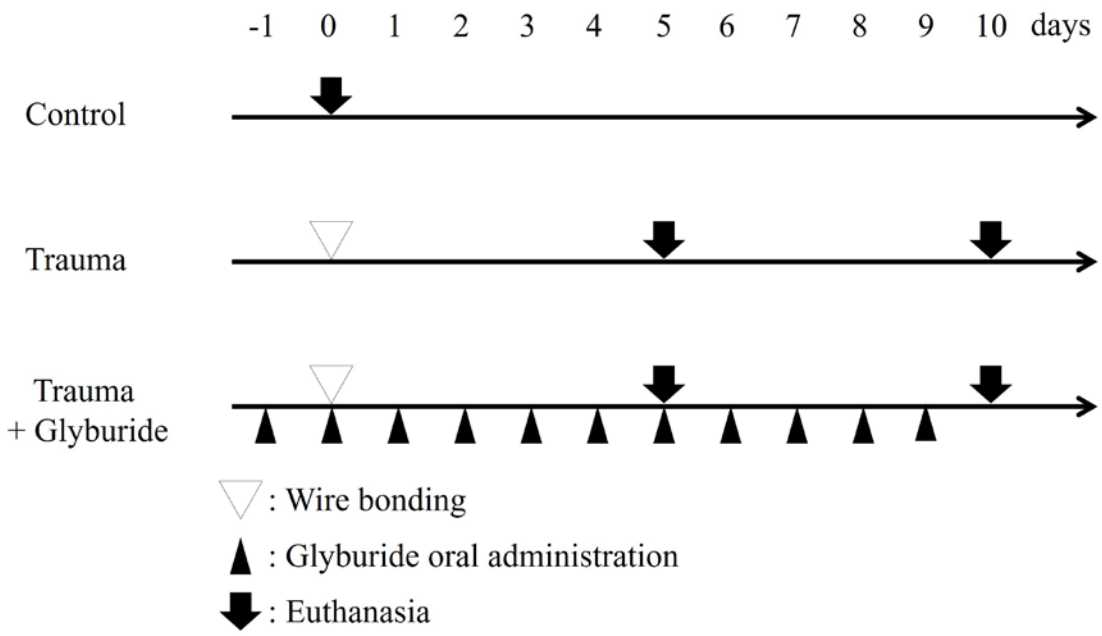

Fig. 1

Experimental schedule. To load excessive occlusive force onto the mandibular first right molar, the occlusal surface of the maxillary first right molar was raised by attaching a cobalt chrome wire 1.0 mm in diameter. Rats were orally administered glyburide at a dose of 20 mg/kg every 24 h. The experimental period was 5 or 10 days after initiation of excessive occlusal loading.

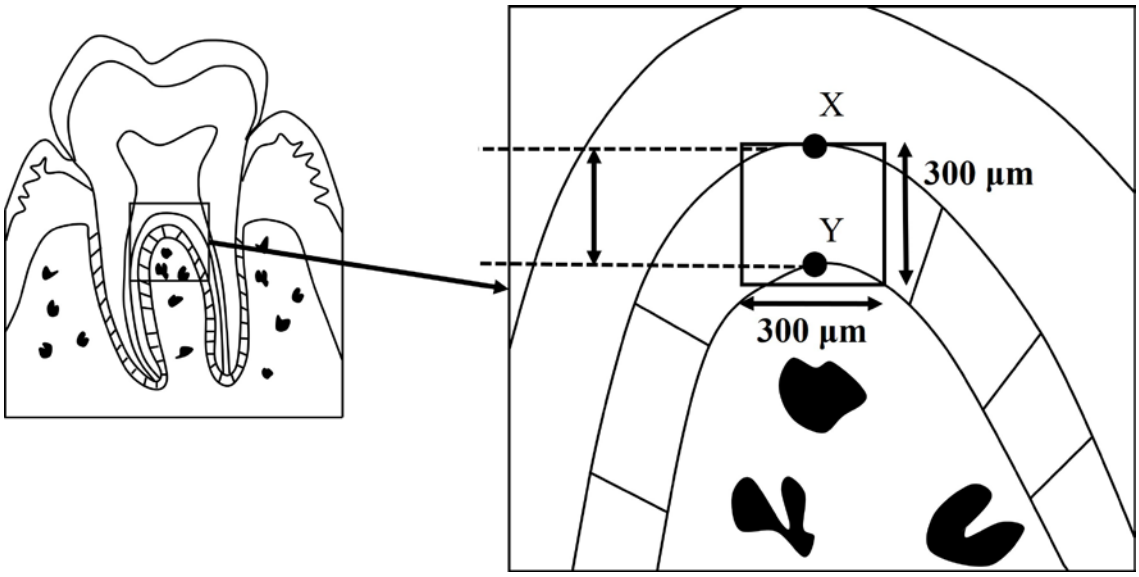

Fig. 2

Buccolingual schema of periodontal tissue around the lower first right molar for histometric analysis. The distance from the root furcation fornix (X) to the alveolar bone crest (Y) was measured to evaluate alveolar bone resorption. The number of TRAP, IL-1β, NLRP3, and RANKL-positive cells was counted in a 300-µm square region of the interradicular septum. X: root furcation fornix, Y: alveolar bone crest.

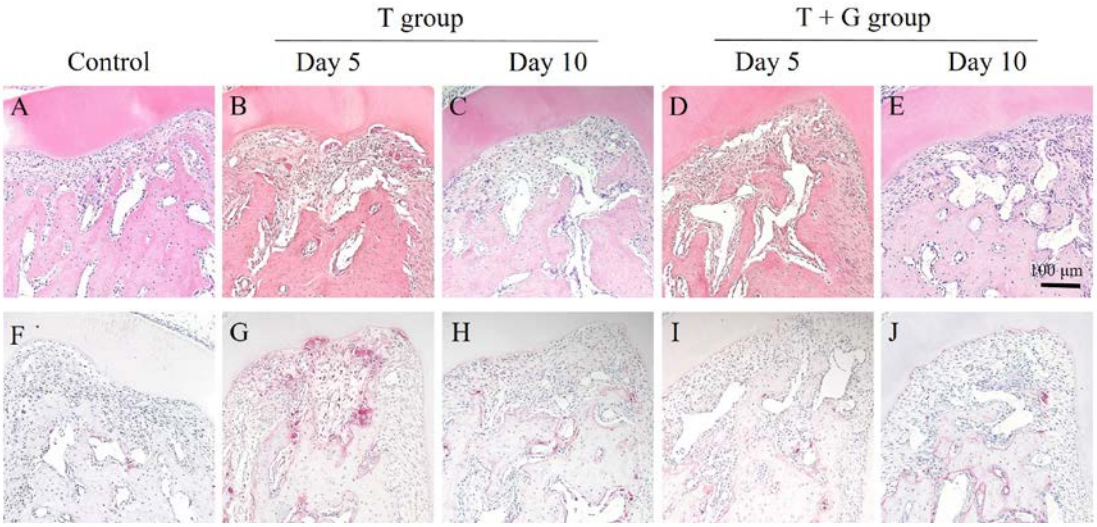

Fig. 3

Histopathological findings on staining with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP). (A, F) Control group. The periodontal ligament (PDL) showed a constant width and few osteoclasts were detected. (B, G) T group on day 5. There is hyaline degeneration and an irregular bone surface with many TRAP-positive cells. (C, H) T group on day 10. The PDL has expanded due to bone resorption, but hyaline degeneration is not observed. There are a few TRAP-positive cells in the furcation area. (D, I) T+G group on day 5. The PDL space is larger than in the control group. (E, J) T+G group on day 10. There were few changes between days 5 and 10. Upper panel: H&E staining. Lower panel: TRAP staining.

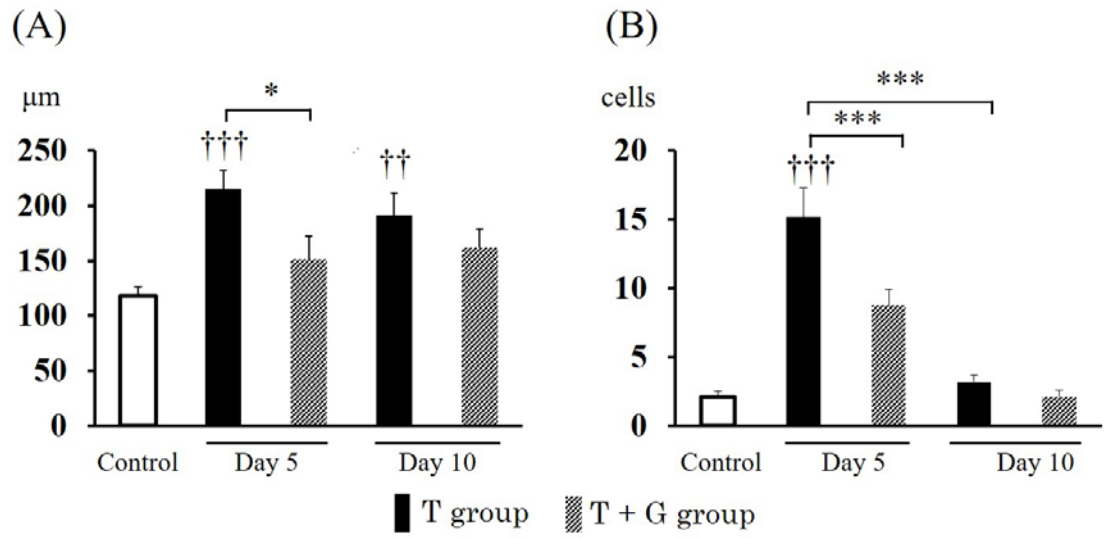

Fig. 4

Histometric analysis of alveolar bone loss and the number of TRAP-positive osteoclasts. (A) The distance from the fornix to the alveolar bone crest (fornix-alveolar bone crest distance) was measured to assess bone loss. (B) The number of TRAP-positive cells was counted in a 300-μm square region of the interradicular septum. Data are shown as the mean ± SD. ††: $P < 0.01$, †††: $P < 0.001$ vs. control, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

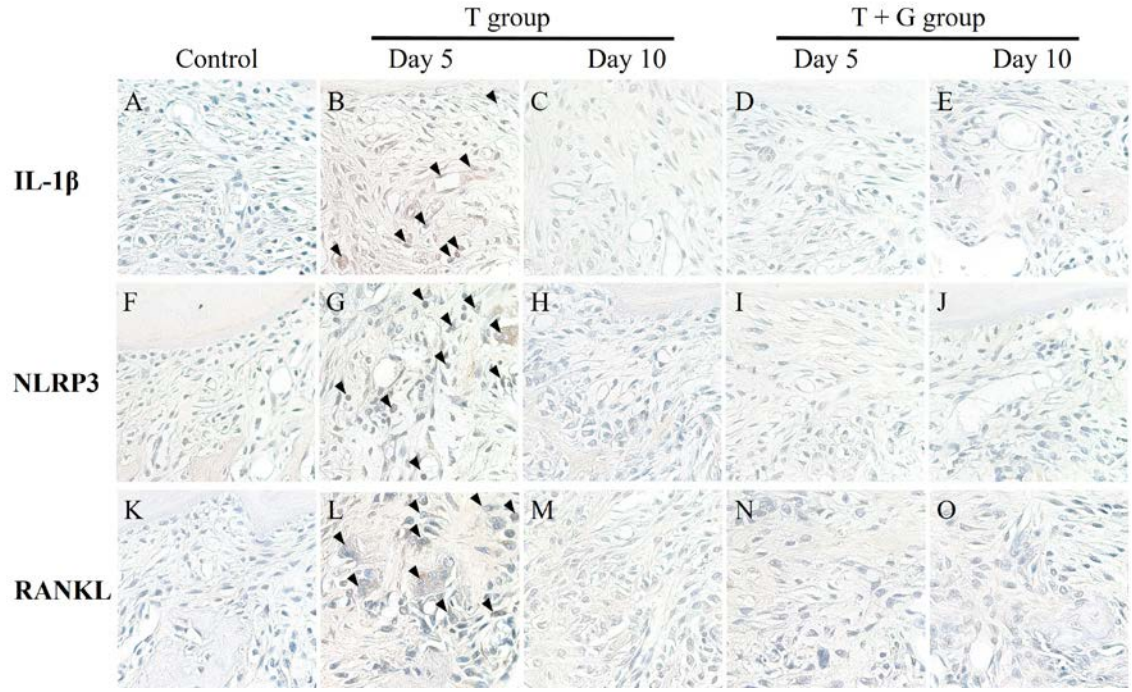

Fig. 5

Immunohistochemical staining of histological sections for IL-1β, NLRP3, and RANKL. (A, F, K) Control group, (B, G, L) T group on day 5, (C, H, M) T+G group on day 5, (D, I, N) T group on day 10, (E, J, O), and T+G group on day 10. In the T group, numerous cells positive for IL-1β, NLRP3, and RANKL were observed on day 5, whereas few such cells were found in the other groups. Cells positive for IL-1β, NLRP3, and RANKL are shown as black triangles.

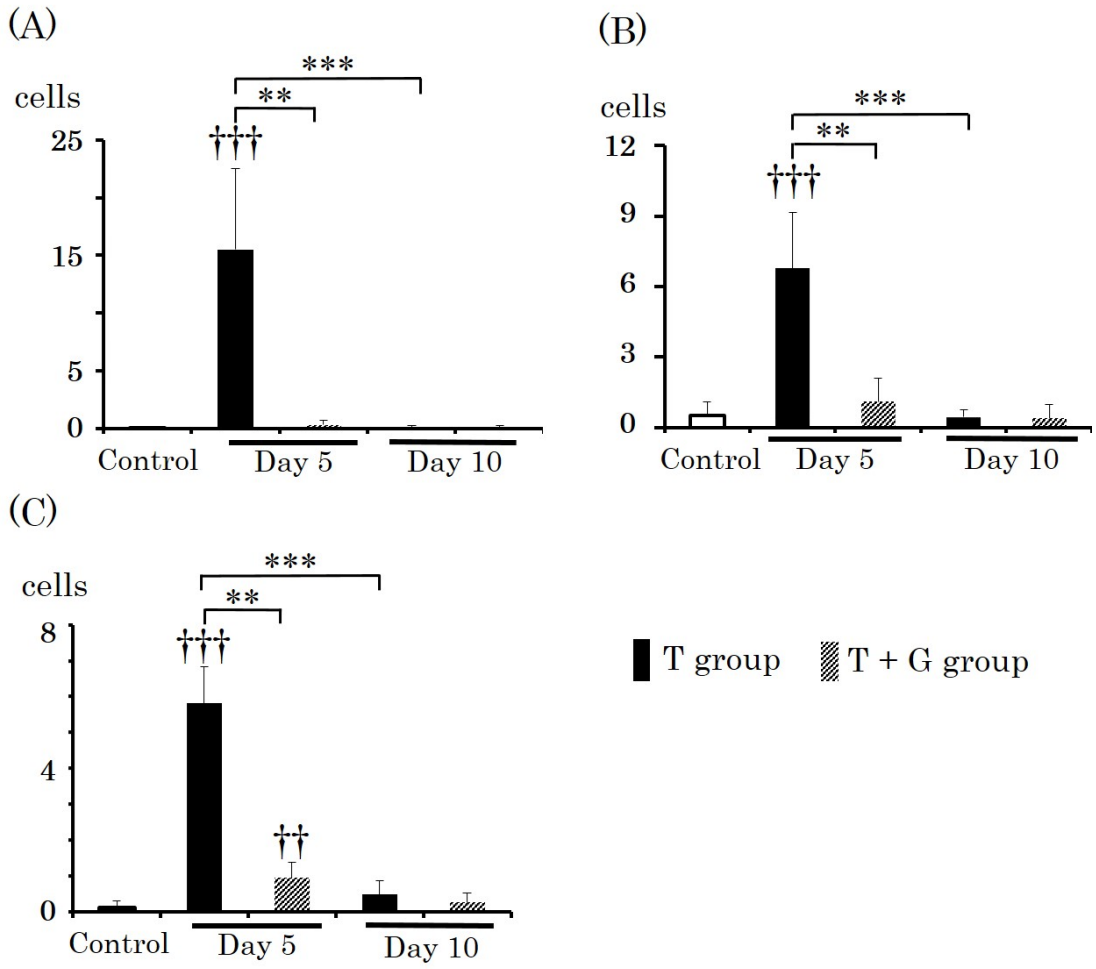

Fig. 6

Cells positive for IL-1β, NLRP3, and RANKL were counted in a 300-μm square region of the interradicular septum. (A) IL-1β, (B) NLRP3, (C) RANKL. Data are shown as the mean ± SD. ††: $P < 0.01$, †††: $P < 0.001$ vs. control, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.