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# TNF-$\alpha$ and RANKL facilitates the development of orthodontically-induced inflammatory root resorption*

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## ABSTRACT

**Background: The objective of this study was to determine the levels of tumor necrosis factor-alpha (TNF-$\alpha$) and receptor activator of NF-kB ligand (RANKL) in the gingival crevicular fluid (GCF) in patients with severe root resorption after orthodontic treatment. Materials and Methods: Ten patients who had been receiving orthodontic treatment (5-control subjects and 5-severe root resorption subjects) participated in this study. GCF was collected from all patients. Subjects with severe root resorption (>1/3 of the original root length) were identified. Control group subjects with no loss of the root structure undergoing orthodontic treatment were also identified. The GCF was collected non-invasively from the mesial and distal sides of each of the upper central and lateral incisors using filter paper strips. The eluted GCF was used for a Western blot analysis with Antibodies against TNF-$\alpha$ and soluble RANKL (sRANKL). Ten male 6-week-old Wistar rats were subjected to orthodontic force of 50 g to induce a mesially tipping movement of the upper first molars for 7 days. The expression levels of TNF-$\alpha$ and RANKL proteins were determined in periodontal ligament (PDL) by immunohistochemical analysis. Results: The Western blot analysis showed that the TNF-$\alpha$ and sRANKL expressions were significantly higher in the severe root resorption group than in the control group. In the experimental tooth movement *in vivo*, resorption lacunae with multinucleated cells were observed in 50 g group. The immunoreactivity for TNF-$\alpha$ and RANKL was detected in PDL tissue subjected to the orthodontic force on day 7. Conclusion: These results suggest that TNF-$\alpha$ and RANKL play important roles in inducing or facilitating the development of orthodontically-induced inflammatory root resorption (OIIRR).**

**Keywords:** TNF-$\alpha$; sRANKL; Orthodontic Root Resorption; Gingival Crevicular Fluid

## 1. BACKGROUND

Orthodontically-induced inflammatory root resorption (OIIRR) is an unavoidable pathological consequence of orthodontic tooth movement. Approximately 5% of orthodontic patients are prone to developing more than 5 mm of resorption during orthodontic treatment with fixed appliances [1]. The condition can be defined as an iatrogenic disorder that unpredictably occurs after orthodontic treatment, whereby the resorbed apical root portion is replaced with normal bone. OIIRR is a sterile inflammatory process that is extremely complex, and involves various disparate components, including mechanical forces, teeth and bone, other types of cells, the surrounding matrix, and certain known biologic messengers [2,3].

With regard to the relationship between OIIRR and receptor activator of NF-kB ligand (RANKL), Yamaguchi *et al.* [4] reported that the compressed PDL cells obtained from patients with severe external apical root resorption exhibit an increased RANKL expression and osteoclastogenesis *in vitro*. Nakano *et al.* [5] reported that rat PDL induces root resorption via the RANKL/RANK expression in response to heavy forces *in vivo*. Therefore, RANKL plays an important role in root resorption during orthodontic tooth movement.

TNF-$\alpha$ is a cytokine generated by a variety of cells including macrophages and PDL cells, and is induced by exogenous stimulation, endotoxins and pathogens. It is a substance whose relationship to conditions involving inflammatory bone resorption such as periodontal disease and rheumatoid arthritis is attracting attention. Ustün *et*

*Competing interests: The authors declare that they have no competing interests.
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*al.* [6] reported that TNF-$\alpha$ is involved in inflammatory bone destruction in patients with periodontal diseases and rheumatisms. Furthermore, Redlich *et al.* [7] reported that the presence of TNF-$\alpha$ aggravates inflammation and consequent bone destruction. However, little is known about the relationships between OIIRR and these cytokines.

The purpose of this study was to determine the expressions of TNF-$\alpha$ and soluble RANKL (sRANKL) in the gingival crevicular fluid (GCF) of patients with radiographic evidence of root resorption. Moreover, the expression levels of TNF-$\alpha$ and RANKL were investigated in rat root resorption during experimental tooth movement due to the application of a heavy force (50 g) using an immunohistochemical analysis.

## 2. MATERIALS AND METHODS

### 2.1. Experimental Subjects

Ten subjects were selected from among patients seeking treatment at the Department of Orthodontics at the Nihon University School of Dentistry at Matsudo. Two groups were established, including a control group and a root resorption group. The control group included five subjects (5 females, mean age: 28.0 ± 5.3 years, mean duration of treatment: 26.4 ± 3.1 months) with no radiographic evidence of root resorption. The root resorption group included five subjects (5 females, mean age: 28.9 ± 6.1 years, mean duration of treatment: 27.8 ± 3.3 months) with radiographic signs of severe root resorption of more than 1/3 of the original root length. Informed consent was obtained from each patient, and the project was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019). All patients providing their written informed consent.

The selection criteria for the subjects were as follows: 1) a Class I malocclusion with mild crowding (≤6 mm; mean 5.4 ± 0.55), 2) four premolar extractions, 3) excellent quality records and, 4) no history or evidence of tooth injury or wear, as shown on the charts and diagnostic records.

All subjects were in good general health with healthy periodontal tissues before the orthodontic treatment; the probing depths were ≤3 mm, and there was no radiographic evidence of periodontal bone loss. Subjects were excluded if they received antibiotic therapy during the treatment or if they had taken anti-inflammatory medication during the month preceding the start of the study.

### 2.2. GCF Collection

The method used in this study has been previously described by Yamaguchi *et al.* [8]. GCF was collected from both the resorption and control groups following orthodontic treatment (debonding). The GCF was collected from the mesial and distal sides of the upper central and lateral incisors using filter paper strips (Periopaper, Oraflow, Smithtown, NY, USA) inserted 1 - 2 mm into the gingival sulcus for 60 seconds (**Figure 1**). After one minute, a second collection was performed. Care was taken to prevent mechanical injury to the soft tissue. The contents were eluted into 1× phosphate buffer saline (PBS) containing a protease inhibitor (0.1 mM phenylmethylsulphonylfluoride) and stored at −30˚C until a further analysis. The volume of GCF on the paper strip was measured with a Periotron 8000 (Harco, Tustin, CA, USA).

For the evaluation of the cytokine expression, the paper strips were placed individually in 100 µl of PBS and then subjected to vortexing 3 times over a 30 minute period. The strip was then removed and the eluate was centrifuged for 5 minutes at 3000 × g. The supernatants were separated and frozen at –30˚C for later use. The protein concentration in the extract was estimated using bovine serum albumin as a standard.

### 2.3. Western Blotting Analysis

The TNF-$\alpha$ and sRANKL expressions in the GCF samples were determined using a Western blotting analysis. The protein content of the samples was measured using the Bradford reagent (BIO-RAD, Tokyo, Japan) according to the manufacturer's protocol. The samples were boiled for 3 minutes with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 3.3% SDS, 30% glycerol, 5% $\beta$-mercaptoethanol and 0.001% bromophenol blue) and the protein (10 µg) samples were then resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 150 V for 1 hour (h). The proteins were electro transferred from the SDS gels onto an Amersham Hybond ECL (GE Healthcare UK Ltd Amersham Place, Little Chalfont, Buckinghamshire, UK) for the immunoblot analyses. Blocking of nonspecific antigen-binding sites was performed with 5% nonfat dry

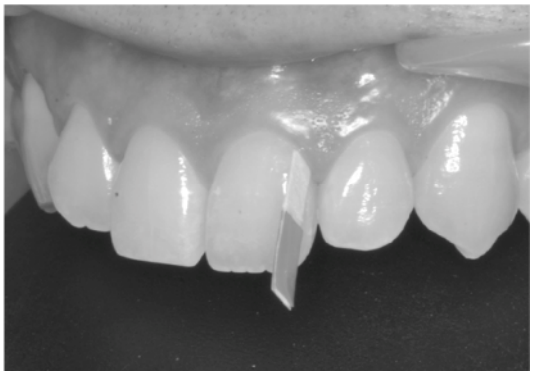

**Figure 1.** The GCF was sampled at the mesial and distal sides of the upper central and lateral incisors.

 

milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween 20 (TBST) buffer (Sigma Chemical Co., St.Lois, MO, USA). The membrane was incubated for 24 h with anti-TNF-$\alpha$ mouse monoclonal antibodies (R & D Systems Inc., Minneapolis, MN, USA) diluted at 1:500 and anti-RANKL rabbit monoclonal antibodies (abcam PLC., Tokyo, Japan) diluted 1:1000 in 5% nonfat dry milk-TBST. Subsequently, the blots were incubated for 2 h with goat anti-mouse IgG (H+L)-HRP conjugate (BIO-RAD) diluted at 1:2500 and goat anti-rabbit IgG (H+L)-HRP conjugate (BIO-RAD) diluted at 1:2000 in 5% nonfat dry milk-TBST, then developed using an ECL system (GE Healthcare Limited). Quantification of the band intensity was performed using the Image J Software program (NIH, Bethesda, MD, USA).

## 2.4. Animal Studies

### 2.4.1. Animals

The animal experimental protocol in this study was approved by the Ethics Committee for Animal Experiments at the Nihon University School of Dentistry at Matsudo (approval No. AP12MD020). A total of ten male 6-week-old Wistar rats (Sankyo Labo Service, Inc., Tokyo, Japan. body weight 180 ± 10 g) were used for the experiments. Animals were maintained at the animal center of Nihon University School of Dentistry at Matsudo in separate cages in a 12-hour light/dark environment at a constant temperature of 23˚C, and were provided with food and water *ad libitum*. The health status of each rat was evaluated by daily body weight monitoring for 1 week before the start of the experiments.

### 2.4.2. Application of Orthodontic Devices and Tissue Harvesting

Animals were anaesthetized with pentobarbital sodium (40 mg/kg body weight) for the application of orthodontic devices. Experimental tooth movement was induced using the method of Fujita *et al*. [9], with a closed-coil spring (wire size: 0.005 inch, diameter: 1/12 inch, Accurate, Inc., Tokyo, Japan) ligated to the maxillary first molar by a 0.008 inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan). The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla with a #1/4 round burr, using the same ligature wire. The upper first molar was moved mesially by the closed coil spring with a force of 50 g (**Figure 2**). The period of the experiment was 7 days.

### 2.4.3. Tissue Preparation

The experimental period was set at 7 days after tooth movement was initiated. The animals were deeply anesthetized by pentobarbital sodium and then were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the maxilla was immediately dissected and immersed in the same fixative for 18 hours at 4˚C. The specimens were decalcified in 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for 4 weeks at room temperature, and the decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin using the usual methods for preparation. Each sample was sliced into 4 μm sections continuous in the horizontal direction, and then was prepared for hematoxylin and eosin (H.E.) staining, and also for immunohistochemical staining. The periodontal tissues in the mesial part of the distal buccal root of a first upper molar were observed. The one that was not moved was defined as the control group.

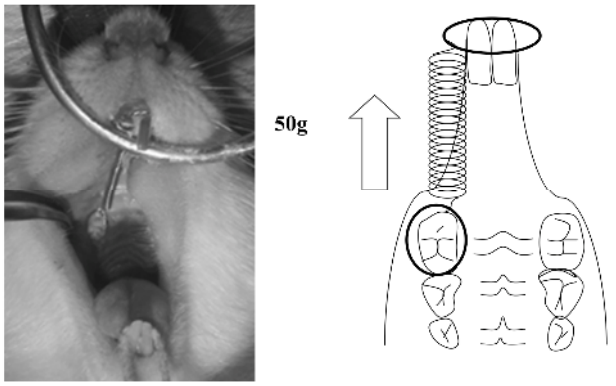


**Figure 2.** Experimental tooth movement was performed with a closed-coil spring (wire size: 0.005 inch, diameter: 1/12 inch) ligated to the maxillary first molar cleat by a 0.008-inch stainless steel ligature wire. The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla with a #1/4 round bur, using the same ligature wire. The upper first molar was moved mesially by the closed coil spring at 50 g. The period of experiment was performed for 7 days.

### 2.4.4. Immunohistochemistry

Immunohistochemical staining was performed as follows. The sections were deparaffinized and the endogenous peroxidase activities were quenched by incubation in 3% $H_2O_2$ in methanol for 30 minutes at room temperature. After washing in tris buffered saline (TBS), the sections were incubated with a monoclonal anti-TNF-$\alpha$ antibody (R & D Systems, Inc., Minneapolis, MN, USA; working dilution, 1:100) and polyclonal anti-RANKL antibody (Santa Cruz Biotechnology, Inc., CA, USA; working dilution, 1:100) for 18 hours at 4˚C. TNF-a and RANKL were stained using the Histofine Simple Stain MAX-Po (G) kit (Nichirei, Co., Tokyo, Japan) according to the manufacturer's protocol. The sections were rinsed with TBS and the final color reactions were performed using the 3, 3'-diaminobenzidine tetra-hydrochloride substrate reagent, and the sections were then counter-stained with hematoxylin. As immunohistochemical controls, several

 

sections were incubated with 0.01 M phosphate buffered saline (PBS) instead of the primary antibody. Negative reactivity was observed for the controls. Positive controls were performed according to the methods of previous studies [9,10].

#### 2.4.5. Statistical Methods

The values in each figure represent the means ± standard deviation (S.D.) for each group. The data are presented as the mean ±S.D. The Mann-Whitney *U*-test was used to compare the means of the groups.

## 3. RESULTS

### 3.1. Patient Samples

In all patients, the degree of plaque accumulation throughout the study was minimal, and the subjects' gingival health was excellent. Furthermore, the probing depths remained less than 3 mm at all times throughout the experimental period, and there was no bleeding on probing.

The mean volumes of GCF obtained from the paper strips were compared. There were no significant differences in the mean volumes of GCF between the root resortion group (mean: 0.41 ± 0.05 μl) and the control group (mean: 0.43 ± 0.05 μl).

### 3.2. Determination of the TNF-*α* and RANKL Expressions Using a Western Blot Analysis

Western blot analysis was performed to detect the sRANKL and TNF-*α* expression in the control and resorption groups. Immunoblotting against TNF-*α* was detected in both group samples. The intensity of band in resorption group showed higher than that observed in the control group (**Figure 3(A)**). Immunoblotting against sRANKL was detected in the resorption group. The control group had less intense bands than the resorption group (**Figure 3(B)**).

### 3.3. Animal Studies

#### 3.3.1. Body Weights during the Experimental Period

The body weights of the rats in both force groups decreased transiently on day 1 and then recovered. No significant differences between the two groups were observed (data not shown). The amount of tooth movement was equal between the 50 g groups during the experimental period (7 days) (data not shown).

#### 3.3.2. Histological Changes in Periodontal Tissues during Tooth Movement (H.E. Staining)

In the control group (0 g), the rat PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that were horizontally aligned from the root

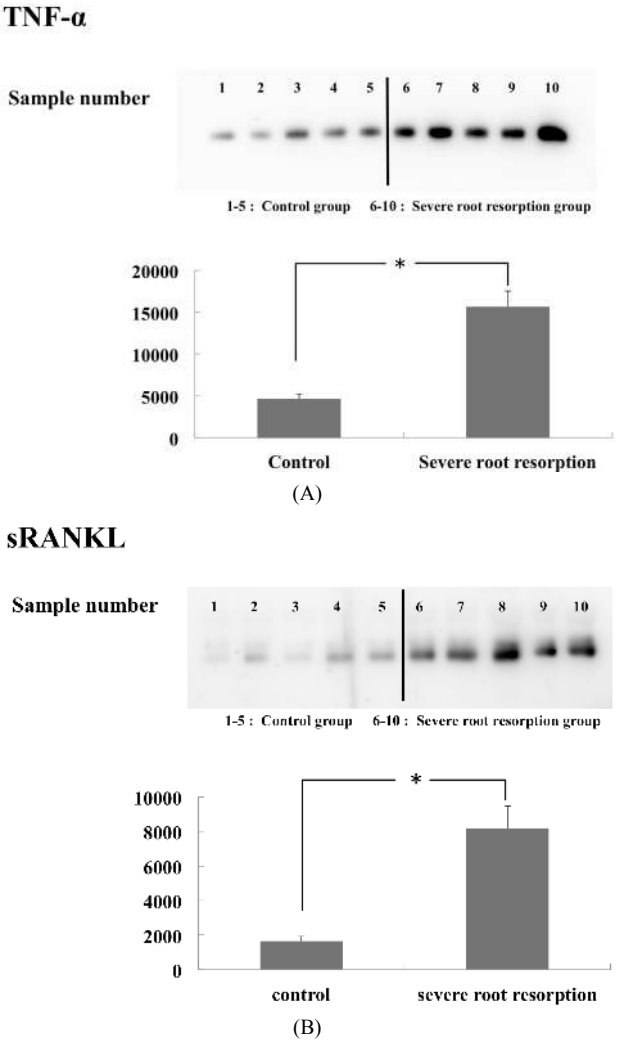


**Figure 3.** Western blot analysis for the immunodetection of TNF-*α* (A) and sRANKL (B) in the gingival crevicular fluid (GCF). Lanes 1 to 5—control group; lanes 6 to 10—severe root resorption group.

cements. The root surface was relatively smooth, with a few mononuclear and multinucleated osteoclasts (**Figure 4(A)**). In the 50 g group, there was a coarse arrangement of fibers and compressed blood capillaries. On day 7, many root resorption lacunae with multinucleated odontoclasts were recognized on the surface of the root (**Figure 4(B)**).

#### 3.3.3. Protein Expression Levels of TNF-*α* and RANKL

The immunorectivity of TNF-*α* and RANKL was examined on day 7 after tooth movement. TNF-*α* and RANKL -positive cells were rarely observed from the control group (**Figures 4(C)** and **(E)**). In the 50 g group, many TNF-*α* and RANKL-positive cells and odontoclasts were observed in the PDL tissues (**Figures 4(D)** and **(F)**).

 

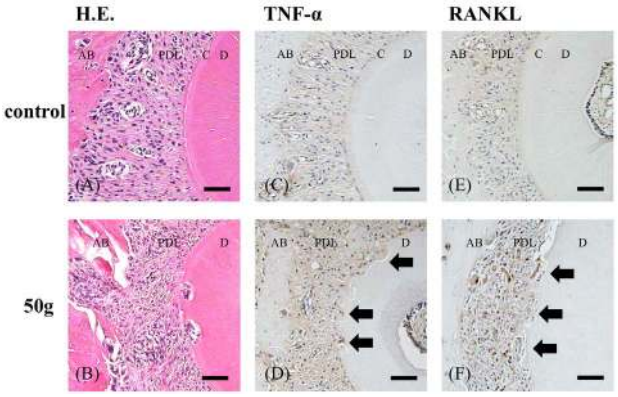


**Figure 4.** Light microscopic images of the effect of orthodontic force (50 g) on the multinucleated osteoclasts (H.E.) (A, B) and the expression of TNF-*α* (C, D) and RANKL (E, F) by odontoclasts as determined by immunohistochemistry. Immunoreactivity for TNF-*α* and RANKL was observed in the odontoclasts (arrows) in the 50 g group on day 7 (D, F). AB: alveolar bone. PDL: periodontal ligament. C: cementum. D: dentine. Original magnification 200×, Bar: 50 μm.

## 4. DISCUSSION

During the process of root resorption, organic matrix proteins and cytokines are released into the gingival crevice. The objective of this study was to determine whether the TNF-*α* and sRANKL expressions could be used as biological markers for root resorption related to orthodontic treatment. The results of this study demonstrate that differences exist between the levels of these proteins in the GCF of subjects with severe root resorption evaluated on radiographs.

GCF was first utilized by periodontists attempting to develop diagnostic tests for detecting periodontal diseases. This fluid is an osmotically-mediated transudate. The aqueous component is derived primarily from the serum; the constituents are derived from the serum, while the gingival tissues through which the fluid passes, and the bacteria present in the tissue and crevice [11]. GCF was chosen for the present study due to its ready accessibility and because its collection poses minimal risk or harm to the patient. Orthodontic forces induce the movement of periodontal ligament fluids and with them any cellular or biochemical products produced from prior mechanical perturbation. During the course of orthodontic treatment, the exerted forces produce distortion of the periodontal ligament extracellular matrix, resulting in the alteration of the cellular shape and cytoskeletal configuration. Such events lead to the synthesis and presence of extracellular matrix components, tissue degrading enzymes, acids and inflammatory mediators in the deeper periodontal tissues, which induce cellular proliferation and differentiation and promote wound healing and tissue remodeling [12]. Dudic *et al.* [13] reported that the GCF composition changes during orthodontic tooth movement. The levels of inflammatory cytokines, such as IL-1 beta, IL-6 and RANKL are elevated in the gingival crevicular fluid during human orthodontic tooth movement [14-16]. Therefore, GCF may be a useful tool for studying OIIRR in a noninvasive manner.

The Western blot results showed that the expressions of TNF-*α* and sRANKL in the GCF were significantly higher in the subjects with severe root resorption than in the subjects without esorption (**Figures 3(A)** and **(B)**). A recent study demonstrated that the concentrations of RANKL in the GCF were significantly higher in the subjects with mild and severe root resorption than in the controls [17]. The RANK/RANKL system has been suggested to play an integral role in osteoclast activation during orthodontic tooth movement [18]. Brooks *et al.* [19] demonstrated that the expression of RANKL during the application of orthodontic forces is involved in osteoclast precursor signaling. The RANK/RANKL system may also regulate the natural process of root resorption in exfoliated primary teeth [20]. Therefore, the RANK/RANKL system may be involved in the process of root resorption resulting from the application of orthodontic forces.

Ren *et al.* [21] reported that the level of TNF-*α* in the GCF increases during orthodontic tooth movement. Kook *et al.* [22] reported that compression forces induce the mRNA expression of TNF-*α* and osteoclastogenesis in human periodontal ligament (hPDL) cells *in vitro*. TNF-*α*-induced osteoclast recruitment is probably central to the pathogenesis of disorders involving inflammation [23]. Therefore, TNF-*α* may stimulate bone resorption during orthodontic tooth movement.

Further, to investigate whether TNF-*α* and RANKL is involved in root resorption during orthodontic treatment or not, we induced root resorption by applying excessive orthodontic force in animal models. The immunoreactivity for TNF-*α* was detected in forced PDL tissues, and the immunoreactions in the 50 g group were higher than those in the control group on day 7, (**Figures 4(C)** and **(D)**). RANKL immunoreactivity was also strongly detected in the PDL and odontoclasts in the 50 g group (**Figures 4(E) and (F)**).

Many investigators have reported that root resorption is aggravated by increasing force magnitudes [24,25]. Previous studies demonstrated osteoclastic resorption of roots on the pressure side surfaced of teeth subjected to heavy orthodontic force (50 g) [25,26]. Therefore, in the present study, 50 g were used as a strong forces model. When 50 g of orthodontic forces were applied to the rat upper first molar for 7 days, many resorption lacunae with odontoclasts appeared on the root surface after tooth movement for 7 days (**Figures 4(A) and (B)**). These results were consistent with previous studies [24-27].

Nakao *et al.* demonstrated that the immunoreactivity

 

for RANKL/RANK was detected in odontoclasts with an orthodontic force of 50 g [5]. Zhou *et al.* [28] reported that the mRNA level of RANKL and the RANKL/OPG mRNA ratio was increased was significantly elevated on the pressure side. These reports support the results in this study. Furthermore, Garlet *et al.* [29] demonstrated TNF-*α* and RANKL in compressed PDL of human teeth subjected to rapid maxillary expansion. Bletsa *et al.* [10] reported that TNF-alpha was expressed in the alveolar bone and PDL along the roots of the orthodontically moved molars and in the gingival of rats. Taken together, these findings and our present results suggest that TNF-*α* and RANKL induced by excessive orthodontic force may activate osteo/odontoclastogenesis.

Considering the relationships between TNF-*α* and RANKL in OIIRR, studies evaluating these correlations are few. However, recent studies have reported that compression forces induce the mRNA expressions of TNF-*α* and RANKL in human periodontal ligament cells *in vitro* [30,31]. Furthermore, direct cell-cell contact between PDL cells and osteoclast precursors synergistically increases the expressions of TNF-*α* and RANKL genes related to osteoclastogenesis. Therefore, these factors may be significant predictive factors for potential inflammatory parameters during treatment, and this induction may contribute to the inflammatory response associated with the ensuing OIIRR. Further studies are needed to investigate the relationships between TNF-*α* and RANKL during root resorption, including studies with an increased number of subjects for the statistical analysis and *in vitro* studies.

## 5. CONCLUSION

These results suggest that TNF-*α* and RANKL play important roles in inducing or facilitating the development of orthodontically-induced inflammatory root resorption (OIIRR).

## 6. ACKNOWLEDGEMENTS

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