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Ultrasound stimulation attenuates root resorption of rat replanted molars and impairs tumor necrosis factor-α signaling *in vitro*

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Background and Objective: A therapeutic protocol to minimize root resorption induced by tooth replantation has not yet been universally established. In this context, noninvasive modality such as ultrasound therapy have been a focus of increased interest. This study aimed to evaluate the inhibitory effect of ultrasound therapy on root resorption of replanted rat molars. In addition, the study aimed to promote insights into the mechanism through which ultrasound mediates the metabolism of periodontal cells *in vitro*.

Material and Methods: An experimental model of tooth replantation in rats, involving luxation and immediate replacement of the maxillary first molars, was used to assess the inhibitory effect of an ultrasound-therapy regimen (15 min of exposure to ultrasound, each day for 21 d) on root resorption. Moreover, the effect of ultrasound on osteoclastogenesis/cementoclastogenesis was examined *in vitro* using a mouse osteoblastic stromal cell line (ST2) and a mouse cementoblastic cell line (OCCM-30).

Results: The area of root resorption lacunae was statistically decreased ($p < 0.01$) in the ultrasound-treated sample. In addition, immunohistochemical staining, using murine TNF-α polyclonal antibody, failed to detect tumor necrosis factor-α (TNF-α) protein in the ultrasound-treated sample compared with the control. An *in vitro* study showed that the lipopolysaccharide (LPS)-induced expression of *Tnfalpha* mRNA was significantly reduced by ultrasound therapy in both osteoblastic and cementoblastic cells. Moreover, the TNF-α-induced up-regulation of *Rankl* mRNA was also inhibited by ultrasound.

Conclusion: Ultrasound may contribute to the reduction of the trauma-induced inflammatory reaction through impairment of the TNF-α signaling pathway. It is therefore suggested that ultrasound shows potential as a therapeutic tool to optimize the regenerative potential of periodontal tissues on replanted teeth.

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Tooth replantation, in general, is an indicated therapeutic approach to traumatic avulsion (1). The success rate is varied and depends crucially on the vitality of periodontal ligament cells that remain attached to the root surface (2). The extra-alveolar time before replantation (3), the storage conditions of the tooth (4), the presence of

contaminants (5) and the root-formation stage (6) have all been reported to be critical factors for the prognosis of replanted teeth. Failure in any of these factors can lead to side effects such as necrosis (7), ankylosis (8) and the subsequent root resorption commonly observed in replanted teeth (9).

In this context, many efforts have been made to improve the regenerative response of periodontal tissues before replantation. Procedures to minimize root resorption induced after tooth replantation are found widely in the literature. Root canal treatment with addition of regenerative substances such as acetozolamide (10) or calcium hydroxide (11) has been reported to be effective. Treatment of the root or of the alveolus surface with alendronate (12), ascorbic acid (13), sodium fluoride (14), propolis (15), sodium hypochlorite (7) or antibiotics (16) has also been proven to exert a positive healing effect. In addition, it has been reported that thyroxine (17), bisphosphonates (18), echistatin (19) and Emdogain® gel (20) enable periodontal regeneration. However, a therapeutic protocol has not yet been universally established, probably because of the application difficulties and/or possible side effects that medicamentous treatment can generate. In this context, attention has been focused on the noninvasive modality, ultrasound therapy, as a promising therapeutic tool for the regeneration of periodontium.

Ultrasound is an acoustic type of radiation that can be transmitted into the body as a pressure wave, resulting in biochemical changes at the cellular level (21). *In vivo* studies have demonstrated that ultrasound, when used therapeutically, can promote bone repair and regeneration, accelerate bone fracture healing and enhance bone formation in osteodistraction sites (22–25). Recent studies have also provided evidence that periodontal tissues are sensitive to ultrasound stimulation (26–30).

Although the role of ultrasound in the osteogenic response of bone has been well documented, the literature still lacks information about its role in tooth support tissues. The main aim of this study was to quantify the amount of root resorption on replanted rat molars exposed to ultrasound. In addition, we also aimed to promote insights into the mechanism through which ultrasound affects the regenerative response of the periodontium by evaluating the role of ultrasound in the tumor necrosis factor-α (TNF-α)-mediated expression of the *Rankl*/osteoprotegerin (*Opg*) gene, a classical mechanism of regulation of osteoclast/cementoclast development and subsequent bone/cementum resorption. The osteoblastic stromal cell line, ST2, and the cementoblastic cell line, OCCM-30, were used in the present study.

It is hypothesized that a possible mechanism responsible for the ultrasound-mediated inhibition of root resorption may be related to attenuation of the inflammatory response.

Material and methods

In vivo experiments

Experimental model— Eight, 10-week-old male Wistar rats (each approximately 400 g in weight) were used in this study. All rats were fed on a solid diet and given water *ad libitum* during the experimental period. The rats were anesthetized intraperitoneally using sodium pentobarbital (Nembutal; Dinabott, Osaka, Japan) at a dose of 50 mg/kg body weight. The right and left maxillary first molars of all animals were extracted and replanted according to the method described by Kvinnsland *et al.* (31) (Fig. 1A). The teeth were extracted using a tissue elevator, rotated once anteriorly so that all roots came out of the socket while leaving part of the attached mesial gingiva intact and then immediately repositioned. No postoperative splinting was used. The procedure protocols were approved by the Animal Care Committee of Hiroshima University.

Ultrasound exposure— The ultrasound exposure system BR-Sonic (ITO Co., Tokyo, Japan), used in this study, consists of a circular transducer of 18 mm in diameter. The sound head of this device has a beam nonuniformity average of 3.6 and an effective radiating area of 0.9 cm^2 (36%). The pulsed ultrasound signal consisted of a frequency of 1 MHz, a spatial-average intensity of 150 mW/cm^2 and a pulse ratio of 1:4 (2 ms on and 8 ms off).

The rats were kept immovable, without any use of anesthesia or sedation, and the ultrasound transducer was placed in contact with one side of the face, in the region corresponding to the upper first molar. The target region

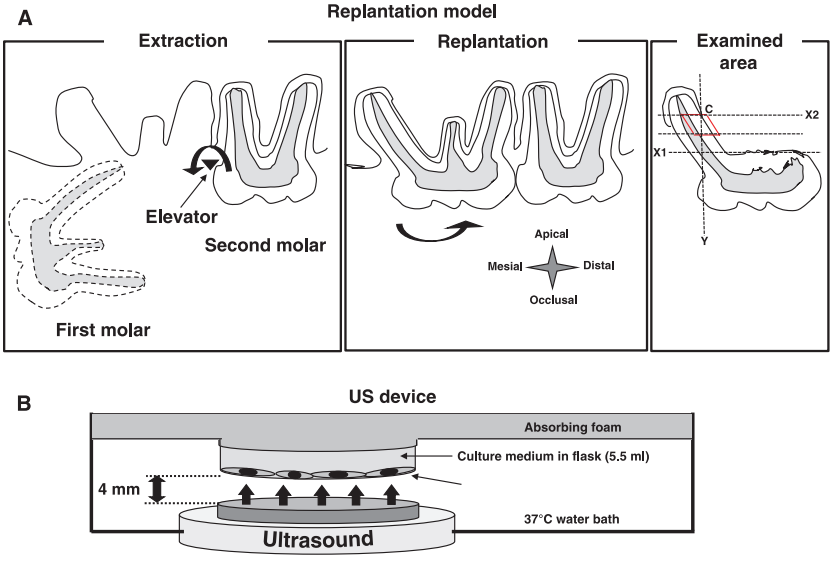

Fig. 1. Schematic representation of an experimental model for extraction, replantation and selection of the examined area (A) and of the ultrasound device (B).

had the fur shaved and coupling gel was constantly in place in order to optimize penetration of the ultrasound waves into the tissues. Aiming to exclude a possible side-related effect, half of the experimental rats received ultrasound stimulation on the right side of the face and the other half received ultrasound stimulation on the left side. Control group refers to the nonultrasound-stimulated side of the rats. The experimental protocol consisted of 15 min of ultrasound exposure each day for 21 consecutive days.

Tissue preparation— The animals were killed under general anesthesia with sodium pentobarbital (Dinabott), 21 d after tooth replantation. The maxillary molar area was removed *en bloc* and fixed in 10% neutral-buffered paraformaldehyde (WAKO Pure Chemical, Osaka, Japan), decalcified in 10% EDTA (pH 7.4) at 4°C for 4 wk and embedded in paraffin according to conventional methods.

Serial, 5-μm-thick sagittal sections of the large mesial root of the first molar were cut (RM2155; LEICA Co. Ltd, Nussloch, Germany) until the complete extension of the root could be observed, including the surrounding tissue. Attention was given during this experimental step to verify the anatomical similarity between all roots examined and to ensure that the selected sections belonged to the correspondent areas. The examined area was defined as follows: the X1 line was drawn tangent to the outline of the tooth crown close to the furcation area. The X2 line was traced parallel to the X1 line, cutting through the most coronal portion of the cellular cementum (point C). The Y line was then traced perpendicular to the X2 line through point C. On the Y line, the segment between point C and the X1 line was divided into two equal segments, and the apical half was taken as the object of evaluation (Fig. 1A). It is believed that the selected area was not mechanically overloaded during the extraction. For histological and histomorphometric analysis, the sections were stained with hematoxylin and eosin and observed under light microscopy.

Histomorphometric analysis of root resorption— Photomicrographs were taken of the periodontal tissues, including the mesial root, under ×100 magnification. To measure the resorption area, outlines of the resorption lacunae were drawn onto a transparent sheet and the areas of resorption were quantified from these outlines, together with a grade mark for compensating the enlargement rate, by transferring the images, via a flat-bed scanner (Scanjet 4C; Hewlett-Packard, Roseville, CA, USA), into a personal computer, following which a custom-made program computed the resorption area from the reproduced image.

Immunohistochemistry— Immunohistochemical staining was carried out as follows. Serial sections fixed in 10% formalin and embedded in paraffin were deparaffinized in xylene and hydrated in a graded alcohol series. The sections were then rinsed in phosphate-buffered saline and immersed, for 45 min, in methanol containing 0.3% hydrogen peroxide, to block the endogenous peroxidase activity. The sections were then washed in phosphate-buffered saline and incubated in normal blocking serum (DAKO Co., Carpinteria, CA, USA) for 20 min at room temperature. Each section was then incubated with murine TNF-α polyclonal antibody (R&D Systems Inc., Mineapolis, MN, USA), diluted 1/150 in phosphate-buffered saline, for 2 h at room temperature in a humid atmosphere. Then, the sections were incubated with mouse secondary antibody (Histofine; Nichirei Biosciences, Tokyo, Japan) in a humid atmosphere at room temperature for 45 min. The color was developed and the antigen–antibody complex was visualized following the application of diaminobenzodine chromogen (DAKO Co.). The specimens were counterstained with hematoxylin and then mounted.

In vitro experiments

Cell culture— The mouse osteoblastic stromal cell line ST2 (32) and the mouse cementoblastic cell line OCCM-30 (33) were cultured in alpha-minimum essential medium (α-MEM; Nissui Pharmaceutical, Tokyo, Japan) and Dulbecco's modified Eagle's minimal essential medium (DMEM; Nissui Pharmaceutical), respectively. Media were supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA) containing 100 U/mL of penicillin and 100 μ/mL of streptomycin (Gibco, Gaithersburg, MD, USA).

Reagents— Confluent cell cultures were treated with 100 ng/mL of lipopolysaccharide (LPS) from *Aggregatibacter actinomycetemcomitans* (strain ATCC29522) or with 10 ng/mL of mouse recombinant TNF-α (R&D Systems Inc.) and then exposed to ultrasound.

In vitro ultrasound exposure system— The ultrasound exposure system BR-Sonic (ITO Co.) is equipped with transducers of a 9.6-cm^2 circular surface area. The sound head of this device has a beam nonuniformity average of 3.6 and an effective radiating area of 4.5 cm^2 (46.8%). The pulsed ultrasound signal has a frequency of 1 MHz, a spatial-average intensity of 30 mW/cm^2 and a pulse ratio of 1:4 (2 ms on and 8 ms off).

A six-well culture plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) was held in place with the top above water level, in a foam-fronted plastic sliding assembly containing an aperture matching the dimensions of the monolayer. The culture plate was placed at a distance of approximately 4.0 mm from the surface of the transducers, which helped to optimize beam uniformity across the target cell region. Unidirectional propagation of the ultrasound signal was accomplished by using absorption foam on top of the culture plate in order to minimize standing waves and unwanted reflection from the flask. The absorber consisted of 1.0-cm-thick silicon-type rubber foam (Exafine; GC Corp., Tokyo, Japan). The space between the monolayer and the absorbing foam was completely filled with 5.5 mL of medium. The water tank was maintained at

37 ± 0.5°C and the ultrasound exposure assembly was maintained in a humidified atmosphere of 5% CO_2 at 37°C during all experiments.

Ultrasound stimulation of confluent cell cultures was initiated 10 min before treatment with LPS or TNF-α, and the cultures were exposed to a further 10 min of ultrasound stimulation after treatment, totaling 20 min of ultrasound exposure. ST2 and OCCM-30 cells were collected 2 h after the initiation of stimulation. Control samples were treated with exactly the same protocol as the test samples, but without ultrasound stimulation. A schematic representation of the ultrasound device is shown in Fig. 1B.

Total RNA isolation and analysis— Total RNA was extracted using Trizol reagent (Invitrogen), digested with DNAse I (Sigma-Aldrich Japan, Tokyo, Japan) and purified using the MiniElute PCR purification kit (Qiagen, Tokyo, Japan). Complementary DNA (cDNA) was produced using the transcriptase PCR kit (ReverTra Dash; Toyobo Biochemicals, Osaka, Japan). Aliquots of cDNA were amplified in a PCR machine (PC701 thermal cycler; Astec, Fukuoka, Japan). To measure the specific RNA level after ultrasound exposure, quantitative PCR analyses were performed using a SYBR Green PCR core reagent kit (SYBR Green PCR master mix; Toyobo Biochemicals). Quantitative real-time PCR analyses were performed using the PCR light cycler 2.0 system (Roche Diagnostics, Mannheim, Germany), as previously described (29).

The quantitative primers used for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TNF-α and RANKL were as follows: GADPH: forward, CCACTCTTCCACCTTCG and reverse, GTGGTCCAGGGTTTC TTAC; TNF-α: forward, CCAAATG GCCTCCCTCTCAT and reverse, GCTACAGGCTTGTCACTCGAATT; and RANKL, forward, CACACCTC-ACCATCAATG and reverse, AGT-CTGTAGGTACGCTTCC. Oligonucleotide primers were purchased from Nihon Gene Research Laboratories Inc. (Sendai, Japan).

Statistical analysis

Statistical analysis for histomorphometric analysis— Data were expressed as mean ± standard deviation. Comparisons between experimental and control groups were performed using the unpaired *t*-test. A *p*-value of < 0.01 was considered statistically significant.

Statistical analysis for gene expression— All data were examined for normal distribution (using the Kolmogorov–Smirnov test) and for uniformity (using the Bartlett test). Results were expressed as mean ± standard deviation, and statistical comparisons of the means were performed using analysis of variance at $p < 0.05$. After a significant difference was determined by analysis of variance, a Bonferroni test was used as the *post hoc* test at the 5% level of significance (Statview®; Abacus Concepts, Inc., Berkerley, CA, USA).

Results

Histological findings

In the control samples, substantial root resorption was observed on the distal side of the mesial root of the upper first molar 21 d after replantation. Root resorption lacunae were evident in most cases, and intense osteoclast and cementoclast/odontoclast activity could be observed along the resorbed root surfaces and alveolar bone (Fig. 2).

Meanwhile, 21 d after the replantation procedure, histological observations of the ultrasound-treated sample revealed substantial inhibition of development of severe root resorption lacunae. In addition, cementum integrity could be observed along the root surface, indicating that ultrasound therapy inhibited cementoclastic activity.

Histomorphometric analysis

According to histomorphometric analysis, the area of resorption lacunae was significantly ($p < 0.01$) decreased by ultrasound therapy (520 ± 315 μm^2) when compared with the untreated specimens (2.885 ± 1.472 μm^2), indicating that ultrasound therapy significantly inhibited the development of root resorption (Fig. 3).

Immunohistochemistry

In the control samples, after the experimental period, positive staining of TNF-α was evident and notably found along the root resorption lacunae. However, in the ultrasound-treated specimens, immunohistochemical staining revealed considerably reduced staining of TNF-α. Positively stained cells could be observed, especially around blood vessels (Fig. 4).

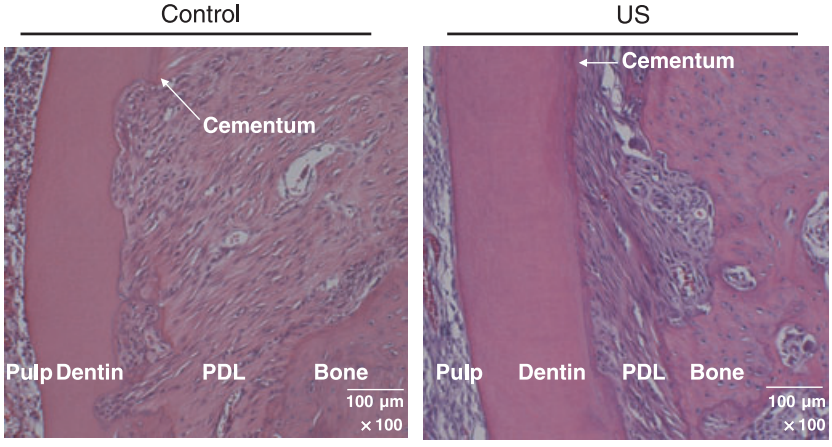

Fig. 2. Histological findings of a replanted tooth. In the control group, substantial root resorption was observed on the distal side of the mesial root of the upper first molar 21 d after replantation. On the ultrasound-treated specimens, the development of severe root resorption lacunae was substantially prevented and cementum integrity could be observed along the root surface. PDL, periodontal ligament.

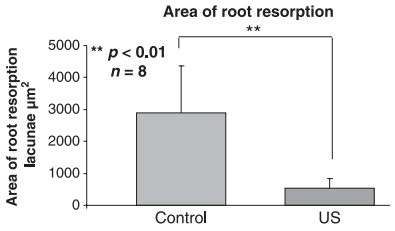

Fig. 3. Histomorphometrical analysis. The area of resorption lacunae was significantly ($p < 0.01$) decreased by ultrasound therapy when compared with the untreated specimens (Control).

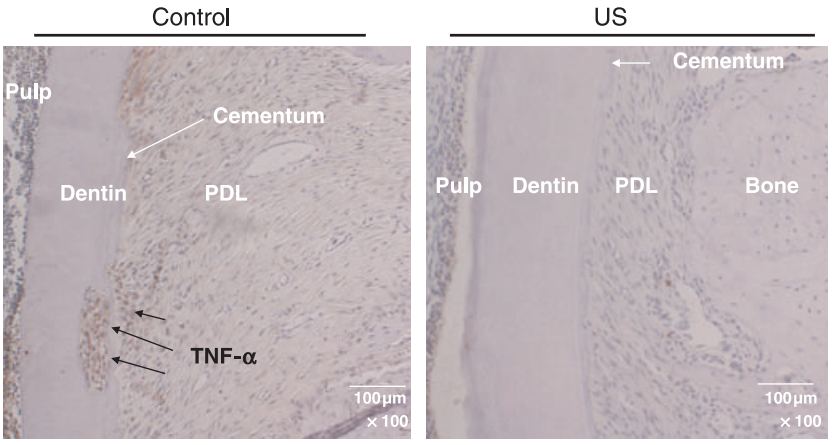

Fig. 4. Immunohistochemical staining. On the control specimen, positive staining of tumor necrosis factor-α (TNF-α) was evident and notably seen along the root resorption lacunae (arrows). On the ultrasound-treated specimens, however, positive staining of TNF-α was almost negligible after the 21-d treatment period.

Effect of ultrasound exposure on expression of *Tnf*alpha mRNA induced by LPS

Treatment with LPS significantly ($p < 0.01$) up-regulated the expression of *Tnfalpha* mRNA in ST2 and OCCM-30 cells. However, ultrasound simulation significantly reduced the LPS-stimulated expression of *Tnfalpha* mRNA in both cell lines examined. Compared with the control, the expression of *Tnfalpha* mRNA after ultrasound exposure was still significantly ($p < 0.01$) higher (Fig. 5).

Effect of ultrasound exposure on the expression of *Rankl* mRNA induced by mouse recombinant TNF-α

Treatment with TNF-α significantly ($p < 0.01$) up-regulated the expression of *Rankl* mRNA in ST2 and OCCM-30 cells. However, ultrasound simulation significantly ($p < 0.01$) reduced the TNF-α-stimulated expression of *Rankl* mRNA in both cell lines examined (Fig. 6).

Discussion

Tooth root resorption is regarded as a major problem encountered after tooth replantation. Eventually, the progression and severity of root resorption cannot be controlled, culminating in complete tooth loss. Approaches aiming to attenuate or inhibit root resorption and restore periodontal integrity after tooth replantation are still the subject of debate and investigation.

Ultrasound stimulation is a noninvasive therapeutic modality for bone regeneration and its efficiency has been widely reported over the years. Interestingly, recent studies have provided evidence that ultrasound also plays an important role in the metabolism of periodontal tissues. El-Bialy *et al*. (26) showed that ultrasound can prevent tooth root resorption during experimental tooth movement in humans. Dalla-Bona *et al*. (27,28) showed that ultrasound can enhance the expression of several genes related to mineral formation in mouse cementoblasts. Furthermore, we also reported that ultrasound promoted early cementoblastic differentiation of immature cementoblasts (29) and that ultrasound induced cementoblastic differentiation and matrix mineralization through the EP2/EP4 prostaglandin receptors pathway (30).

The present results showed that ultrasound stimulation significantly inhibits root resorption in replanted teeth. However, we should note that the experimental conditions were close to the ideal suggested for tooth replantation. In our *in vivo* experiment, the tooth was extracted and rapidly replanted. Therefore, the experiment excluded failure factors that lead to periodontal cell death, such as a long extra-alveolar time before replantation, compromising storage conditions of the tooth or the presence of contaminants. Future studies with different experimental conditions are highly anticipated.

Although the mechanisms involved in low-intensity ultrasound-stimulated tissue repair have not yet been elucidated, it is recognized that the anabolic biophysical effects caused by ultrasound are most likely to be the result of mechanical stress and/or fluid micro-streaming impacting on the cellular plasma membrane, focal adhesion and on cytoskeletal structures to trigger intracellular signal transduction and subsequent gene transcription (34,35). Among the genes related to mineral metabolism, it is well documented that ultrasound plays an important role in the *Rankl*/*Opg* mechanism. The *Rankl* gene is essential for osteoclast differentiation, playing a critical role in bone resorption. Furthermore, OPG is a soluble decoy receptor for RANKL and can inhibit its effects, thereby preventing osteoclast development and subsequent bone resorption (36). A study also

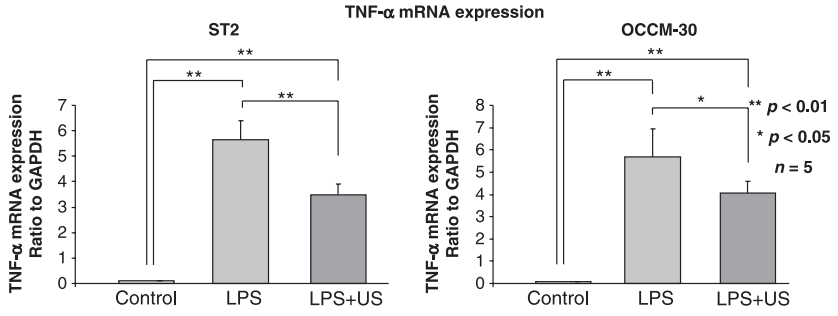

Fig. 5. Lipopolysaccharide (LPS) treatment significantly ($p < 0.01$) up-regulated the expression of tumor necrosis factor-α (*Tnfalpha*) mRNA in ST2 and OCCM-30 cells. In contrast, ultrasound simulation significantly reduced the expression of *Tnfalpha* mRNA, induced by LPS treatment, in both cell lines examined. Compared with the initial level, the expression of *Tnfalpha* mRNA after treatment with ultrasound was still significantly ($p < 0.01$) higher.

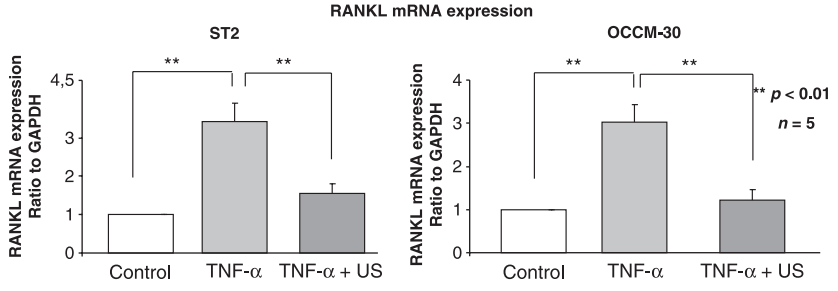

Fig. 6. Tumor necrosis factor alpha (TNF-α) treatment significantly ($p < 0.01$) up-regulated the expression of *Rankl* mRNA in ST2 and OCCM-30 cells. On the other hand, ultrasound simulation significantly ($p < 0.01$) reduced the expression of *Rankl* mRNA, induced by TNF-α treatment, in both cell lines examined.

reported that cementoblasts expressed RANKL and OPG and that the ratio of *Rankl*/*Opg* mRNA expression was a determinant for cementoclast formation (37).

It is well known that TNF-α, a potent inflammatory mediator, induces osteoclast differentiation and proliferation, playing a critical role in bone resorption (38). It has been reported that TNF-α stimulates osteoclast formation, both directly and indirectly. TNF-α acts directly on osteoclast precursors, inducing the formation of multinuclear cells positive for TRAP. Because this induction cannot be inhibited with OPG, it is assumed to be a direct effect (39,40). Nevertheless, it has also been reported that knockout of the TNFR I receptor on TNF-α significantly inhibits RANK expression and impairs osteoclast differentiation, thus implying an indirect effect of TNF-α on osteoclast formation. It is considered that a considerable overlap exists in the signal transduction pathways transmitted by RANKL and TNF receptors (41).

We thus hypothesized that a possible mechanism regarding the ultrasound-mediated inhibition of root resorption may be related to attenuation of the inflammatory response. In the present study, TNF-α expression was induced by pretreatment with LPS, a potent pathological molecule involved in inflammatory bone destruction. Our findings showed that ultrasound stimulation decreased the LPS-induced expression of *Tnfalpha* mRNA in both bone and cementoblast cells. Compared with the control level, however, ultrasound-stimulated cells still showed a high level of expression of *Tnfalpha* mRNA. It is important to point out that LPS treatment might play a dramatic effect *in vitro*. In addition, the present results represent the outcome of a single treatment with ultrasound. In contrast, the *in vivo* results represent the cumulative effects of 21 d of ultrasound treatment. In this regard, we evaluated, using a murine TNF-α polyclonal antibody, the expression of TNF-α after 21 d of ultrasound therapy. The results showed that in the ultrasound-treated sample, TNF-α expression was not commonly observed, in contrast to the untreated specimens.

Subsequently, in order to verify the indirect effect of TNF-α on osteoclastogenesis or cementoclastogenesis, the expression of *Rankl* mRNA, induced by preincubation with recombinant TNF-α, was evaluated with or without concomitant ultrasound stimulation. Interestingly, the up-regulation of *Rankl* mRNA induced by TNF-α was abolished by treatement with ultrasound, suggesting that ultrasound impairs osteoclastogenesis/cementoclastogenesis through a TNF-α signaling pathway. Using the same experimental conditions, the expression of *Opg* mRNA was not influenced by either treatment with TNF-α or ultrasound exposure (data not shown).

In conclusion, it is suggested that ultrasound may contribute to the reduction of the trauma-induced inflammatory reaction through impairment of the TNF-α signaling pathways, thus decreasing the severity of root resorption on replanted teeth.

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