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Preventive effects of trehalose on osteoclast differentiation in rat periodontitis model

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Key words: Trehalose; Periodontitis; Osteoclast; Animal studies

Running title: trehalose and periodontitis

Conflict of interest: The authors report no conflicts of interest related to this study.

Grant: This work was supported by Grants-in-Aid for Scientific Research (23792513) from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

ABSTRACT

Aim: Trehalose, which is a disaccharide formed by a 1,1 linkage of two glucose molecules, was suggested to have a suppressive effect on bone resorption. In this study, we examined the effects of topical application of trehalose on osteoclast differentiation in a rat periodontitis model.

Material and Methods: Rats were divided into four groups. One group received no treatment. In the other groups, experimental periodontitis was induced by ligature placement. These rats with experimental periodontitis received topical application of pure water (vehicle group), 30 mg/ml trehalose solution (30 mg/ml trehalose group) or 60 mg/ml trehalose solution (60 mg/ml trehalose group) to the gingival sulcus, respectively.

Results: The vehicle group showed higher numbers of polymorphonuclear leucocytes, receptor activator of nuclear factor kappa B ligand (RANKL)-positive cells and osteoclasts compared to the no treatment group, respectively. Trehalose-applied groups exhibited lower numbers of these cells compared to the vehicle group. Gene expressions of tumor necrosis factor-α, RANKL and toll-like receptor 4 were suppressed by trehalose. In addition, protein expressions of RANKL inducing pathway were less activated by trehalose.

Conclusion: Topical application of trehalose could suppress osteoclast differentiation by inactivation of RANKL inducing pathway in the rat periodontitis model.

Clinical Relevance:

Scientific rationale for the study: Trehalose has been reported to have a suppressive effect of bone resorption. It is possible that trehalose has preventive effect on bone resorption in periodontal lesion. However, it is still unclear whether topical application of trehalose offers clinical benefits on periodontal health.

Principal findings: Topical application of trehalose inhibited osteoclast differentiation by inactivation of PKC/ERK pathway in the periodontal lesion.

Practical implications: Trehalose could reduce differentiation of osteoclasts, and this effect might suppress periodontal bone resorption.

Introduction

Periodontitis is a chronic inflammatory disease characterized by gingival inflammation and resorption of alveolar bone (Williams 1990). The principal cell that is responsible for bone resorption is the osteoclast (Boyle et al. 2003). Receptor activator of nuclear factor kappa B ligand (RANKL), a member of the superfamily of tumor necrosis factors (TNFs), is a key mediator in the process of osteoclast formation. This membrane-bound protein, which is expressed by a variety of cells including osteoblasts, binds to receptor activator of nuclear factor kappa B (RANK) on the surface of osteoclast precursors and results in osteoclast differentiation (Bartold et al. 2000).

Lipopolysaccharide (LPS), a major constituent of gram-negative bacteria, is considered to be a potent stimulator of bone resorption in inflammatory diseases (Nair et al. 1996). Oral bacterial LPS stimulate osteoblasts in periodontal tissue to produce RANKL. Recently, the molecular mechanism of RANKL expression in osteoblasts has been clarified. LPS binds to toll-like receptor (TLR) 4 seen on the biomembrane of osteoclasts and activates the receptor. TLR 4 is a critical receptor and signal transducer for LPS (Kikuchi et al. 2001). On activation, myeloid differentiation factor 88 (MyD88) is recruited to TLR4 (Li et al. 2006), and this activates the protein kinase C (PKC) and extracellular signal regulated kinase (ERK) pathway. The activation of PKC/ERK

pathway plays an important role in the activation of RANKL expression (Kikuchi et al. 2001).

Trehalose is a nonreducing disaccharide in which two glucose molecules are linked together by a 1,1-glycosidic linkage (Patist et al. 2005). It has been shown to have protective properties against stress induced by freezing, heating, drying and oxidative damage (Elbein et al. 2003). Since it can maintain the stability of proteins and nucleic acids in cold or dry conditions, trehalose has been utilized as a stabilizer in foods, drugs, cosmetics (Richards et al. 2002) and transplant surgery (Kang et al. 2011). In addition, previous studies indicate that trehalose has a suppressive effect on pathological bone resorption. For example, consumption of trehalose decreased the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in the femurs of LPS-stimulated mice (Arai et al. 2001) and of ovariectomized mice (Nsishizaki et al. 2000). In human intestinal cells, trehalose increased production of osteoprotegerin (OPG), a decoy receptor of RANKL (Ariyasu et al. 2002). Furthermore, application of trehalose after LPS injection into the mouse jugular vein suppressed the gene expression of TLR4 in lung and liver (Minutoli et al. 2008). These findings suggest that trehalose has a suppressive effect on bone resorption during progression of periodontitis. Although it has been shown that trehalose treatment can also inhibit inflammation by

suppressing inflammatory cytokine production and nuclear factor kappa B expression (Taya et al. 2009, Echigo et al. 2012), the effect of trehalose on osteoclast differentiation in a periodontal lesion is still unclear.

The placement of ligatures around the teeth has been widely used to induce experimental periodontitis in animal models (Ebersole et al. 1999, Irie et al. 2008). A previous study demonstrated that ligature placement causes an increase in LPS (Ebersole et al. 1999). In the present work, we hypothesized that topical application of trehalose would suppress osteoclast differentiation by inhibiting the interaction between LPS and TLR4 in a ligature-induced periodontal lesion. Therefore, the purpose of the present study was to investigate the preventive effects of trehalose on osteoclast differentiation and TLR4 expression in ligature-induced periodontitis in rats. To gain better insight of the mechanism of action, we also analyzed the histological changes, and the changes in gene expression and intracellular signaling pathway (PKC/ERK pathway) activation of RANKL production.

Material and methods

Animals

Twenty-four male Wistar rats (8 weeks old) were housed in an air-conditioned room (23–25°C) with a 12-h light-dark cycle. They had free access to powdered food (MF, Oriental Yeast Co. Ltd., Osaka, Japan) and drinking water. The experimental protocol was approved by the Animal Research Control Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Experimental design

The rats were randomly divided into four groups of six rats each. One group received no treatment for 2 weeks (no treatment group). In the other groups, a 3/0 cotton ligature (Alfresa Pharma Co., Osaka, Japan) was placed in a subgingival position at the maxillary second molars to induce periodontitis (Irie et al. 2008) for 2 weeks. During the experimental period, these rats received topical application of deionized water (vehicle group), 30 mg/ml trehalose (30 mg/ml trehalose group) or 60 mg/ml trehalose (60 mg/ml trehalose group). Anhydrous trehalose (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) was dissolved in deionized water at a concentration of 30 or 60 mg/ml. In the experimental groups, 5 μl experimental solution was applied

three times into the palatal gingival sulcus of both maxillary second molars once a day. After application, the excess liquid remaining in the oral cavity was removed using a cotton ball (Maruyama et al. 2011).

After the experimental period, the animals were sacrificed under general anesthesia. For histological analysis, maxillary molar regions were resected *en bloc* from each rat and were immediately fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 1 day. Gingival biopsy samples of maxillary molar regions were homogenized by the frozen cell crusher (Microtec Co., Chiba, Japan). The homogenized samples were used for real-time reverse transcription polymerase chain reaction (RT-PCR) and for western blot analysis.

Histopathological analysis

After fixation with paraformaldehyde, the maxillary samples were decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 8 weeks at 4°C. Formalin-fixed tissue samples were embedded in paraffin following dehydration with ethanol (70%, 80%, 90%, and 100%) and immersion in xylene. The paraffin-embedded bucco-palatal sections (4 μm thick) were then stained with hematoxylin and eosin or other stains, as described below.

Immunohistochemical staining for lipid A (a lipid component of LPS), phosphorylated-ERK (p-ERK), RANKL and TRAP staining were carried out. The monoclonal antibody against lipid A (Abcam, Tokyo, Japan) and polyclonal antibodies against p-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and RANKL (Santa Cruz Biotechnology) were diluted at 1/100, 1/50, and 1/100 in phosphate buffered saline, respectively. The color was developed by placing sections in a solution of 3-3'-diamino bentizine tetrahydrochloride. To identify osteoclasts, TRAP activity was detected using the azo dye method (Sanbe et al. 2009). Sections were counterstained with Mayer's hematoxylin. In addition, to show that the labeling is specifically due to the primary antibody, similarly diluted normal serum from the same species was used instead of the primary antibody as a negative control.

The polymorphonuclear leucocytes in the connective tissue subjacent to the junctional epithelium were counted in two standard areas [0.05 mm (depth) × 0.1 mm each] at a magnification of ×400 (Endo et al. 2010). RANKL-positive osteoblasts and TRAP-positive osteoclasts on the surface of alveolar bone were counted using a light microscope at ×400 magnification and reported as number/millimeters (Sanbe et al. 2009). A single blinded examiner (Y.E.) performed the histometric analyses. We evaluated intra-examiner reproducibility by double-scoring 10 randomly selected

sections at two-week intervals. Agreement with one cell of polymorphonuclear leucocytes, RANKL-positive cells and TRAP-positive cells was more than 90%, respectively.

Real-time RT-PCR

Total RNA was isolated from the gingival biopsy samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The isolated RNA was quantified by measuring the absorbance at 260 nm and the purity was determined by the 260/280 nm absorbance ratio. Only samples with a ratio greater than 1.8 were used (Endo et al. 2010). Total RNA was reverse transcribed by AMV reverse transcriptase (Takara Bio Inc., Shiga, Japan) at 42°C for 30 min. Real-time PCR was performed using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) in a Real-time QPCR system (Agilent Technologies, Tokyo, Japan). The primer sequences are shown in Table 1 (Irie et al. 2012).

Amplification cycling conditions were as follows: 50 cycles at 95°C (30 s), 55°C (30 s), 72°C (6 s) for TNF-α; 45 cycles at 95°C (15 s), 54°C (20 s), 72°C (20 s) for RANKL; 45 cycles at 95°C (15 s), 59°C (20 s), 72°C (20 s) for TLR4; 45 cycles at 95°C (10 s), 54°C (20 s), 72°C (10 s) for β-actin. The mRNA levels were calculated in terms

of the relative copy number ratio of each mRNA to β-actin for each sample.

Western blot analysis

Total proteins were extracted from gingival tissue samples using a detergent-based extraction buffer (Invitrogen) containing a protease inhibitor cocktail (Bio-Rad Laboratories, Hercules, CA, USA). The tissue samples were macerated in buffer (20 μl/mg of tissue) and centrifuged at 12,000 rpm × *g* for 10 min at 4°C. Aliquots were mixed with sample buffer (125 mM Tris-HCl (pH 6.8) with 20% glycerol, 4% sulfate polyacrylamide gel, 0.04% bromophenol blue, and 10% 2-mercaptoethanol) and heated at 95°C for 3 min. The samples and controls along with marker were loaded onto 10% polyacrylamide gel for SDS-PAGE, and proteins were separated in 30 min at 200 V. After gel electrophoresis, the separated proteins were electroblotted onto a 100% Triton-free nitrocellulose membrane (pore size = 0.2 μm) in a transfer buffer (0.3% Tris-HCl, 1.44% glycine) using 24 V for 100 min. The membrane was blocked with 5% (W/V) non-fat milk in 0.1% Tween-20 in Tris-buffered saline for 1 h at room temperature. The membranes were probed with antibody to TLR4 (1:5,000), MyD88 (1:5,000), PKC-α (1:5,000), phospho-PKC-α (1:5,000), ERK (1:5,000), phospho-ERK (1:5,000) (Cell Signaling Technology, Beverly, MA, USA) and α-tubulin (1:10,000)

(SIGMA-Aldrich, St. Louis, MO, USA) overnight at 4°C or for 1 h at room temperature. Membranes were washed in a washing buffer three times for 10 min each and incubated with secondary antibodies conjugated to horseradish peroxidase (1: 30,000) (Pierce Chemical, Rockford, IL, USA) for 1 h at room temperature, and finally washed in buffer. Subsequently, signals were visualized with ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd, Amersham, Buckinghamshire, UK). The images of bands were analyzed using Image Quant LAS 4000 mini (Fujifilm, Tokyo, Japan) and quantified by densitometry using an Epson perfection scanner (Epson Europe, Amsterdam, the Netherlands). α-Tubulin was used as internal control.

Statistical analysis

The differences in histological and gene expression data among the groups were analyzed by one-way ANOVA followed by Tukey's method. *T*-test was used for statistical comparison of the data of western blot analysis between the vehicle group and 60 mg/ml trehalose group. $P < 0.05$ was considered statistically significant.

Results

Histopathological analysis of periodontal tissue

The vehicle group showed 3.35, 2.48 and 5.48 times higher numbers of polymorphonuclear leucocytes, RANKL-positive cells and TRAP-positive osteoclasts compared to the no treatment group, respectively ($p < 0.05$). On the other hand, the 30 mg/ml trehalose group and the 60 mg/ml trehalose group exhibited 0.56 and 0.43 times lower numbers of polymorphonuclear leucocytes compared to the vehicle group, respectively ($p < 0.05$). These groups also showed 0.62 and 0.64 times lower numbers of RANKL-positive cells and 0.45 and 0.32 times lower numbers of TRAP-positive osteoclasts compared to the vehicle group, respectively ($p < 0.05$). There was no significant difference among the no treatment group, the 30 mg/ml trehalose group and the 60 mg/ml trehalose group in terms of the number of polymorphonuclear leucocytes, RANKL-positive cells and TRAP-positive osteoclasts (Table 2, Figure 1).

Gene expressions of TNF-α, RANKL and TLR4

The vehicle group showed 4.79, 2.63 and 2.27 times higher mRNA expressions of TNF-α, RANKL and TLR4 compared to the no treatment group, respectively ($p < 0.05$). On the other hand, the 60 mg/ml trehalose group showed 0.71, 0.55 and 0.41 times

lower mRNA expressions of TNF-α, RANKL and TLR4 compared to the vehicle group, respectively ($p < 0.05$) (Figure 2).

Protein expressions of PKC/ERK pathway and lipid A

The results of western blot analysis are shown in Figure 3. The signals from the TLR4 and MyD88 protein in the 60 mg/ml trehalose group were significantly weaker than those in the vehicle group, respectively. In addition, PKC/ERK pathway was less activated in periodontal tissues obtained from the 60 mg/ml trehalose group than in those obtained from the vehicle group. There were some p-ERK-positive cells on the surface of alveolar bone in the vehicle group, but not in the 60 mg/ml trehalose group (Figure 4). In addition, expression of lipid A in the periodontal tissue was more evident in the vehicle and the 60 mg/ml trehalose groups than that in the no treatment group (Figure 5).

Discussion

Previous studies have shown that ligature placement in the sulcus of rat molar induces experimental periodontitis (Endo et al. 2010, Kasuyama et al. 2011). In this study, the number of polymorphonuclear leucocytes and TRAP-positive osteoclasts in the vehicle group was higher than that of the no treatment group. These findings indicate that experimental periodontitis was actually induced by ligature placement. In this pathogenic situation, application of trehalose decreased the number of polymorphonuclear leucocytes in gingiva and TRAP-positive osteoclasts on the surface of alveolar bone. In addition, the expression of RANKL in gingiva was also suppressed by trehalose. These results indicate that gingival inflammation and the consequent osteoclast differentiation were inhibited by application of trehalose. In our model, trehalose-diluted water was applied while induction of periodontal inflammation was continued. Therefore, these results reveal a preventive aspect of trehalose on inflammation and osteoclast differentiation in the periodontium.

In this study, trehalose did not decrease lipid A expression in the periodontal lesion. This indicates that trehalose had little effect on LPS production in our model. However, both protein and gene expressions of TLR4 were decreased by application of trehalose. These findings are consistent with those of a previous study (Minutoli et al. 2008).

Previous studies showed that trehalose altered the interaction between endotoxin and the cellular membrane, by causing significant changes in the binding of LPS to its specific receptors (Minutoli et al. 2007, Minutoli et al. 2008). Another study demonstrated that ligature placement in the sulcus of rat molar induces proliferation of subgingival bacteria (Meulman et al. 2011). As shown in our results (Figure 5), LPS produced by bacteria was increased in rat periodontal lesions in the ligature model. Therefore, the present results indicate that trehalose might inhibit the interaction between TLR4 and LPS, which was increased by ligature placement.

PKC/ERK pathway is one of the RANKL-producing pathways, which transmits signals via MyD88 after activation of TLR4 in osteoblasts (Kikuchi et al. 2001, Li et al. 2006). In this study, MyD88 and PKC/ERK pathway were inactivated by trehalose application. These findings indicate that inhibition of RANKL expression by trehalose were associated with inactivation of the PKC/ERK pathway.

It is reported that MyD88 promotes not only activation of PKC/ERK pathway but also expression of inflammatory cytokines (Szabo et al. 2011). In the present study, trehalose induced gene expression of TNF-α, one of the inflammatory cytokines. The cytokine promotes differentiation of osteoclasts both directly and indirectly (Yang et al. 2005). Therefore, the suppressive effect of trehalose on differentiation of osteoclasts

may be caused by inhibition of inflammatory cytokines as well as by inactivation of the PKC/ERK pathway.

Some previous studies demonstrated association between trehalose and bone resorption. For example, consumption of trehalose decreased the number of TRAP-positive osteoclasts and inhibited bone loss in femurs of ovariectomized mice (Nsishizaki et al. 2000). In addition, a report showed that trehalose promotes the production of OPG by intestinal epithelial cells (Ariyasu et al. 2002). In this study, we proved that the number of TRAP-positive osteoclasts on the surface of alveolar bone was decreased by direct application of trehalose to gingiva. The result is in agreement with previous findings that trehalose suppresses bone loss.

The concentration of trehalose solution was determined based on a previous report (Cejková et al. 2011). In an in vitro study, 100 mmol/ml trehalose inhibited the activation of mitogen-activated protein kinase family, such as ERK, produced by LPS-stimulated macrophages (Minutoli et al. 2007). In addition, 10% trehalose inhibited oral dryness in humans (Mori et al. 2010). Although the concentration of the trehalose solution we prepared in this study was within the range of these studies, more studies are needed to determine an appropriate concentration to prevent periodontitis.

In dentistry, utilization of a substitute sweetening agent is recommended to prevent

oral diseases. For example, xylitol, which is a sugar alcohol that has no cariogenicity, is added to chewing gums instead of sucrose to prevent dental caries (Seki et al. 2011). Trehalose has been reported to be a low cariogenic disaccharide (Neta et al. 2000) and it inhibits oral dryness (Mori et al. 2010). In addition to these effects, we found that trehalose might have a suppressive effect on bone resorption in this study. Therefore, it would be useful as a component of dentifrices or some other oral care products to prevent dental caries as well as periodontal disease.

The bacterial flora in the sulcus of a ligature-placed rat is different from that of human. Therefore, we did not investigate how trehalose directly affected the bacterial flora. However, since it is possible that trehalose has direct effects on bacteria, more studies need to conducted to clarify this issue.

In the present study, we analyzed the differentiation of osteoclasts, but not degree of bone resorption. Therefore, it remains to be determined whether trehalose actually suppresses bone resorption. In addition, the experimental period was only 2 weeks, so we need to examine the long term results to clarify a true effect of trehalose on bone resorption. These are the limitations of our study.

In conclusion, trehalose could suppress osteoclast differentiation in alveolar bone via inactivation of TLR4 and PKC/ERK pathway in the rat periodontitis model.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (23792513) from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan. The authors report no conflicts of interest related to this study.

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Table 1. Primer sequences for TNF-α, RANKL, TLR4 and β-actin

	sense (5'-3')	antisense (3'-5')	Length (bp)	Accession No.
TNF-α	GTGATCGGTCCCA ACAAG	ATCGGGTGCAGCA TCGTT	112	NM012675
RANKL	GCTCACCTCACCA TCAATGCT	ATTTCAGTCAGAC AGGAGAACCATGG	70	NM057149
TLR4	GTGAGCATTGATG ATGAGTTCAG	CATCTAATGATTG ATAAGGATT	170	NM019178
β-actin	TGTTGCCCTAGAC TTCGAGCA	GGACCCAGGAAG GAAGGCT	155	NM007393

Table 2. Histopathological Evaluation in Periodontal Tissues at 2 weeks (Mean ± SD)

	No treatment (N= 6)	Vehicle (N= 6)	30mg/ml trehalose (N= 6)	60mg/ml trehalose (N= 6)
Polymorphonuclear leucocytes (numbers/0.05mm×0.1 mm)	1.5±0.5	5.1±0.6 *	2.9±1.3 †	2.2±0.7 †
RANKL positive cells (numbers/mm)	7.1 ± 2.2	17.7 ± 2.0 *	11.1 ± 2.3 †	11.3 ± 1.1 †
TRAP-positive osteoclasts (numbers/mm)	0.6 ± 0.5	3.5 ± 1.1 *	1.6 ± 0.9 †	1.1 ± 0.8 †

* Significantly different from the no treatment group, $p < 0.05$ (Tukey's method).

† Significantly different from the vehicle group, $p < 0.05$ (Tukey's method).

Figure regends

Fig. 1. Hematoxylin and eosin (A-C) or tartrate-resistant acid phosphatase (TRAP) and receptor activator of nuclear factor kappa B ligand (RANKL) stains (D-J) of rat periodontal tissue at 2 weeks. No pathological changes were observed in the periodontium in any of the samples in the no treatment group (A, D and H). The vehicle group showed higher number of polymorphonuclear leucocytes (B), TRAP-positive osteoclasts (arrows) (E) and the number of RANKL positive cells (arrow heads) (I) more than the 60 mg/ml trehalose group (C, F and J), respectively. Control stains with normal serum did not show any positive cells (G). CEJ, cement-enamel junction; AB, alveolar bone; CM, cementum. Scale bar = 50 μm (A-F) and 30 μm (G-I).

Fig. 2. Fold changes of gene expression on rat gingival tissue. The mRNA levels were calculated in terms of the relative copy number ratio of each mRNA to β-actin for each sample (n=3 per group). The vehicle group showed 4.79, 2.63 and 2.27 times higher mRNA expressions of TNF-α, RANKL and TLR4 compared to the no treatment group, respectively. The 60 mg/ml trehalose group showed 0.71, 0.55 and 0.41 times lower mRNA expressions of TNF-α, RANKL and TLR4 compared to the vehicle group, respectively. * $p < 0.05$, compared with the no treatment group (Tukey's method). † $p <$

0.05, compared with the vehicle group (Tukey's method).

Fig. 3. Gingival protein expression of TLR4, MyD88, PKC-α, and ERK in the vehicle and 60 mg/ml trehalose groups (A) and calculated protein levels (B). The α-tubulin was used as internal control. Values are presented as means of 6 rats ± SD. The expressions of TLR4, MyD88, p-PKC, p-ERK in the 60 mg/ml trehalose group were lower than those in the vehicle group. * $p < 0.05$, compared with the vehicle group (*T*-test).

Fig. 4. Immunohistochemical staining for p-ERK in the periodontal tissue. Control stains with normal serum did not show any positive cells (A). There were some p-ERK-positive cells (arrows) on the surface of alveolar bone in the vehicle group (C), but not in the no treatment (B) and 60 mg/ml trehalose groups (D). AB, alveolar bone. Scale bar =30 μm.

Fig. 5. Immunohistochemical staining for lipid A in the periodontal tissue. Control stains with normal serum did not show any positive cells (A). Expression of LPS (arrows) in the vehicle (C) and the 60 mg/ml trehalose group (D) were greater than that in the no treatment group (B). JE, junctional epithelium. Scale bar =30 μm.

Figure1

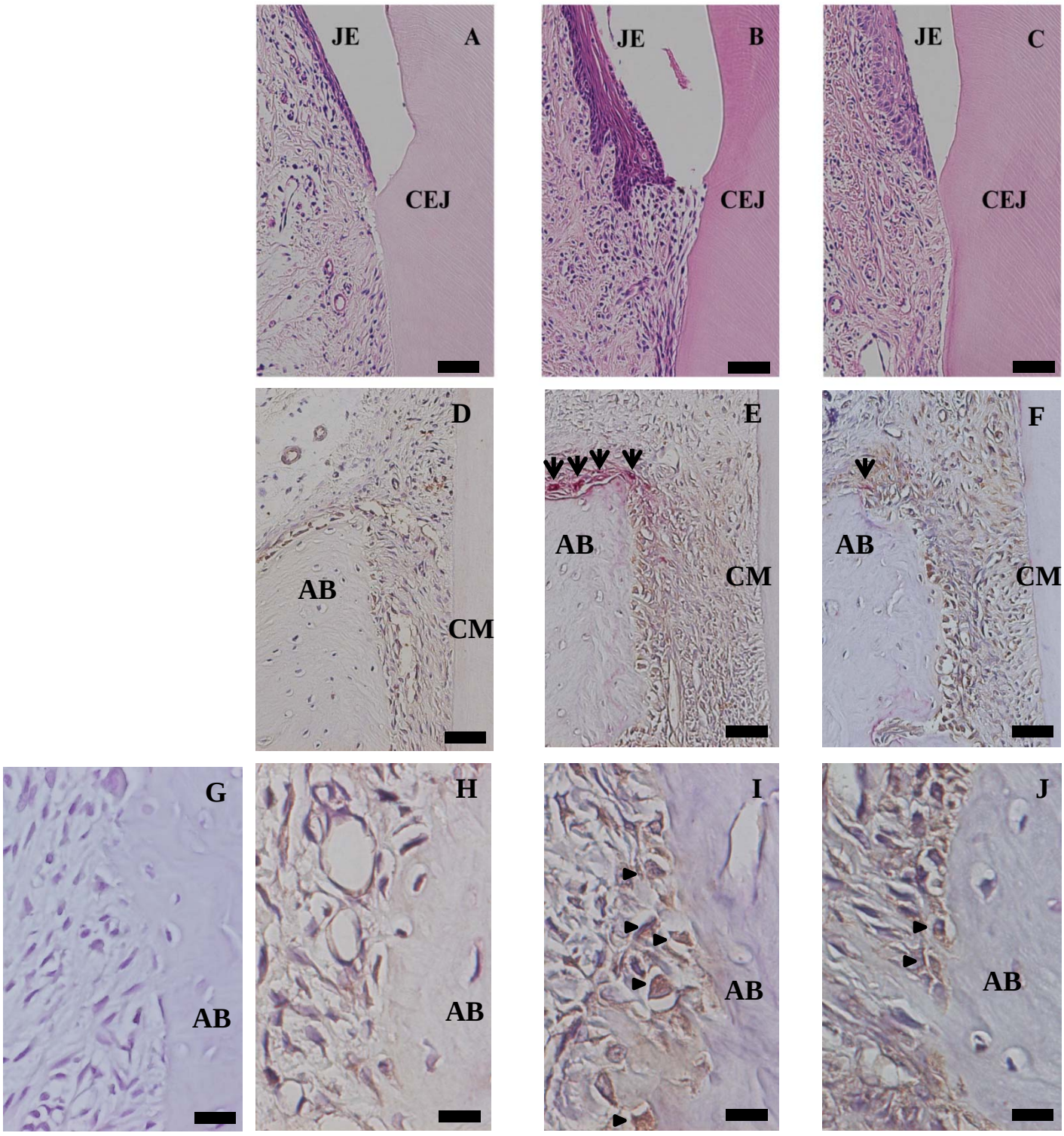


figure2

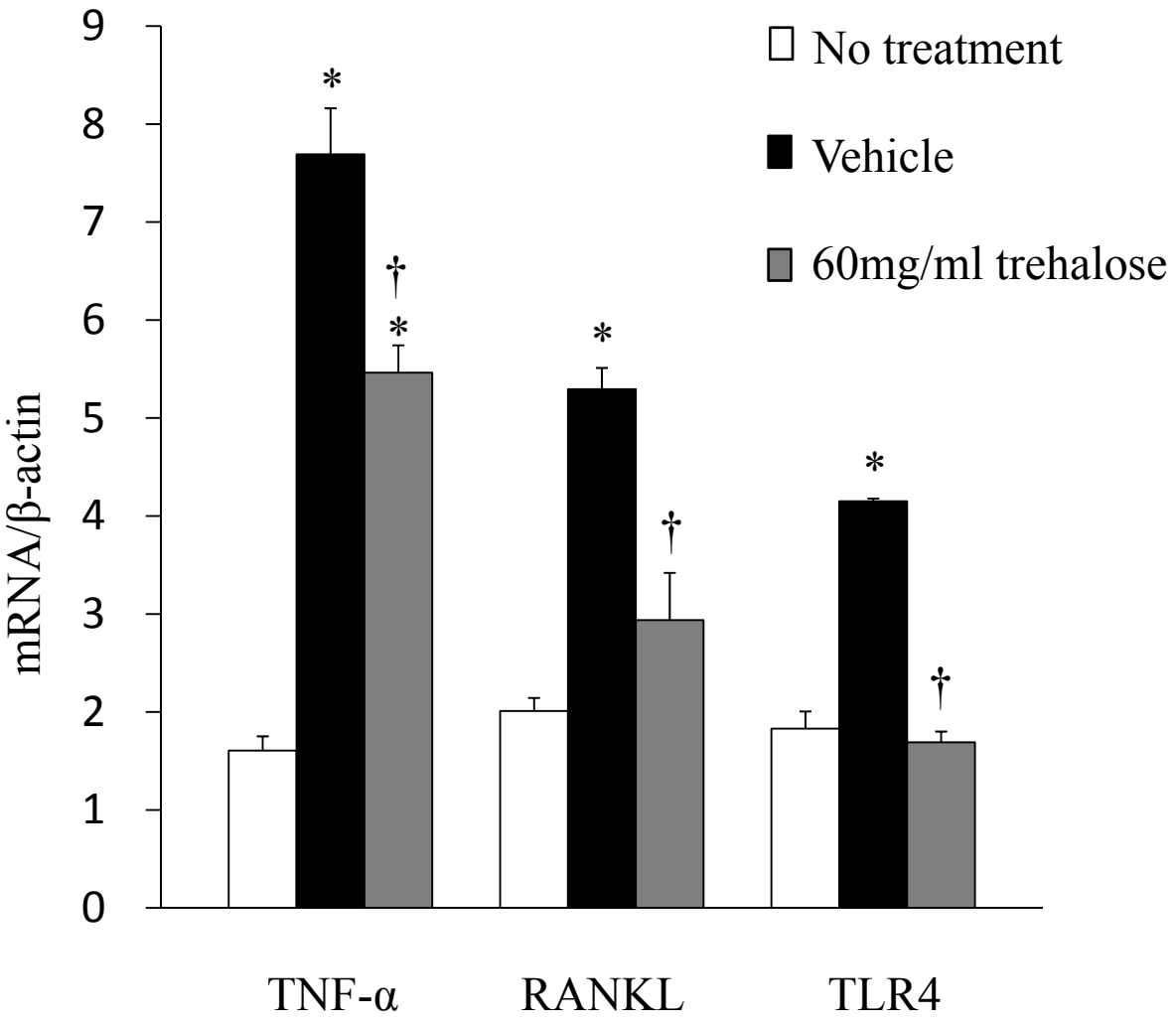
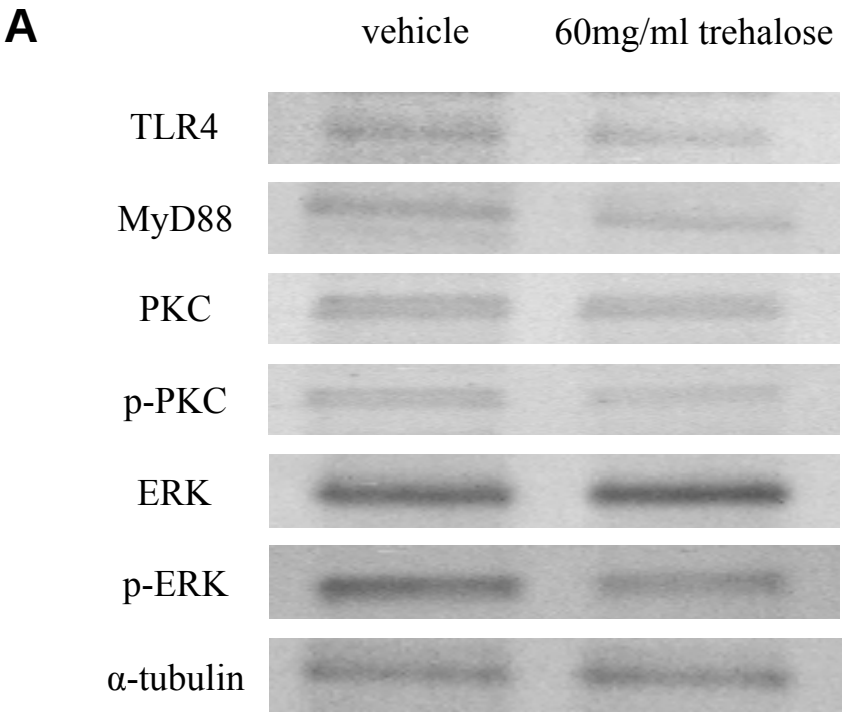

mRNA/β-actin
0
1
2
3
4
5
6
7
8
9
No treatment
Vehicle
60mg/ml trehalose
*
†
*
*
†
*
†
TNF-α
RANKL
TLR4

Figure3


A
vehicle
60mg/ml trehalose
TLR4
MyD88
PKC
p-PKC
ERK
p-ERK
α-tubulin

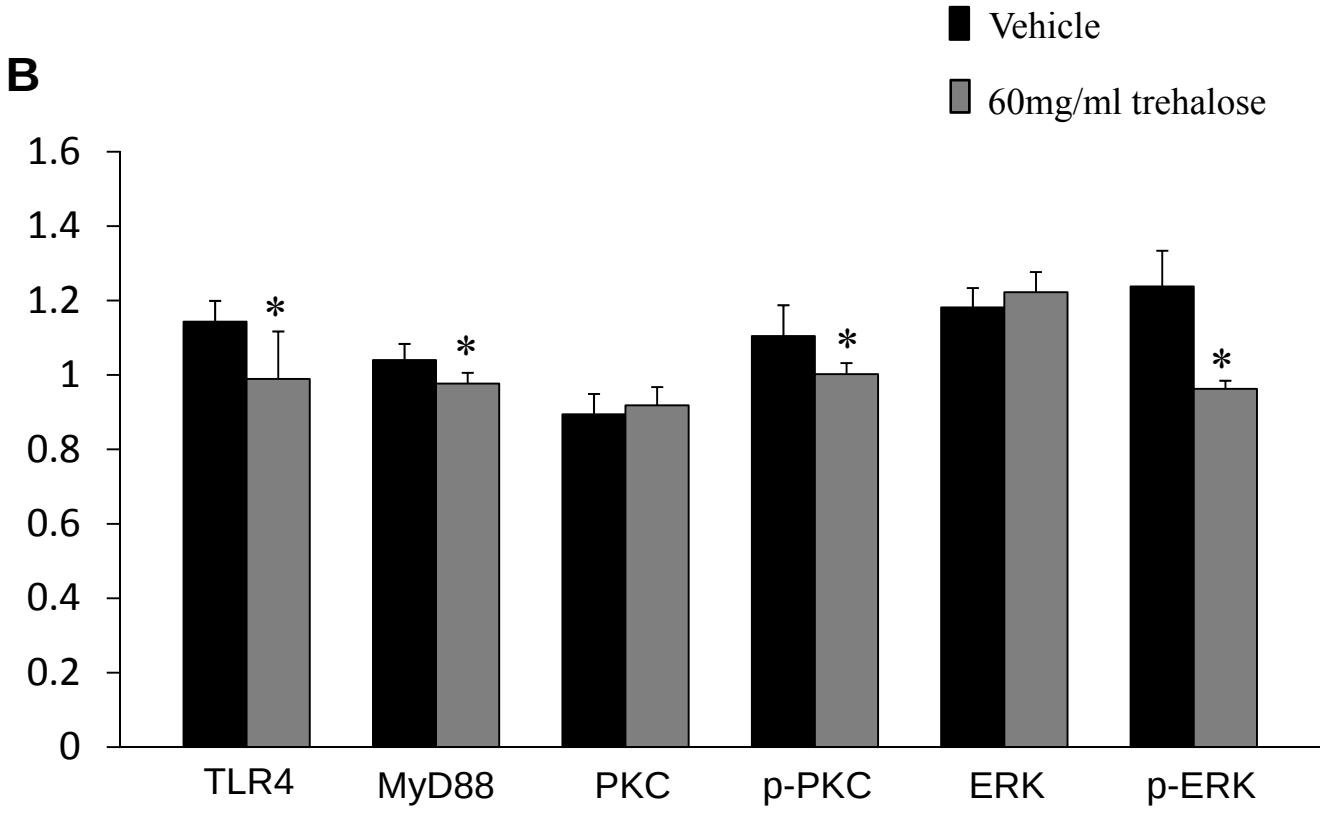

B
Vehicle
60mg/ml trehalose
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
TLR4
MyD88
PKC
p-PKC
ERK
p-ERK

Figure4

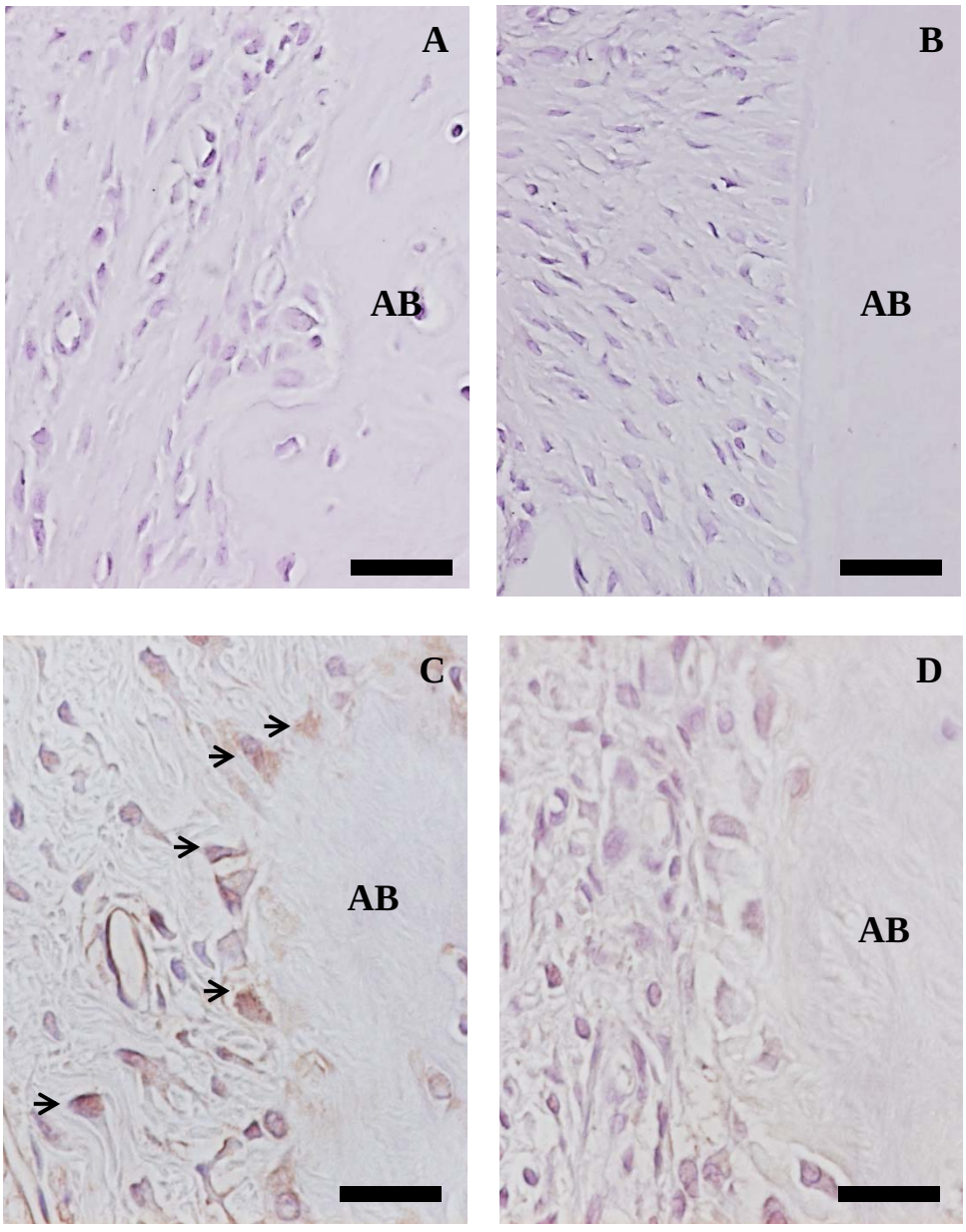
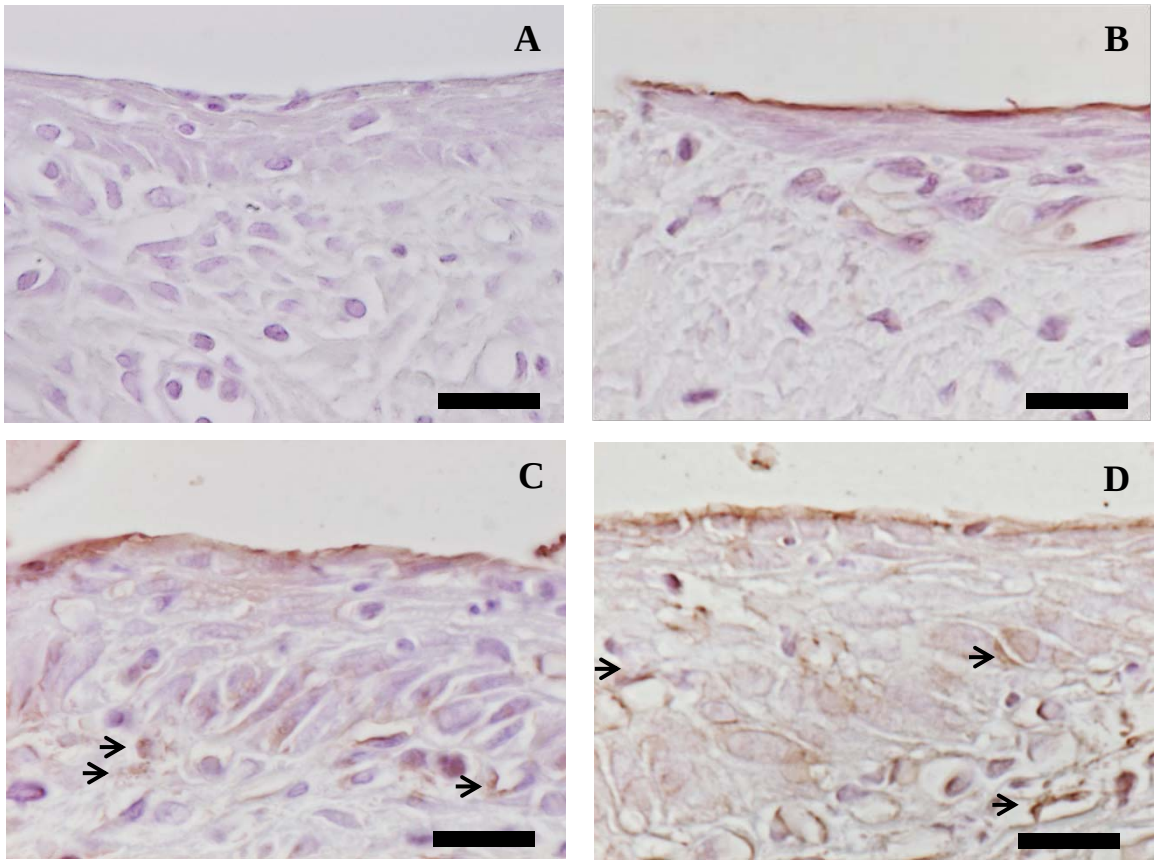



Figure5