**Notch signaling response to heavy compression force induces orthodontic root resorption via RANKL and IL-6 from cementoblasts**

Takehiro Iwane,[1] Jun Kikuta, [1]

[1] Department of orthodontics, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba, Japan

Corresponding author: Takehiro Iwane

Department of Orthodontics, Nihon University School of Dentistry at Matsudo

2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan

Tel & Fax: +81-47-360-9414

E-mail: mata16005@g.nihon-u.ac.jp

**Abstract**: One of the serious accidents in orthodontic treatment is orthodontic root resorption (ORR). As recently clarified, Notch signaling was involved in ORR deterioration via the receptor activator of NF-Kappa B ligand (RANKL) and interleukin (IL) -6 expression in human periodontal ligament (hPDL) cells. Human cementoblasts may also participate in ORR occurrence. In this study, we focused on Notch signaling in human cementoblast and examined the expression of Notch2, Jagged1, RANKL and IL-6 when optimal compression force (CF) and heavy CF were applied.

In vivo, an experimental tooth movement model was used to examine the expression levels of Jagged1, Notch2, RANKL, and IL-6 after 7 days.

In vitro study, we examined the effects of Jagged1, Notch2, RANKL, and IL-6 from human cementoblast-like cells (HCEM) . In addition we examined the effects of CF with/without GSI (an inhibitor of Notch signaling) on RANKL and IL-6 production from HCEM.

On day 7, tartrate-resistant acid phosphatase (TRAP), Jagged1, Notch2, RANKL, and IL-6 positive cells were detected in root surface exposed to heavy CF. Meanwhile, the expression levels of Jagged1, Notch2, RANKL, and IL-6 were found to significantly increase by the heavy force than that by the optimal force in vitro study. In addition, the addition of GSI to suppress Notch signaling reduced RANKL and IL-6 expression levels. Therefore, when excessive heavy CF is applied to HCEM, Notch signal may cause ORR through RANKL and IL-6.

## Introduction

Orthodontic root resorption (ORR) occurs in tooth movements during orthodontic treatment, although the degree varies depending on the patient. Many ORRs recorded in orthodontic clinics are not severe and are localized in the root surface and apex. Eventually, the repair mechanism of the absorption site occurs by cementogenesis, without any clinical problem. However, in orthodontic practice, cases of tooth roots that are absorbed extensively and cause tooth sway, which greatly affects tooth function and safety, are rarely reported.

Kaley et al.[1)] showed that ORR occurred in most patients who received orthodontic treatment, of which 3% had severe ORR (absorption of more than a quarter of the root length) in the maxillary central incisors. Many factors are involved in the process of ORR.

Risk factors in orthodontic treatment include prolonged treatment and abnormal root morphology [2)], strong orthodontic power [3)] and tooth movement [4)]. Some of the patient's risk factors include genetic factors [5)], history of dental trauma [6)], and allergies [7)], but the cause is still unclear.

In the study of Yamaguchi et al. [8)], receptor activator of NF-Kappa B ligand (RANKL) was received when compression force (CF) was added to human periodontal ligament (hPDL) cells derived from patients with severe ORR. RANKL, as well as inflammatory cytokine expression in the periodontal ligament, is reportedly involved in the development of ORR. In addition, Kikuta et al. [9)] induced RANKL and interleukin (IL) -6 through Notch signaling of Jagged1 and

Notch2 in hPDL cells with strong orthodontic force, suggesting the possibility of worsened ORR by promoting odontogenic differentiation.

Therefore, the relationship between Notch signal and RANKL is deeply related to ORR in tooth movement in orthodontic treatment. Cementum is the tissue that covers the surface of the tooth root and is classified into cellular cementum and acellular cementum. Cementoblast have a round or polygonal cell morphology and are adjacent to the cementum. These cells have tubules and communicate with adjacent cement and bone cells [10,11]. In addition, cementoblasts are similar to bone cells in matrix proteins such as type I collagen, osteopontin, and osteocalcin [12]. Considering the biological similarity between cementum and bone, the same situation can occur in the cementoblasts on the compression side. Huynh et al. [13] demonstrated that cementoblasts have the capacity to induce osteoclastogenesis. Furthermore, Yao et al. [14] suggests that inflammatory cytokines may impair the cementum remodeling under mechanical stimulation. However, the effect of Notch signal in the cementoblasts on the compression side of tooth during orthodontic treatment has not been elucidated.

In this study, we focused on Notch signals of Jagged1 and Notch2 in cementoblasts. Jagged1, Notch2, RANKL, and IL-6 were immunohistochemically stained using a rat experimental tooth movement model in vivo. We also examined the gene expression levels of Jagged1, Notch2, RANKL, and IL-6 when CF was added using cultured human cementoblast-like cell (HCEM) line in vitro.

## Materials and Methods

### A. In vivo studies

#### 1) Experimental animals

The animal experiments conformed to the guideline by the ethics committee of Matsudo School of Dentistry at Nihon University (Approval No. AP17MD 007-2).

Wistar male rats (Sankyo Labo Service, Tokyo, Japan) were carried at 5 weeks of age, and at 6-weeks- old Wistar male rats (n=18, body weight, 120.1 ± 10.5 g), they were used for 1 week of preliminary breeding. The rats were randomly assigned to the following three groups: control group, where no force was applied (n = 6); 10 g group, where an optimum force of 10 g was applied (n = 6); and 50 g group, where a heavy force of 50 g was applied (n = 6).

#### 2) Experimental tooth movement model

The rats were intraperitoneally injected with three anesthetics agents (medetomidine hydrochloride, 0.375 mg/kg; mitazolam, 2 mg/kg; butorphanol tartrate, 2.5 mg/kg). The experimental tooth movement model was created as per the method of Asano et al. [15] After deep anesthesia, mesial movement of the maxillary right first molar was achieved with a closed coil spring ligated to the maxillary first molar using a 0.008-inch stainless steel

ligation (Tommy International Inc., Tokyo, Japan) wire (wire size: 0.005 inch, diameter: 1/12 inch; Accurate Sales Co, Tokyo, Japan). Forces of 10 and 50 g were applied in the 10 and 50 g groups for 7 days. The other side of the coil spring was ligated using the same ligation wire to a hole drilled with a 1/4 round bur in the maxillary incisor, just above the gingival papilla. The molar was moved mesially with 10 and 50 g forces for 7 days (Fig 1).

**3) Tissue preparation**

The rats applied with orthodontic force underwent deep anesthesia by using the abovementioned three types of mixed anesthesia. Moreover, perfusion fixation was performed with saline and 10% neutral buffered formalin. Immediately after the maxilla was dissected and immersed in the same fixative for 18 h at 4°C, the sample was decalcified with a 10% disodium ethylenediaminetetraacetic acid (pH 7.4) solution for 4 weeks at room temperature. Then, we washed the decalcified sample and generated a paraffin-embedded block. Each sample was continuously sliced horizontally at 4 μm thickness and prepared for hematoxylin and eosin (H.E.) and immunohistochemical staining procedures.

**4) Immunohistochemical staining**

We deparaffinized each tissue section and quenched the endogenous peroxidase activity via incubation in 0.5% $H_2O_2$ in methanol for 30 min at room temperature. After being

washed in Tris- buffered saline (TBS), the sections were incubated with rabbit polyclonal anti tartrate-resistant acid phosphatase (TRAP), rabbit polyclonal anti Jagged1, rabbit polyclonal anti Notch2, goat polyclonal anti RANKL, and goat polyclonal anti IL-6 (individual working dilution, 1:100; Santa Cruz Biotechnology, Inc., CA, USA) antibodies for 18 h at 4°C. Then, TRAP, Jagged1, Notch2, RANKL, and IL-6 were stained using the Histofine Simple Stain MAX-Po (G) and (R) kit (Nichirei Co., Tokyo, Japan) according to the manufacturer‘s protocol. Each section was washed with TBS, stained with 3,3′-diaminobenzidine tetrabasic acid (Takara Co., Shiga, Japan), counterstained with Mayer's hematoxylin solution, and dehydrated in alcohol xylene series. Then, they were sealed with marineor. The observation site was referred to the method of Kikuta et al. [9] (Fig 2). Furthermore, the periodontal tissue around the compression side of the efferent distal buccal root of the maxillary right first molar was observed with a light microscope at 200 ×, the brown-stained cells were considered positive.

**B. In vitro studies**

**1) Culture of HCEM**

HCEMs were acquired from Professor Takata Takashi (Hiroshima University).

HCEMs were cultured according to the method described by Kitagawa et al. [16] The medium used was alpha minimum essential medium (α-MEM; Wako, Osaka, Japan) supplemented

with 100 μg/ml of penicillin G (Sigma Chemical Co., St. Louis ), 0.3 μg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA), 10% fetal calf serum (Cell Culture Laboratories, OH, USA), and 50 μg/ml of gentamicin sulfate (Sigma Chemical Co., St Louis). The culture solution was then stored at 37°C in a humidified incubator (Forma $CO_2$ incubator MIP-3326; Sanyo Electric Medical System Co., Tokyo, Japan) under 95% air and 5% $CO_2$ conditions.

**2) CF application of compression force**

We reproduced continuous CF at orthodontic tooth movements in HCEM, according to the method described by Kikuta et al.[9] (Fig 3).

At first, HCEMs were seeded in 100-mm cell culture dishes with 10% FCS. After overnight incubation, the medium of approximately confluent cells was changed to 1% FCS and cover glass plate was placed on top. Furthermore, we prepared a compression model by placing a weight of 1.0 g /cm² as the optimum CF and 4.0 g/cm² as the heavy CF harvest at 0, 1, 3, 6, 9, 12 and 24 h. Cells in the control group were treated with a thin cover glass plate without weight, producing a CF of 0.032 g/cm².

**3) Notch inhibitor addition**

GSI (R&D Systems Co., MN, USA) was added to the medium at 5.0 μM concentration to inhibit Notch before CF application according to the method reported by Osathanon et al. [17]

and Fukushima et al. (Fig .3).

**4) Real-time polymerase chain reaction (PCR)**

We extracted the total RNA from HCEMs by using the RNeasy Mini Kit (Qiagen Co., Tokyo, Japan) and performed real-time PCR on aliquots containing equal mRNA amounts. The Prime Script RT Reagent Kit (Takara Co., Shiga, Japan) was used to reverse- transcribe the mRNA into cDNA according to the manufacturer's protocol. Real-time PCR was amplified using the SYBR Premix Ex Taq (Takara Co., Shiga, Japan) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara Co., Shiga, Japan) PCR primers for Jagged1, Notch2, RANKL, IL-6, and GAPDH were purchased from Takara Co. and designed with reference to the respective cDNA sequences (Table 1).

**5) Statistical processing**

Each value represents the mean ± standard deviation of each group. Group means were compared using Mann-Whitney U test. P values of $<0.05$ and $<0.01$ were considered statistically significant.

## Results

### A. In vivo studies

**1) Changes in body weight of experimental animals**

No significant difference was observed in the body weight changes in the experimental animals during the experiment. (data not shown).

**2) Change of periodontal tissue in H.E. staining of experimental tooth movement model**

The PDL tissue of the control rats consisted of root cementum and fibroblasts. There were no resorption pits on the root surface, it was almost smooth and no osteoclasts were found around it. (Fig 4a). In the 10 g group after 7 days, the root cementum almost did not change, but many resorption pits were observed on the alveolar bone surface (Fig 4b). In the 50 g group after 7 days, coarse arrangement of fibers with dilated vessels was observed, and many root resorption pits with multinucleated osteoclasts were identified on the root surface (Fig 4c).

**3) Findings for TRAP**

After 7 days, TRAP-positive multinucleated cells were not found on the root surface and alveolar bone of the control group (Fig 4d). Conversely, in the 10 and 50g groups such cells were found in the bone resorption pit on the alveolar bone surface (Fig 4e) and in the root resorption pit, respectively (Fig 4f).

**4) Findings for Jagged1, Notch2, RANKL, and IL-6**

Immunoreactivity for Jagged1, Notch2, RANKL, IL-6 was examined on day 7 after tooth movement. In the control group, bone resorption and root resorption were not observed, and Jagged1, Notch2, RANKL, IL-6 positive cells were diffusely observed in the PDL tissue (Fig 5g, j, m, p). Conversely, in the 10 g groups, Jagged1, Notch2, RANKL and IL-6 positive cells were observed around osteoclasts in bone resorption pits on the alveolar bone surface (Fig 5h, k, n, q). In the 50 g group, Jagged1, Notch2, RANKL, and IL-6 positive cells were observed around odontoclasts in the root resorption pit (Fig 5i, l, o, r).

**B. In vitro studies**

**1) Changes in the gene expression of Jagged1, Notch2, RANKL and IL-6 by CF in HCEM cells**

The gene expression of Jagged1 had a higher tendency in the CF group (1.0 and 4.0g/cm$^2$) than in the control group. Furthermore, in the CF group, it increased in the 4.0 g/cm$^2$ group compared with that in the 1.0 g/cm$^2$ group, showing its peak at 6 h after the compression began. The gene expression of Notch2 also increased in the CF 4.0 g/cm$^2$ group, showing its peak at 9 h after the start of compression. Similarly, RANKL and IL-6 gene expression increased in the CF 4.0 g/cm$^2$ group, showing its peak at 12 h after the start of compression (Fig 6).

**2) Changes in RANKL and, IL-6 gene expression addition of Notch inhibitor in HCEM cells**

For inhibition experiments, the Notch inhibitor GSI was added to HCEM cells for 24 h. At that time, the gene expression of RANKL decreased by approximately 58% at 12 h in the CF 4.0 g/cm$^2$ group with GSI addition compared with that in the group without GSI addition. The gene expression of IL-6 also decreased by approximately 42% at 12 h in the CF 4.0 g/cm$^2$ group with GSI addition compared with that in the group without GSI addition (Fig 7).

## Discussion

In this study, we investigated the effect of Notch signal of Jagged1and Notch2 by two different orthodontic forces, namely, optimal force and heavy force on cementoblasts and examined whether such effect is involved in ORR during orthodontic treatment.

Huynh et al. [13] demonstrated that cementoblasts can induce osteoclastogenesis in relation to ORR and cementum. Furthermore, Liao et al. [18] stated that cementoblasts differentiate from periodontal progenitor cells and share many common features with PDL and osteoblasts. In addition, Kikuta et al. [9] induced RANKL and IL-6 through Notch signaling of Jagged1 and Notch2 in hPDL cells with strong orthodontic force, suggesting possible worsening of ORR by promoting odontogenic differentiation. Therefore, we investigated how Notch signal affects

ORR in cementoblasts.

In vivo, an experimental tooth movement model using rats was used to investigate the periodontal tissue response when two different orthodontic forces (10 g and 50 g) were applied to rats for 7 days. In the 10 g group after 7 days, the root cementum slightly changed, but many resorption pits were observed on the alveolar bone surface. In the 50 g group after 7 days, ORR occurred, reaching the cementum and dentin, additionally, immunoreactivity of Jagged1 and Notch2 was detected in the PDL tissue, and osteoclasts were observed. In addition, RANKL and IL-6 immunoreactivity was detected in the root resorption pits. Duan et al. [19] suggested that Notch signaling may be involved in pathological bone remodeling. Therefore, ORR may be involved with PDL, as well as cementum. Thereafter, to elucidate the mechanism of ORR occurrence of in the cementum, we utilized HCEM-like cells with two different CF (CF 1.0 and 4.0 g/cm$^2$) in vitro, and examined the gene expression levels of Jagged1, Notch2, RANKL, and IL-6. Results showed that the peak of Jagged1 was at 6 h, which increased in the CF 4.0 g/cm$^2$ group compared with that in the control group and CF 1.0 g/cm$^2$ group. Meanwhile, the peak of Notch2 was at 9 h, which increased in the CF 4.0 g /cm$^2$ group. Furthermore, the peaks of RANKL and IL-6 were at 12 h, which increased significantly in the CF 4.0 g/cm$^2$ group. In addition, in the inhibition experiment using GSI (Notch inhibitor), RANKL and IL-6 decreased in the CF 4.0 g/cm$^2$ group with GSI addition than in the CF 4.0 g/cm$^2$ group without GSI addition. At 12 h, RANKL decreased by approximately 58%, and IL-6 decreased by

approximately 42%.

These results, suggest that Notch signal may promote production of RANKL and IL-6 and cause ORR by applying excessive CF to cementoblasts.

For future research, it is necessary to investigate the relationship between Notch signal and ORR using Notch knockout mouse. Sethi et al. [20] reported that Jagged1 advances tumor growth by stimulating IL-6 release from osteoblasts and directly activating osteoclast differentiation. Furthermore, Nakao et et al. [21] reported that Jagged1 advances RANKL-induced osteoclastogenesis, whereas Fukushima et al. [22] reported that RANKL induces the production of Jagged1 and Notch2 in bone marrow macrophages during osteoclastogenesis. Taken together, the increased Jagged1 expression observed in HCEM cells treated with excessive CF activates osteo/odontoclastogenesis.

However, there are studies in recent years that contradict this study. For example, Li et al. [23] reported that Notch signaling enhances the osteogenic differentiation of periodontal ligament stem cells in osteoporotic rats, and Ugarte et al. [24] documented that Notch signaling enhances osteogenic differentiation in primary human bone marrow stromal cells. The result of Notch signal changes may be related to the difference in cell type and the stimulus applied to the cell.

In conclusion, applying excessive CF to HCEM may activate Notch signaling, promote production of RANKL and IL-6, and stimulate the process of ORR. Therefore, There are several pathways related to ORR, but the Notch signal in cementum plays an important role in the

process of ORR. We suggested that Notch signaling may be important for the development of ORR in cementum although the involvement of other pathways cannot be ruled out.

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**Figure Legends**

Fig 1. Experimental tooth movement model

After deep anesthesia, mesial movement of the maxillary right first molar was achieved with a closed coil spring ligated to the maxillary first molar using a 0.008-inch stainless steel wire. Forces of 10 g and 50 g were applied for 7 days.

Fig 2. Observation site

The mesial part of the region formed by the line connecting the center of the mesial root (MR) and the center of the distal buccal root (DBR) and the perpendicular line of the maxillary right first molar A quarter of periodontal tissue was observed. The positive cells of each staining and root resorption were examined in the mesial plane of the DBR root on the compression side in the region located in the 300 μm apical direction from the bifurcation.

Fig 3. Applications of compression force (CF)

HCEM were continuously compressed using different forces (1.0 or 4.0 g/cm$^2$). Briefly, cells were grown in wells to confluency, then covered with cover glass. Subsequently, lead weights were placed on the confluent cell layer in each well. The number of weights determined the amount of CF. The effects of CF on Jagged1, Notch2, RANKL and IL-6 expression were investigated. In addition, we examined the gene expression of RANKL, IL-6 when GSI (Notch

inhibitor) was added.

Fig 4. Histopathological staining (H.E staining) and, TRAP immunohistochemical staining results

In the 10 g group after 7 days, the root cementum slightly changed, but many resorption pits were found on the alveolar bone surface (b) . In the 50 g group, root resorption was observed, the root resorption fossa extended to cementum and dentin, and multinucleated cells were observed in the resorption fossa (c) . TRAP immunoreactivity was found in the bone resorption fossa on the alveolar bone surface in the 10 g group and in the root resorption fossa in the 50 g group (e, f) .

Fig 5. Immunohistochemical staining results for Jagged1, Notch2, RANKL, and IL-6

In the control group, bone resorption and root resorption were not observed, and Jagged1, Notch2, RANKL, IL-6 positive cells were diffusely observed in the PDL tissue (Fig 5g, j, m, p). In the 10 groups, Jagged1, Notch2, RANKL and IL-6 positive cells were observed around osteoclasts in bone resorption pits on the alveolar bone surface (Fig 5h, k, n, q). In the 50 g group, Jagged1, Notch2, RANKL, and IL-6 positive cells were observed around odontoclasts in the root resorption pit (Fig 5i, l, o, r).

Fig 6. Changes in the gene expression of Jagged1, Notch2, RANKL, and IL-6 by CF in HCEM

cells

The gene expression of Jagged1 had a higher tendency in the CF group (1.0 and 4.0 g/cm² groups) than in the control group. Furthermore, expression increased in the CF 4.0 g/cm² group compared to with that in the CF 1.0 g/cm² group, showing its peak at 6 h after the start of compression. The gene expression of Notch2 also increased in the CF 4.0 g/cm² group, showing its peak at 9 h after the start of compression. Similarly, RANKL and IL-6 gene expression, increased in the CF 4.0 g/ cm² group, showing its peak at 12 h after the start of compression. **P <0.01 and *P <0.05 indicate significant difference from the control.

Fig 7. Changes in RANKL and IL-6 gene expression with addition of Notch inhibitor in HCEM cells

For inhibition experiments, Notch inhibitor GSI was added to HCEM cells for 24 h.

At that time, RANKL gene expression decreased in the CF 4.0 g/cm$^2$ group with GSI addition compared with that in the group without GSI addition. The gene expression of IL-6 also decreased in the CF 4.0 g/cm$^2$ group with GSI addition compared with that in the group without GSI addition. **P <0.01 and *P <0.05 indicates significant difference from the control.

TABLE LEGEND

| | |
|---|---|
| Jagged1 | F:5′-CACCCTGCTGAGTCTGTTCTGG-3′<br>R:5′-CCAGGTCTTTGAGAACTCCAGATG-3′ |
| Notch2 | F:5′-CIGAATCCCACAAACCCTAGCA-3′<br>R:5′-CCTTGTCCCTGAGCAACCATC-3′ |
| RANKL | F:5′-TGGATGCCTTGAATAATAAGCAGGA-3′<br>R:5′-AATTTGCGGCACTTGTGGAA-3′ |
| IL-6 | F:5′-AAGCCAGAGCTGTGCAGATGACTTA-3′<br>R:5′-TGTCCTGCAGCCACTGGTTC-3′ |
| GAPDH | F:5′-GCACCGTCAAGGCTGAGAAC-3′<br>R:5′-TGGTGAAGACGCCAGTGGA-3′ |

Table 1.

Real-time PCR cDNA layout design

PCR primers for Jagged1, Notch2, RANKL, IL-6 and GAPDH were designed with reference to the respective cDNA sequences.

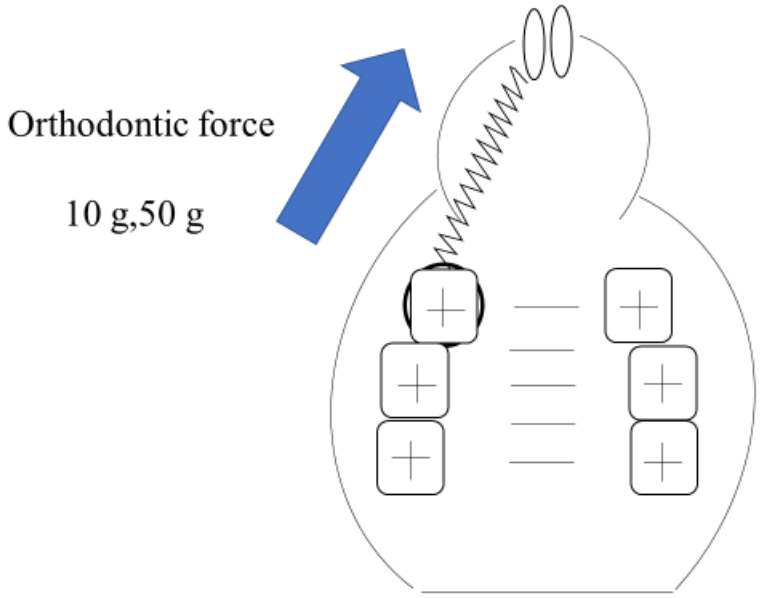
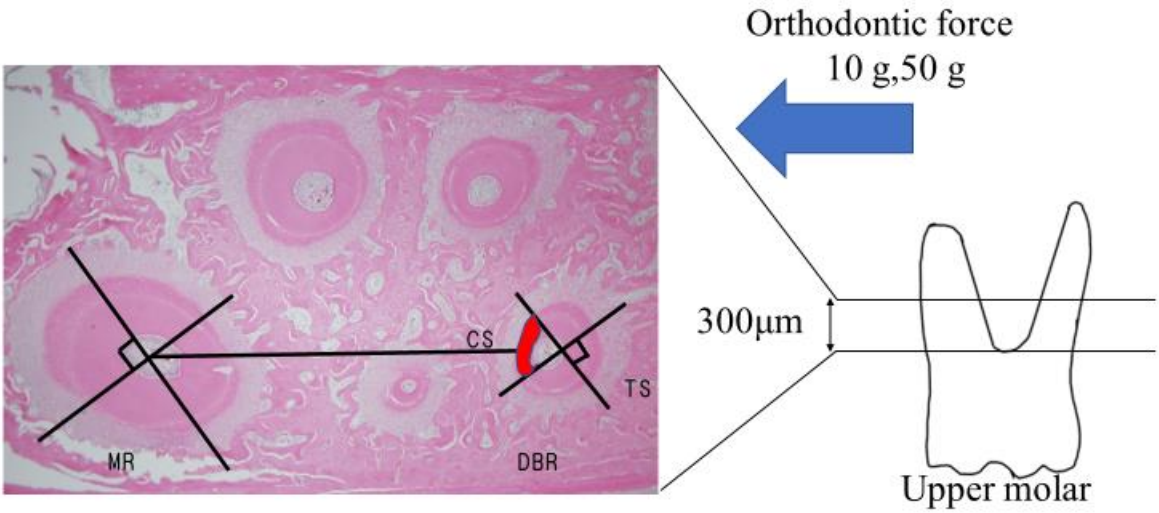



Fig 1.




MR : mesial root DBR : distal buccal root
CS : compression side TS : tension side

Fig 2.

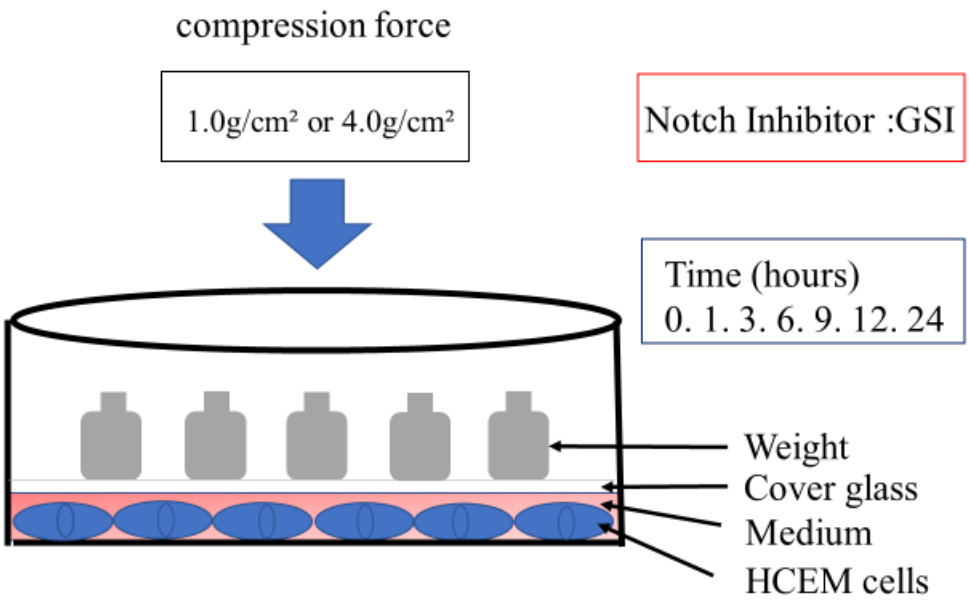
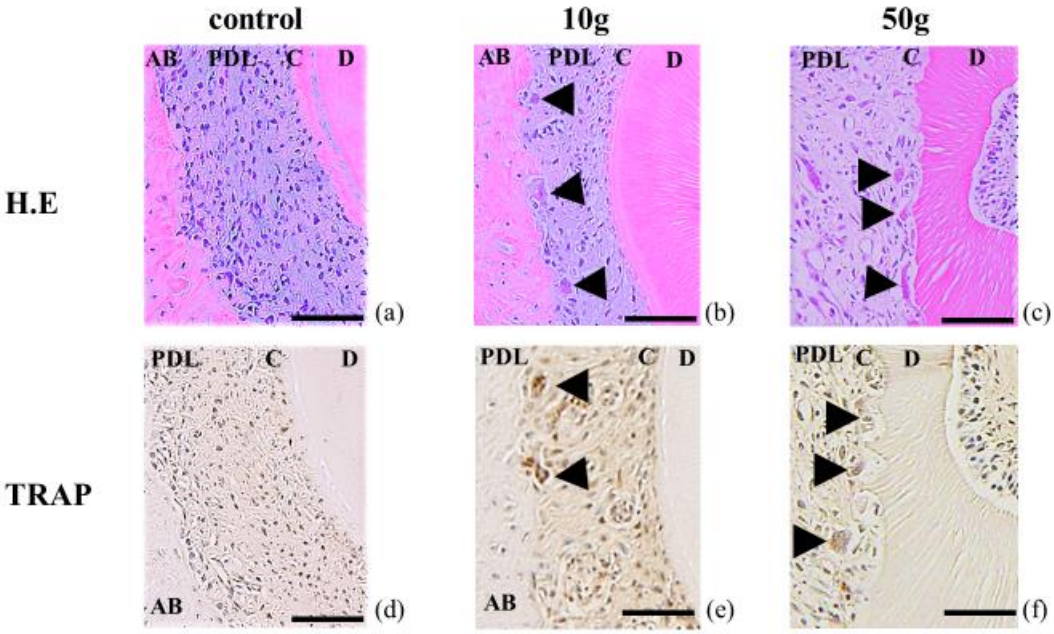

compression force
1.0g/cm² or 4.0g/cm²
Notch Inhibitor :GSI
Time (hours)
0. 1. 3. 6. 9. 12. 24
Weight
Cover glass
Medium
HCEM cells

Fig 3.




AB:Alveolar bone, D:Dentin, C: Cementum, PDL: Periodontal ligament
▶ : positive cell Bar:50μm

Fig 4.

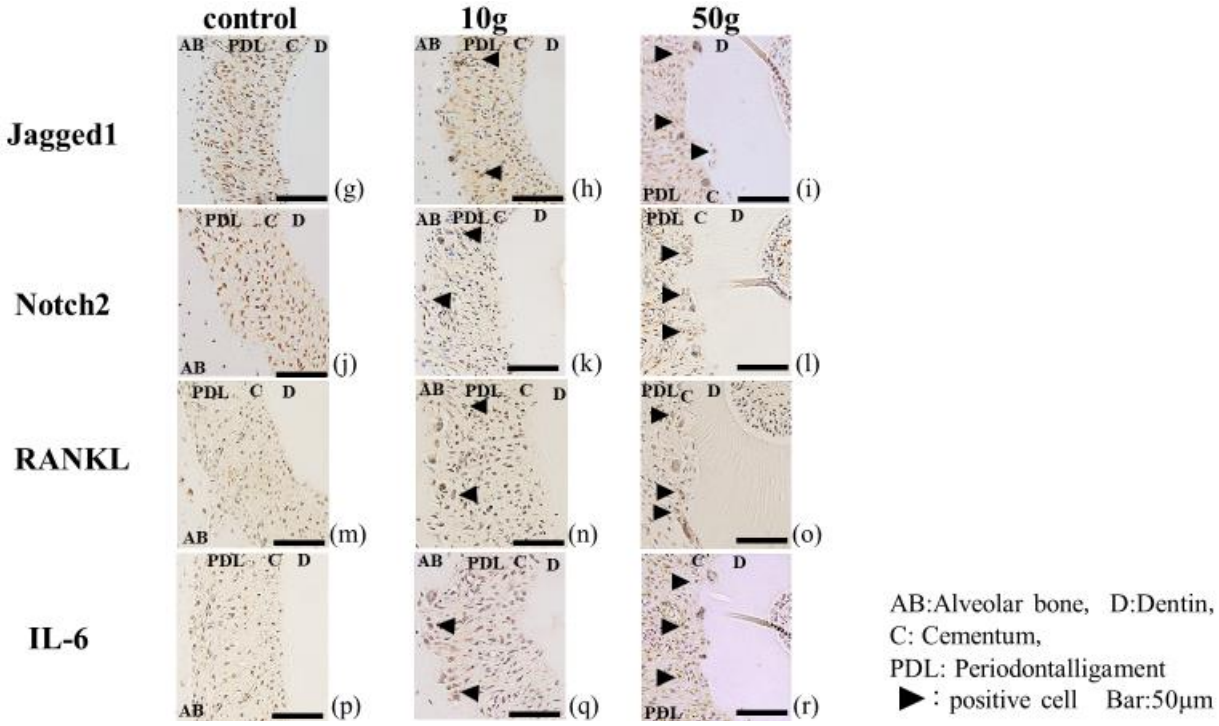

control
10g
50g
Jagged1
Notch2
RANKL
IL-6
AB PDL C D
PDL C D
AB
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)
AB:Alveolar bone, D:Dentin,
C: Cementum,
PDL: Periodontalligament
▶: positive cell Bar:50μm

Fig 5.

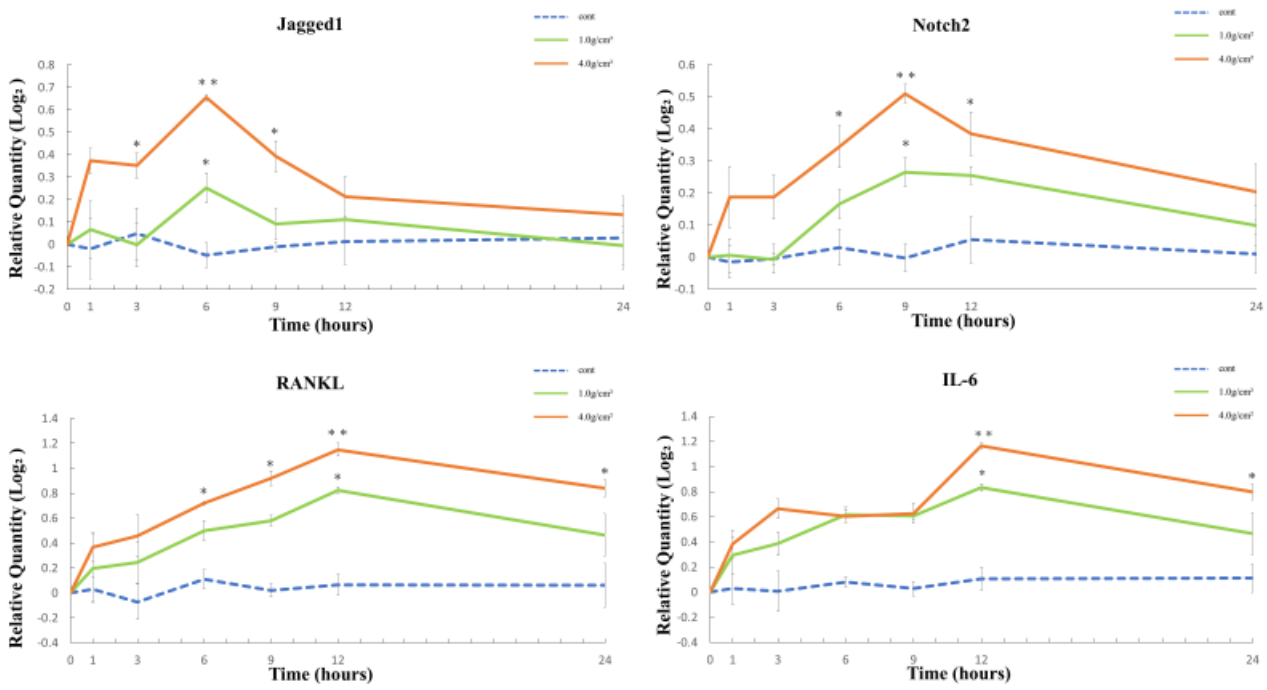

Jagged1
Notch2
RANKL
IL-6
Relative Quantity (Log2 )
Time (hours)
cont
1.0g/cm²
4.0g/cm²

Fig 6.

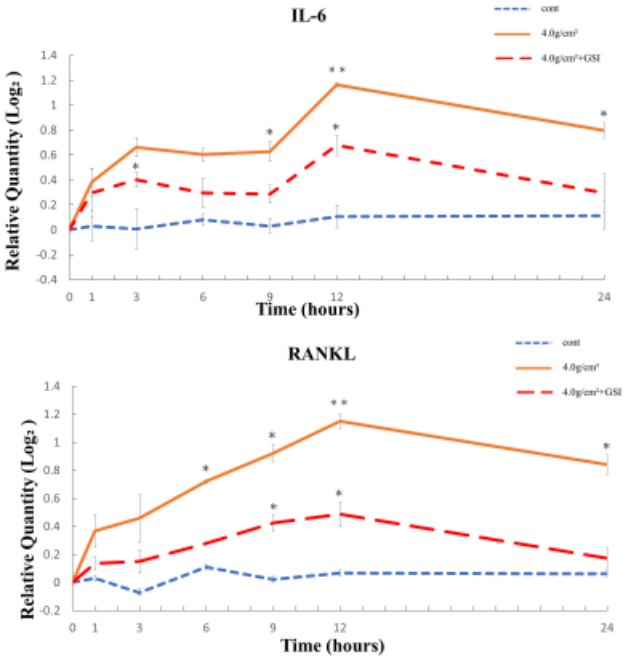

IL-6
cont
4.0g/cm²
4.0g/cm²+GSI
Relative Quantity (Log₂)
Time (hours)
RANKL
cont
4.0g/cm²
4.0g/cm²+GSI
Relative Quantity (Log₂)
Time (hours)

Fig 7.