Morphological Resolution of Periodontal Mechanoreceptors in Mouse Maxillary Incisors Following Appliance-induced Crossbite

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Abstract

This study aimed to investigate whether the morphology of Ruffini endings (RE) in mouse maxillary incisors' periodontal ligament (PDL) recover completely following experimentally-induced crossbite. Appliances causing an anterior crossbite were placed in 24 three-week old mice for four weeks; an identical number of mice were used as controls. Equal numbers of control and experimental animals were anesthetized and perfused with 4 per cent paraformaldehyde at seven (week 0), eight (week 1), nine (week 2), and 11 (week 4) weeks of age. Frozen sagittal cryostat sections of the decalcified maxillary incisors were prepared and double-stained by immuno-histochemistry for protein gene product 9.5 to reveal the neural elements, followed by histochemistry for tartrate-resistant acid phosphatase activity to show bone resorption sites.

The results showed that the resorption sites seen in the experimental groups at week 0 differed from those in the untreated controls. By week 1, fewer RE with vague outlines and unusually long microprojections were found in the experimental group's PDL, and the numbers continued to decline throughout the remainder of the experiment. A reduction in the number of club-shaped endings in the middle third of the lingual PDL was also observed. By week 4, the RE had recovered to control levels. It was concluded that a complete resolution of RE in mouse maxillary incisor PDL occurs following removal of the appliance-induced crossbite.

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Introduction

Anterior crossbite affects speech and chewing patterns,[1] and has been associated with abnormal activity of the perioral muscles,[2] muscle pain,[3] and a significantly deeper periodontal pocket.[4,5] Furthermore, mandibles in untreated Class III patients with anterior crossbites may become more protrusive with time causing a more severe skeletal discrepancy to develop.[1,6]

Many oral reflexes are induced by periodontal mechanoreceptors, which morphologically are Ruffini endings (RE).[7,8] They have been observed in the periodontal ligament (PDL) of several mammals, and in mouse PDL[9] their presence has been confirmed with immunohistochemistry for protein gene product 9.5 (PGP 9.5).[10]

Histochemistry for tartrate-resistant acid phosphatase (TRAP) activity using the azo dye technique[11] reveals resorptive sites in the labial and lingual alveolar bone (AB) of mice. Lateral skull radiographs of mice have shown that the masticatory force from mandibular incisors causes a counterclockwise rotation of maxillary incisors, and the axis of rotation is located within the lingual AB.[12]

A physiological study has shown that the responses of motor units in the contralateral temporal muscle to the direction of mechanical stimulation applied to a tooth differ between subjects with normal occlusion and subjects with a crossbite.[13] After orthodontic correction of an anterior crossbite jaw muscle activity improves.[14] We have reported abnormal RE in the PDL of mouse maxillary incisors after appliance-induced anterior crossbite.[12] Short-term crossbites resulted in swollen and vague contoured nerve terminations with unusually long and

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pedunculated microprojections. The endings were club-shaped with a smooth outline. However, the morphology of the periodontal mechanoreceptors following removal of an appliance causing a crossbite has not been reported.

The aims of the present study were to determine changes in the morphology of the periodontal mechanoreceptors after an appliance-induced crossbite has been relieved. Changes in morphology were observed by staining for PGP 9.5 and TRAP activity.

Materials and methods

This study was approved by Ethical Committee for Animal Experimentation, Naresuan University. Forty-eight three week-old male and female C3H/HeSlc mice were used in this study, and handled according to the World Health Organization guidelines.[15,16] The method of attaching an anterior guiding plane to mouse mandibular incisors has been described elsewhere.[12] The appliances were removed after four weeks, and the mandibular incisors were cleaned. Feeding, drinking, grooming behavior, and body weight of the mice were monitored throughout the experiment. Equal numbers of unoperated control and experimental mice were killed immediately after removal of the appliances, after one, two and four weeks later. There were six control and six experimental mice at each stage of the experiment. The mice were anesthetized with diethyl ether, and perfused through their left ventricles with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The maxillae were removed *en masse* and stored in the same fixative solution at 4ºC for an additional 14 hours. Following decalcification in 10% EDTA-2Na solution, pH 7.4 at 4ºC for 3 weeks, the specimens were saturated in 30% sucrose solution overnight at 4°C, and processed for cryostat sections (Leica CM3000, Nussloch, Germany). Serial sagittal 20 μm sections were cut, mounted on poly-L-lysine-coated glass slides (Matsunami, Osaka, Japan) and processed for indirect immunostaining using the avidin-biotin-complex (ABC) method.

Immunohistochemistry

Rabbit polyclonal anti-human PGP 9.5 antibody (Ultraclone, Cambridge, UK) was used as the primary antibody to identify the nerve elements. After inactivation of endogenous peroxidase with 0.3% hydrogen peroxide in absolute methanol for 30 minutes, non-specific immunoreactivity was blocked with 2% normal goat serum (Vector, Burlingame, Calif) in 0.01 M phosphate-buffered saline (PBS), pH 7.4. The sections were incubated overnight at 37ºC with the primary antibody diluted 1:10,000 with 2% normal goat serum, followed by two consecutive incubations with a biotinylated goat anti-rabbit immunoglobulin G (Chemicon, Calif) and ABC, according to the manufacturers' instructions (Vector). At each step, the sections were washed several times in 0.01 M PBS containing 0.3% Triton X-100 (Sigma Chemical, St. Louis, Mo).

Histochemistry

After treatment by the ABC method, histochemistry for TRAP activity was performed using the azo dye technique.[9] Briefly, the staining solution consisted of naphthol 3-hydroxy-2-naphtho-2, 4-xylidide (AS-MX) phosphate sodium salt (Sigma Chemical, USA) as the substrate, fast red violet 5-chloro-4-benzamido-2-methylbenzene diazonium chloride, hemi (zinc chloride) (LB) salt (Sigma) as the diazonium salt, and 50 mM l (+)-tartric acid (Wako Pure Chemical, Tokyo, Japan) as the inhibitor of acid phosphatase for TRAP activity. Incubation was conducted at 37ºC for 12 minutes and at pH 5.3 with an acetate buffer.

For the final visualization of immunoreactive sites, the sections that had already been incubated for TRAP activity were treated with 0.02% 3,3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6. The sections were then counterstained with 1% methyl green, dehydrated in a graded ethanol, rinsed in xylene, and finally mounted with Entellan new (E. Merck, Darmstadt, Germany).

Specificity controls

To check the specificity of the immunoreactions, the primary antibody was replaced with non-immune rabbit serum or treatment with anti-rabbit immunoglobulin G or ABC complex was omitted.

Analyses

Examination and photography of the sections were performed by using a conventional light microscope. A pixel image analysis was carried out to quantify the histological changes in RE in the lingual PDL and the following parameters were measured:

1. The surface area of the lingual PDL of maxillary incisors (Table 1).

2. The surface area occupied by normal RE as a percentage of the total lingual PDL surface of maxillary incisors (Table 2).
3. The surface area occupied by abnormal club-shaped RE as a percentage of the total lingual PDL surface of maxillary incisors (Table 3).
4. The surface area occupied by abnormal RE with swollen and hazy contours as a percentage of the total lingual PDL surface of maxillary incisors (Table 4).

Area observed	Weeks after appliance removal			
	Week 0	Week 1	Week 2	Week 4
Marginal third				
Experimental group	5,732.33[a] ± 183.98	6,322.00 ± 185.22	6,672.00 ± 171.66	6,746.83 ± 207.53
Control	6,956.33[a] ± 163.83	6,326.67 ± 177.21	6,658.83 ± 174.22	6,788.50 ± 184.07
Middle third				
Experimental group	6,867.33[b] ± 98.44	5,911.50 ± 87.50	5,607.17 ± 134.11	6,160.17 ± 112.23
Control	6,011.67[b] ± 126.15	5,891.67 ± 73.75	5,600.83 ± 186.05	6,113.17 ± 156.82
Basal third				
Experimental group	5,970.83[c] ± 54.63	5,808.67 ± 85.07	5,612.50 ± 68.68	5,856.83 ± 101.38
Control	5,418.83[c] ± 105.96	5,823.80 ± 46.74	5,624.83 ± 61.31	5,847.00 ± 76.41

Table 1. Surface area (pixels) of mouse maxillary incisor lingual periodontal ligament. (All values are expressed in mean ± standard deviation.)
Identical uppercase letters indicate significant intracolumn differences between the experimental groups and their controls in the respective areas using analysis of variance and post hoc comparison by Newman-Keuls test at $p < 0.05$.

For the surface analysis, five sections were randomly selected from each control and experimental animal. There were 6 control and 6 experimental animals at each stage. Photographic slides of the sections were converted to digital images at a resolution of 1,200 pixels per inch at a scan pitch of 2 pixels. The surface within the described areas was measured using an image analysis software (NIH Image Version 1.55, Bethesda. Mass) and the data were expressed in terms of the number of pixels. Since nerve endings had been immunohistochemically stained dark brown, the image was first converted to cyano-magenta-yellow, black for color separation to leave only stained nerves. The areas occupied by the apparently abnormal nerve elements described in parameters 3 and 4 above were analyzed. The total PDL surface area was obtained by selecting 16 color gradients within the white to light yellow range and subsequent erasure of remaining unwanted non-PDL tissues. The percentage of the surface area occupied by club-shaped RE or those with swollen and hazy contours was then calculated as: 100 x number of pixels occupied by the respective nerve endings/number of pixels occupied by the lingual PDL.

Area observed	Weeks after appliance removal			
	Week 0	Week 1	Week 2	Week 4
Marginal third				
Experimental group	0.64[a] ± 0.02	0.58[a] ± 0.02	0.79 ± 0.01	0.93 ± 0.02
Control	0.90[a] ± 0.03	0.95[a] ± 0.01	0.84 ± 0.02	0.90 ± 0.02
Middle third				
Experimental group	1.68[b] ± 0.02	1.84[b] ± 0.03	2.21 ± 0.01	2.61 ± 0.03
Control	2.52[b] ± 0.02	2.47[b] ± 0.02	2.69 ± 0.03	2.57 ± 0.02
Basal third				
Experimental group	0.96 ± 0.02	1.23 ± 0.02	1.21 ± 0.01	1.12 ± 0.02
Control	1.06 ± 0.02	1.17 ± 0.03	1.12 ± 0.02	1.06 ± 0.02

Table 2. Percentage of surface area occupied by normal Ruffini endings (pixels) in relation to total lingual periodontal ligament surface area (pixels). (All values are expressed in mean ± standard deviation)
Identical uppercase letters indicate significant intracolumn differences between the experimental groups and their controls in the respective areas using analysis of variance and post hoc comparison by Newman-Keuls test at $p < 0.05$.

Area observed	Weeks after appliance removal			
	Week 0	Week 1	Week 2	Week 4
Marginal third				
Experimental group	0.04 ± 0.02	0.01 ± 0.01	0.02 ± 0.01	0.06 ± 0.04
Control	UO	UO	0.03 ± 0.01	0.06 ± 0.03
Middle third				
Experimental group	0.67 ± 0.11	0.46 ± 0.07	0.28 ± 0.08	0.36 ± 0.07
Control	UO	UO	0.13 ± 0.04	0.37 ± 0.09
Basal third				
Experimental group	0.08 ± 0.03	0.04 ± 0.03	0.07 ± 0.04	0.06 ± 0.03
Control	UO	UO	0.06 ± 0.04	0.05 ± 0.03

Table 3. Percentage of surface area occupied by club-shaped Ruffini nerve endings (pixels) in relation to total lingual periodontal ligament surface area (pixels). (All values are expressed in mean ± standard deviation.)
Abbreviation: UO, unobservable.
No significant difference is observed between the experimental groups and their controls in the respective areas using analysis of variance and post hoc comparison by Newman-Keuls test at $p < 0.05$.

All measurements were performed blind by three observers, duplicated, and the overall

means used in all calculations. An analysis of variance (ANOVA) and post hoc comparisons using the Newman-Keuls (NK) test were carried out to examine all numerical data. A probability value of less than 5% ($p < 0.05$) was considered significant.

Area observed	Weeks after appliance removal			
	Week 0	Week 1	Week 2	Week 4
Marginal third				
Experimental group	$0.20^{a} \pm 0.04$	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
Control	$0.02^{a} \pm 0.01$	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02
Middle third				
Experimental group	$0.70^{b} \pm 0.06$	0.09 ± 0.02	0.12 ± 0.02	0.17 ± 0.05
Control	$0.11^{b} \pm 0.02$	0.10 ± 0.03	0.13 ± 0.06	0.15 ± 0.01
Basal third				
Experimental group	$0.63^{c} \pm 0.03$	0.12 ± 0.04	0.12 ± 0.02	0.12 ± 0.02
Control	$0.09^{c} \pm 0.04$	0.10 ± 0.01	0.10 ± 0.09	0.11 ± 0.03

Table 4. Percentage of surface area occupied by swollen and hazy contoured nerve endings (pixels) in relation to total lingual periodontal ligament surface area (pixels). (All values are expressed in mean ± standard deviation pixels.)

Identical uppercase letters indicate significant intracolumn differences between the experimental groups and their controls in the respective areas using analysis of variance and post hoc comparison by Newman-Keuls test at $p < 0.05$.

Results

Both experimental and control mice gained weight steadily after the appliances were removed. No remarkable changes in the animals' behavior were detected. ANOVA and post hoc comparisons indicated between week 0 and week 4, and week 1 and week 4, both control and experimental mice gained weight significantly (Table 5). The specificity controls for immunohistochemistry of PGP 9.5 revealed no immunolabelled elements. In addition, the immunoreactive sites showed no interference between the immunohistochemistry for PGP 9.5 and the subsequent histochemistry for TRAP activity, indicating that both neural elements and TRAP-positive areas were different between the controls and the experimental groups.

Incisor relationships

By week 0, the maxillary incisors in all experimental mice were in crossbite. The incisor crown lengths in the experimental mice were shorter than those in the control mice and the incisors were edge-to-edge by week 1. By the end of week 2, the incisor relationships in both experimental and control mice were similar. Throughout the experiment, no palatal inflammation could be seen by the naked eyes.

Histochemistry and immunohistochemistry - Control group

Histochemistry for TRAP activity with the azo dye technique disclosed a large number of osteoclasts on the labial AB surface adjacent to the root of the maxillary incisor, but none on the lingual AB surface (Figure 1a). New AB was deposited in the lingual PDL of the older control mice.

PGP 9.5-immunoreactive neural elements with expanded terminations, regarded as RE, displayed irregular outlines and numerous fine microprojections (Figure 2a). They were found throughout the lingual PDL, notably in the middle region (Table 2). Club-shaped RE with few, if any, microprojections (Figure 2b) were found in the control mice, aged 9 weeks and older. The number of club-shaped endings increased with age (Table 3).

Experimental group

At week 0, osteoclasts were restricted to the area between the middle and basal thirds of labial AB adjacent to the maxillary incisor, and in the marginal third of the lingual AB, suggesting active bone resorption (Fig. 1b). The lingual PDL space was narrow and numerous Howship's lacunae were observed (Table 1).

Immunohistochemistry for PGP 9.5 showed morphological changes in the periodontal nerve endings at week 0. Compared to those in the controls, the RE possessed differently shaped microprojections with longer stalks, and the outlines of some endings were hazy and swollen (Fig. 2c). These nerve terminals were distributed throughout the lingual PDL, but were more commonly seen in the middle and basal thirds of the PDL (Table 4). Club-shaped RE (Fig. 2b) were also found throughout the lingual PDL; with a higher frequency in the middle third of the PDL (Table 3).

By week 1, the distribution of osteoclasts was similar to that found in the control mice. Osteoclasts were found between the marginal and middle thirds of the labial AB lining the PDL, but not on the lingual side of the PDL (Fig. 1a). There were no statistically differences in the surface areas of the lingual PDL in the control and experimental mice (Table 1). RE with hazy contours and unusually long stalks were less

frequent than at week 0 (Table 4).

By weeks 2 and 4, the distribution patterns of osteoclasts in the lingual and labial PDL were similar to those found in the controls (Fig. 1a). No statistically significant group differences in the surface areas of the lingual PDL were found (Table 1). Two weeks post-removal of the appliance, new AB had been deposited in the lingual PDL. From week 2, RE with numerous microprojections and visible stalks, as well as those with swollen and hazy contours, were scarce (Table 4). By week 4, the RE displayed the same morphological features as those found in the control PDL. Quantitative analyses between the experimental and control groups revealed no significant differences (Tables 2, 3, and 4).

Discussion

This investigation demonstrates that an appliance-induced crossbite causes changes in the nerve endings. It also shows that the nerves return to normal after the appliance causing the crossbite is removed.

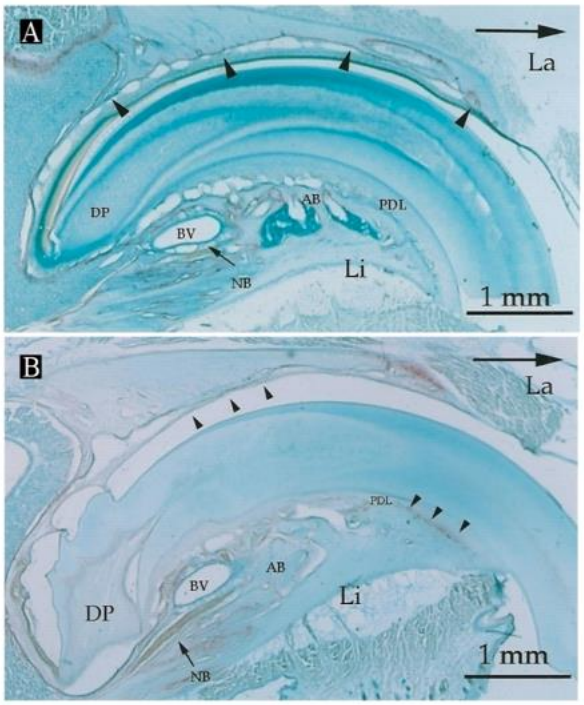

Figure 1. Histochemical reactions (red) for tartrate-resistant acid phosphatase activity using the azo dye technique in sagittally cut frozen sections of the mouse maxillary incisors' periodontal ligament (PDL). A, Control, week 1. Osteoclasts (arrowheads) extend from the marginal third to the middle third in labial alveolar bone (AB), while none were observed in lingual AB. B, Experimental, week 0. Osteoclasts (arrowheads) are located in the resorptive lacunae in the marginal third of lingual AB and in a narrow area between the middle and basal thirds of labial AB. BV, blood vessel; DP, dental pulp; La, labial side; Li, lingual side; NB, nerve bundle. Large arrow indicates the incisal edge's direction. Methyl green counterstaining (x 5.0).

Resorption of the AB lining mouse maxillary incisors was confirmed by the presence of osteoclasts, stained for TRAP activity using the azo dye technique. In this investigation, resorption was found in the marginal third of the lingual AB in the experimental mice only immediately following removal of the appliance. By week 1 no further resorption was found. Because rodent incisors erupt continuously throughout life, and are worn down by attrition,[17] the incisor relationship returned to that observed in the control mice. The absence of control - experimental differences on the lingual side from week 1 indicates that the lingual PDL was the tension side.

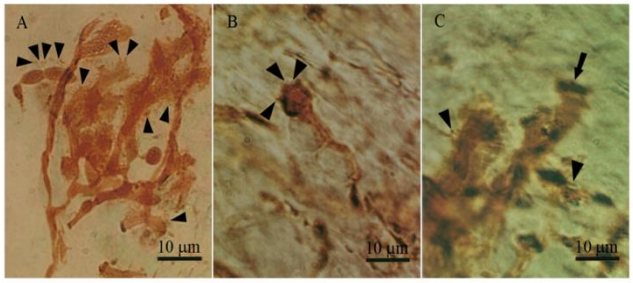

Figure 2. PGP 9.5 immunoreacted Ruffini nerve endings (brown). A, Fine structure of typical Ruffini endings with several microprojections (arrowheads) found in all control groups. B, Club-shaped Ruffini nerve endings with fewer microprojections (arrowheads) observed in the older mice. C, Ruffini nerve endings with longer microprojections (arrowheads) and those with hazy and swollen outlines (arrow) observed in the experimental mice at week 0 (x 40).

During an orthodontic tooth movement, alterations in the morphology[18,19] and number[18,20,21] of periodontal nerves have been reported. The changes are particularly rapid: following placement of a separating elastic

periodontal innervation increases rapidly for three days and returns to normal in two weeks.[22] The periodontal innervation of teeth moved buccally return to normal four weeks after removal of the displacing appliance.[23] The density and distribution of periodontal nerves in maxillary molars moved mesially are close to the density and distribution found in control animals after 3 weeks.[21] In the present study at week 0, when the appliance was removed, some RE possessed a hazy contour, while others had unusually long microprojections, presumably in reaction to the orthodontic force.

At appliance removal, the RE in the PDL had an irregular contour, which some consider to be due to degeneration or endings in the process of regenerating.[24] Long *et al*.[25] reported significantly fewer myelinated axons in the PDL of cat canines, subjected to an orthodontic force for 12 weeks, which had not recovered 8 weeks later. In the present investigation, the incisal relationship in the experimental mice was identical to that in the control mice two weeks after removal of the appliance. The restoration of neural configuration by week 1 agrees with others.[26] The declining number of nerve terminations with vague contours or numerous microprojections indicates that the force on the maxillary incisors had lessened, and the nerve terminals were returning to control levels.

Club-shaped RE became trapped between old and new AB in oldest control mice. These endings, which were found in PDL on the lingual side in the experimental mice, had fewer mitochondria,[27] indicating a regressive change in the endings. There were significantly fewer RE following removal of the appliance. It could be postulated that this is natural resolution and regression of the nerve endings after removal of an orthodontic force.

Periodontal nerves are involved in the early stages of periodontal remodeling and in the regenerative processes of the PDL.[21] The normal appearance of periodontal mechanoreceptors is restored about 8 weeks after removal of an orthodontic force.[28] However, they still possess a raised threshold to forces applied slowly, lower discharge frequencies, and respond to a narrower range of force directions than previously. This may be due to disorganization of the collagen matrix and/or direct injury to the nerve endings. A neurophysiological study in patients[14] has revealed that alterations in occlusal load occur during correction of an anterior crossbite, and concluded that orthodontic tooth movement influences periodontal mechanosensory input which, in turn, may modify the trigeminal motor output and thus, eventually, jaw muscle activity. It is, however, unlikely that the altered pattern of chewing found in patients with anterior crossbites can be mediated by a peripheral neural mechanism alone. The results in the present study have demonstrated a close relationship between the remodeling of RE and the periodontal tissues during and after orthodontic tooth movement. It could be argued that the findings support an orthodontic correction of anterior crossbite.

Area observed	Weeks after appliance removal			
	Week 0	Week 1	Week 2	Week 4
Experimental group	$20.43^{a} \pm 0.89$	$20.97^{b} \pm 0.45$	21.35 ± 0.61	$21.92^{a,b} \pm 0.37$
Control	$20.50^{c} \pm 0.94$	$20.78^{d} \pm 0.39$	21.73 ± 0.35	$22.07^{c,d} \pm 0.45$

Table 5. Body weights (in gram) of the experimental mice after appliance removal and their controls. (All values are expressed in mean ± standard deviation.)
Identical uppercase letters indicate significant intragroup differences between the experimental periods using analysis of variance and post hoc comparison by Newman-Keuls test at $p < 0.05$.

Conclusions

The lingual PDL supporting mouse maxillary incisors was under tension following removal of the crossbite appliance. Immunohistochemistry for PGP 9.5 revealed that there were fewer RE with vague contours and/or unusually long microprojections one week after removal of the appliance. Compared to those in the untreated controls, the number of RE with a hazy configuration and/or a club-shape appearance in the experimental mice declined after 1 week ($p < 0.05$). The morphology of RE recovered following removal of the crossbite appliance. A close relationship is indirectly implied between the remodeling of RE and the periodontal tissues during and after orthodontic tooth movement.

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Declaration of Interest

The authors declare no conflict of interest.

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