Effects of Relaxin on Relapse After Experimental Tooth Movement in Rats

Yurie Hirate,1 Masaru Yamaguchi,1 Tadahiko Utsunomiya,2 Hirotugu Yamamoto,2 and Kazutaka Kasai1

Departments of 1Orthodontics, and 2Oral Pathology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271–8587, Japan

Correspondence to:
Masaru Yamaguchi
E-mail: yamaguchi.masaru@nihon-u.ac.jp

Abstract

Long-term stability is the major goal of orthodontic treatment. However, long-term observation of treated cases after retention often reveals a disturbing degree and frequency of relapse. Relaxin is a member of the insulin/relaxin family of structurally related hormones. This hormone is produced in many mammals during pregnancy, and has been shown to promote cervical softening and elongation of interpubic ligaments in mice and cattle. Furthermore, relaxin is related to many other physiologic processes, such as collagen turnover, angiogenesis, and antifibrosis in both males and females. So far, no study concerning the effect of relaxin on orthodontic tooth movement has been done. The purposes of this study were to investigate whether local administration of relaxin in rats has an effect on relapse after orthodontic tooth movement and to explore the molecular mechanism of its role. In the present study, the upper first molars were moved mesially in 16 male, 6-week-old, Wistar strain rats using a coil spring with a force of 10 g. After 14 days, the appliance was removed, animals in the experimental group were given attachment gingival injections of relaxin at 20 μl for 7 days, and animals in the control group received phosphate-buffered saline. The results were evaluated by micro-computed tomography (μCT). At days 0, 14 and 21 after tooth movement, histopathological features were examined by immunohistochemistry based on proliferating cell nuclear antigen (PCNA). Relapse distances and percentages were significantly lower in the experimental group compared to those of the control group. Relapse in the control group was greater and faster than that in the experimental group. The ratio of PCNA–positive cells increased at day 14 after tooth movement and then decreased at day 21. Our results indicated that relaxin inhibited the relapse of experimentally moved rat molars, and this hormone might prevent relapse following orthodontic treatment.

Keywords:
relapse, periodontal ligament, relaxin, tooth movement, PCNA

Introduction

Orthodontic tooth movement is regulated by the application of mechanical orthodontic force. The relapse of moved teeth, an undesirable outcome of orthodontic treatment, is a major clinical issue. After removal of the orthodontic appliance, stresses are released, and the teeth begin to relapse to their original positions (1, 2). Relapse is the physiologic response of supporting tissues to force application and can be attributed mainly to occlusal stability and increased mechanical tension exerted by the transseptal fiber system (3, 4). The origin of this tendency is mostly unknown, but generally a distinction is made between relapse due to intrinsic factors within the periodontal ligament (PDL) and alveolar bone, and extrinsic factors, such as growth of facial structures, soft tissue pressure and interdigitation. The turnover or remodeling of extracellular matrix (ECM) components is apparently a prerequisite for relapse, just as it is for active tooth movement.

The PDL is a complex, vascular and highly cellular connective tissue localized between the tooth and
alveolar bone, and serves as a cushion against occlusal forces during mastication (5). Parallel bundles of collagen fibers attached to the root of the tooth and to alveolar bone mainly constitute the periodontal ligament. An additional component of the ligament is the network of elastic system fibers (6). PDL homeostasis involves both intensive and subtle transcriptional and translational regulation of collagen and matrix metalloproteinase (MMP) genes, which are a family of zinc-dependent enzymes that have the capacity to degrade nearly all components of ECM (7). The principal components of ECM, collagens and proteoglycan, exert mechanical resistance to tensile and compression stresses, respectively. The precise regulation of MMP gene expression in relation to collagen gene expression is critical for tissue repair and homeostasis, and dysregulation can lead to various pathological events (8).

Relaxin is a member of the insulin/relaxin family of structurally related hormones and has been shown to bind to receptors that are part of the leucine-rich repeat-containing G-protein receptor family (LGR7 and LGR8) (9). Relaxin was previously shown to induce a matrix-degrading phenotype in human lung fibroblasts in vitro and to inhibit pulmonary fibrosis in vivo. A novel peptide that targets the relaxin RXFP1/LGR7 receptor was recently identified using our computational platform designed to predict novel G protein-coupled receptor peptide agonists. Relaxin, a pleiotropic hormone with known extracellular matrix remodeling capabilities, also exhibits antifibrotic and anti-inflammatory activities (10). In a previous study, Stewart et al. (11) reported that relaxin had an influence on orthodontic tooth movement through alterations of PDL.

A previous study indicated that orthodontic force affects the proliferative activity of PDL cells (12). However, the correlation between this activity of PDL cells and collagen synthesis in the PDL during orthodontic tooth movement remains to be elucidated. Yoshimatsu et al. (14) suggested that collagen was metabolized predominantly on the tension side, and that PDL cells actively proliferate on both the tension and pressure sides during orthodontic tooth movement in mouse. Since proliferating cell nuclear antigen (PCNA) synthesis occurs in the latter part of the G1 phase and throughout the S phase, but is virtually undetectable in M phase cells (13), it is a useful marker for evaluating the proliferative activity of cells. So far, no study concerning the effect of relaxin on orthodontic tooth movement has been done. The purpose of this study was to investigate whether local administration of relaxin in rats had an effect on relapse after orthodontic tooth movement or not.

Materials and Methods

Animals

The animal experimental protocol in this study was approved by the Ethics Committee for Animal Experiments of Niho University School of Dentistry at Matsudo (approval No. AP09MD032). A total of 16 male, 6-week-old, Wistar strain rats (Sankyo Lab Service Co., Tokyo, Japan) weighing 180±10 g were used for the experiment. The rats were divided into two groups (eight rats each), according to the magnitude and duration of the applied force. The contralateral molars served as controls (0 g). The rats were kept in the animal center of Niho University School of Dentistry at Matsudo in separate cages, with a 12-hour light/dark environment at a constant temperature of 23°C, and provided with food and water ad libitum. The health status of each rat was evaluated by monitoring the body weight daily for 1 week before the experiments.

Application of orthodontic devices and relaxin injection

The animals were anaesthetized with pentobarbital sodium (35 mg/Kg body weight) for fixation of the orthodontic devices. Experimental tooth movement was induced using the method reported by Fujita et al. (15), with a closed-coil spring (wire size: 0.005 inch, diameter: 1/12 inch, Accurate, Inc., Tokyo, Japan) ligated to the maxillary first molar by a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan). The other side of the coil spring was also ligated, with holes in the maxil-
lary incisors drilled laterally just above the gingival papilla with a #1/4 round bur, using the same ligature wire. The upper first molar was moved mesially by the closed coil spring with a force of 10 g (Fig. 1). The period of the experiment was 14 days. At the end of the experimental period, the spring appliances were removed. The rats were then randomly divided into 2 groups: a control group and an experimental group that received relaxin injections. The rats in the experimental group were given attachment gingivae injections of relaxin (R&D Systems, Inc., MN, USA) at 500 ng/ml (volume of 20 μl) every other day for 7 days from the last day of tooth movement. Four attachment gingivae injections were given surrounding the tooth (mesial, distal, buccal and palatal sides) (Fig. 2). Rats in the control group received injections of phosphate-buffered saline (PBS) during the same period (Fig. 3).

Micro-CT scanning and measurement of tooth movement
To quantify tooth movement, the distance between the distal grooves of the first and second molars was measured on the μCT of each animal. A quantitative image analysis of the amount of tooth movement was performed using an in vivo micro-CT system (Rigaku-μCT®, Tokyo, Japan). The rat molars were scanned using μCT with an X-ray source of 90 KV/88 μA at 0, 14, 17, 19 and 21 days after surgery. After the rats were deeply anesthetized with intraperitoneally injected sodium pentobarbital (35 mg/Kg), each rat was set on the object stage and imaging was performed on the sample over a full 360° rotation with an exposure time of 17 seconds. An isotropic resolution of 30×30×30 μm voxel size was selected, which displayed the microstructure of the interdental distances between the first (M1) and second (M2) molars (Fig. 4). The original 3D images were displayed, and the data were analyzed with the I–View® software program (J. Morita, Kyoto, Japan).

Tissue preparation
The evaluation periods were set at days 0, 14 and 21 after these experiments. The rats were deeply anesthetized by pentobarbital sodium and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the maxilla was immediately dissected and immersed in the same fixative for 18 hours at 4 °C. The specimens were decalcified in 10% disodium ethylenediamine tetracetic acid (EDTA, pH 7.4) solution for 4 weeks, and the decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin using the usual methods for preparation. Each sample was sliced into 4-μm sections continuous in the horizontal direction, and prepared for hematoxylin and eosin staining (H.E.). The periodontal tissues in the mesial part of the distobuccal root and the distal part of the mesiobuccal root of the upper first molar were observed. The side that was not moved was defined as the control group (Fig. 5).

Immunohistochemistry
Immunohistochemical staining was performed as follows: first, the sections were deparaffinized and endogenous peroxidase activity was quenched by incubation in 3% H2O2 in methanol for 15 minutes at room temperature (RT). After washing in Tris-buffered saline (TBS), they were incubated with monclonal anti–mouse Proliferating Cell Nuclear Antigen (PCNA) (Dako, Glostrup, Denmark; working dilution: 1:100) for 1 hour at RT. PCNA was stained using ChemMate ENVISION kit/HRP (DAB) (Dako, Glostrup, Denmark), according to the manufacturer’s protocol. They were rinsed with TBS and final color reactions were performed using 3,3’–diaminobenzidine tetra–hydrochloride and aminoethyl carbazole, and then counter–stained with hematoxylin. As immunohistochemical controls, a few sections were incubated in the same way, and then incubated with either non–immune rabbit IgG or 0.01 M PBS alone, instead of the primary antibody. To evaluate the effect of tooth movement on the expressions of PCNA, we determined the numbers of PCNA–positive cells. Results were standardized as follows: the number of PCNA–positive cells/the number of all stained cells (%).
Statistical methods
The values in each figure represent the means ± standard deviation (S.D.) for each group. A Mann–Whitney’s U-test was used to compare the means of the groups. The values of (*) P < 0.05 and (**) P < 0.01 were considered statistically significant (Figs. 6B, 8 and 9).

Results
Body weight and tooth movement during the experimental period
The body weight of the rats in both treatment groups decreased transiently on day 1 and then recovered. No significant differences in the body weight or tooth movement between the two groups were observed (data not shown).

Relapse distances and percentages after administration of relaxin
The mean total amount of active tooth movement during the experimental period was 0.40 ± 0.07 mm. As shown in Fig. 6, after relaxin administration for 7 days, the relapse distances in the experimental group were less than those in the control group. After relaxin administration from days 14 to 21, the relapse distances in the experimental group were lower than those in the control group (P < 0.01) (Fig. 6). At days 17, 19 and 21, the relapse percentages of the experimental group were 8.7%, 16.8% and 33.0% compared to the value at day 14, respectively. On the other hand, the relapse percentages of the control group were 12.6%, 46.6% and 68.1%, respectively. The relapse percentage in the experimental group was significantly lower than that of the control group.

Histological changes in periodontal tissues during tooth movement (H.E.)
For the control group at days 14 and 21 after tooth movement, PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that regularly ran in a horizontal direction from the root cementum towards the alveolar bone. Blood capillaries were mainly recognized near the alveolar bone in the PDL. The alveolar bone and root surfaces were relatively smooth. In the experimental group, the arrangement of the fibers and fibroblasts became coarse and irregular (Fig. 7).

Immunohistochemical findings of PCNA
PCNA–positive cells were distinguished based on brown staining of their nuclei upon immunohistochemistry (Figs. 8 and 9). PCNA–positive cells were found in the PDL on the pressure and tension side in all groups. On the pressure side, the ratio of PCNA–positive cells at day 14 was significantly higher than that at day 0. However, there was no difference in the ratio of the PCNA–positive cells between day 0 and day 21 in the PBS group. In addition, the ratio of PCNA–positive cells in the relaxin group was significantly lower than that in the PBS group (Fig. 8).

Similarly, on the tension side, the ratio of PCNA–positive cells at day 14 was significantly higher than that at day 0. On the other hand, there was no difference in the ratio of PCNA–positive cells between day 0 and day 21 in the PBS group. In addition, the ratio of PCNA–positive cells in the relaxin group was significantly lower than that in the PBS group (Fig. 9).

Discussion
In the present study, we examined whether local administration of relaxin in rats had an effect on relapse after orthodontic tooth movement. The novel finding of this study is that relaxin stimulation significantly decreased the relapse distance and percentage.

We first examined the effects of relaxin on relapse and periodontal tissue remodeling after experimental tooth movement in rats. A force of 10 g was applied because previous studies had demonstrated that a 10–g force stimulated substantial molar tooth movement in rats (16). Tooth movement occurs by alveolar translocation, which is a unique type of remodeling, featuring simultaneous bone formation and resorption on opposite sides of the alveolus. Taken together, these processes result in drift of the entire alveolus parallel to tooth drift to maintain tooth support (17) and require remodeling of the principal
fibers of PDL, alveolar bone matrix, and the embedded Sharpey's fibers. A previous study suggested that stress/strain forces from adjacent teeth determine the mineralization pattern, diameter, and density of Sharpey's fibers in the alveolar wall. For any tooth movement to occur, remodeling of Sharpey's fibers at the PDL interface with the alveolus is required (18). In addition, Johnson (19) reported that attachment of PDL fibers to bone required continuous and coordinated synthesis of collagenous fibers of both the alveolar bone matrix and adjacent Sharpey's fibers in response to the experimental force. The amount of separation achieved was 0.30–0.40 mm for animals sacrificed at day 0. In this study, we found that the relapse was the fastest in the control group, amounting to approximately half of the total distance. The control group showed a relapse value of 12.6% at day 17. Thereafter, the control group showed significantly increased amounts of relapse at days 19 and 21 after application of the force. On the other hand, the experimental group had a relapse value of 8.7% at day 17, and they showed significantly lower amounts of relapse compared to the control group at days 19 and 21 (Fig. 6). Han et al. (20) reported that the relapse energy stored in the collagenous periodontal and transseptal fiber systems was gradually released after spring removal, resulting in faster and greater relapse within 1 week. Moreover, Gibson et al. (21) showed that there was distal molar drift in rats without appliances, and that dental relapse from an applied force is rapid. In addition, van Leeuwen et al. (4) found that relapse started immediately, not only without retention, but also after retention. These reports supported the results of the present study.

Examination of the H.E. stained specimens revealed that the PDL specimens in the control group were composed of relatively dense connective tissue fibers and fibroblasts that regularly run in a horizontal direction from the root cementum towards the alveolar bone. The alveolar bone and root surface were relatively smooth. On the other hand, in the experimental group, the arrangement of the fibers and fibroblasts became coarse and irregular (Fig. 7). This study was designed to investigate the correlation between relaxin injection and PCNA in PDL during tooth movement using immunohistochemistry. Immunohistochemical staining showed that the expression of PCNA significantly increased at day 14 after tooth movement in the experimental group (Figs. 8 and 9). However, there was no difference in the ratio of PCNA-positive cells between day 0 and 21 (Fig. 9). In a previous study, Yoshimatsu et al. (14) reported that PDL cells actively proliferate on both the tension and pressure sides during orthodontic tooth movement. In addition, Mabuchi et al. (12) observed that the ratio of PCNA-positive cells on the tension side was the highest at day 3 after insertion and then decreased for the remaining experimental period. Moreover, the ratios of cell proliferation and cell death are closely related to regeneration and reconstruction of PDL, which reflect the orthodontic force. In agreement with our results, these previous studies have shown the activation of PCNA expressed by orthodontic tooth movement.

Several studies have demonstrated that relaxin is able to act at multiple levels to inhibit fibrogenesis and collagen overexpression. It is important to note that while several actions of relaxin are consistently identified in multiple organs, some of the actions are tissue-specific or vary between organs, suggesting that relaxin inhibits fibrosis through common and specific mechanisms, depending on the organ to which it is applied. The ability of relaxin to inhibit fibroblast proliferation was demonstrated in the late 1980s, when it was shown to prevent the normal course of mitosis and inhibit the proliferative response during the inductive phase of differentiation of the 3T3-L1 fibroblast cell line without directly affecting fibroblast differentiation (22). More recently, relaxin was shown to directly inhibit fibroblast differentiation into myofibroblast expression by inhibiting alpha-smooth muscle actin (α-SMA) expression in a dose-dependent manner in rat cortical fibroblasts (23). Bennett et al. (24) reported that relaxin inhibited α-SMA expression in rat hepatic stellate cells and inhibited fibroblast proliferation and differentiation in rat cardiac fibroblasts. These
Fig. 1.

Fig. 2.

Tooth movement
(Coil set and activation)

0 1 3 5 7 14 17 19 21

Control group

Relaxin group

○: μCT examination

↑↓: Sacrifice

Fig. 3.

Relaxin injection

Fig. 4.
Fig. 5.

A

0 day  14 days  21 days PBS  21 days relaxin

B

![Graph showing tooth movement](image)

Fig. 6.
Fig. 1. Demonstration of orthodontic appliance used for experimental tooth movement. The upper first molar was moved mesially by a closed coil spring using 10 g of orthodontic force.

Fig. 2. Attachment gingivae injection points. The injections were in the mesial, buccal, and palatal sides of the gingiva, located in the area of the moved upper right first molar. The volume of each injection was 5 µl. Black arrows, attachment gingivae injection points.

Fig. 3. The experimental time schedule for each group.

Fig. 4. The measurement area of tooth movement. The image reconstruction software was the I-View® software program (J. Morita, Kyoto, Japan). We measured the distance between the first molar and second molar to determine the tooth movement.

Fig. 5. A photograph of labeled alveolar bone taken under a light microscope. Immunopositive reactions for PCNA-positive cells, MMP-1 and 8 were measured in the pressure side quarter of the distal area facing the mesial root, and in the tension side quarter of the mesial area facing the distal root. Arrow, relapse direction; MR, mesial root; DBR, distal buccal root; PDL, periodontal ligament; PS, pressure side; TS, tension side.

Fig. 6. The effects of attachment gingivae injection on tooth movement. (A) After the spring appliances removal, the interdental spaces between molars showed a rapid decerese in width. The relapse distances in the experimental group were less than those in the control group. (B) The relapse distances in the experimental group were less than those in the control group (Mann-Whitney's U test, a significant difference from the corresponding control. (*P < 0.05, **P < 0.01). The values were the means ± SD for eight rats.

Fig. 7. Histological changes in the periodontal tissues during tooth movement (H-E staining) (bar : 50 µm). (a-d) Pressure side. (e-f) Tension side. The alveolar bone and root surface distances were narrowed in the control group compared with day 14. On the other hand, in the experimental group, the distance to the tooth root surface of the alveolar bone was not significantly different from that at day 14. PDL, periodontal ligament; C, cementum; D, dentin.
findings are consistent with the ability of relaxin to inhibit collagen synthesis and deposition (25). Moreover, Lekgabé et al. (26) demonstrated that relaxin selectively and rapidly inhibited collagen overexpression in the affected myocardium and kidney of spontaneously hypertensive rats without noticeable side effects. Takano et al. (27) reported that relaxin decreased the release and gene expression of type I collagen. In short, the findings of our study suggest that relaxin down-regulates PDL proliferation on both the tension and compression sides during orthodontic tooth movement.

Conclusions
The results of our study indicate that orthodontic tooth movement increased the number of PCNA-positive PDL cells, whereas relaxin decreased the number of PCNA-positive PDL cells. In addition, relaxin reduced the occurrence of relapse, and may be useful in preventing orthodontic relapse following orthodontic treatment.

Acknowledgments
This research was supported by a Grant-in-aid from the Japan Society for the Promotion of Science (C: 22592297). We are grateful to Mr. Takashi Matsumoto (Division of Diagnostic Pathology, Nihon University Hospital School of Dentistry at Matsudo) for his expert technical advice and kind help in the study.

References
12. Mabuchi R, Matsuoka K, Shimono M: Cell prolifera-