Daidzein Inhibits Relapse after Rat Experimental Tooth Movement

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Abstract

**Objectives:** Relapse is a major clinical problem in orthodontic treatment, and long-term observation of treated cases often reveals relapse. Collagen metabolism in the periodontal ligament (PDL) is thought to be involved in relapse. Daidzein stimulates skin collagen synthesis in cultured skin fibroblasts *in vitro*. We investigated the inhibitory effects of daidzein on relapse after orthodontic tooth movement. **Methods:** After a force of 10 g induced tooth movement for 14 days, the appliance was removed, and the experimental group was injected with daidzein while the control group was injected with phosphate-buffered saline for 1 week. The distance of relapse was measured by micro-computed tomography. In addition, hematoxylin and eosin staining and the histopathological features were examined by immunohistochemistry using collagen type I (COL-I), matrix metalloproteinase (MMP)1, and proliferating cell nuclear antigen (PCNA). **Results:** The distances and ratio of relapse in the daidzein group were significantly lower than in the control group. Immunohistochemistry showed marked positive staining of COL-I and MMP1 in the daidzein group. The ratio of PCNA-positive cells in the daidzein group increased at day 7. **Conclusion:** These results indicated that daidzein activated the collagen metabolism in the stretched PDL and may inhibit relapse after orthodontic treatment.

Keywords: tooth movement, daidzein, rat

Introduction

Tooth movement in orthodontic cases has been studied to evaluate the long-term stability. Relapse is a major clinical problem and an undesirable outcome of orthodontic treatment. The cause is not obvious (1). Tooth movement using an elastic band for 21 days showed that the periodontal ligament (PDL) tended to expand and PDL consisted of irregular the human periodontal ligament (hPDL) cells. Tooth relapse after rat experimental tooth movement may therefore involve rapid remodeling of the PDL (2). The arrangement of these PDL fibers indicates their need and function in maintaining relationships between neighboring teeth and in stabilizing the tooth against movement forces. The PDL responds to stress caused by orthodontic movement with increased resistance of the transseptal fibers when they seek to return and maintain the original positions of the tooth (3). These reports suggest, we thought that extension of PDL after tooth movement is one of the important factors related to relapse.

The PDL is composed of an abundance of type I and III collagen (COL-I and III). The PDL homeostasis causes both intensive and subtle transcriptional and translational regulation of collagen and matrix metalloproteinase (MMP) genes. Our previous studies (4) demonstrated the effects of relaxin, a peptide hormone, on the collagen metabolism in hPDL cells. The relaxin affected the collagen metabolism of stretched hPDL cells *in vitro*. Specifically, it reduced the expression of COL-I and induced the expression of MMP-1 in stretched PDL cells. In addition, after rat experimental tooth movement, relaxin inhibited relapse by increasing the expression of COL-I, MMP-1 and MMP-8 (5).

Daidzein is a compound extracted from soybean with one

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isoflavone. The structure of daidzein resembles that of the female hormone estrogen. In the cosmetic field, collagen from skin fibroblasts is applied in anti-wrinkle treatment. Daidzein has been shown to stimulate skin collagen synthesis in cultured skin fibroblasts via the TGF-β/smad signaling pathway in vitro (6). In addition, daidzein modulated against solar ultraviolet light-induced matrix metalloproteinase (MMP)1 in normal human dermal fibroblasts (7). Therefore, as daidzein exerts estrogen-like activities, similar to relaxin, it may inhibit relapse after orthodontic treatment via the stimulating of the collagen metabolism.

The purposes of this study were to investigate whether or not the local administration of daidzein inhibits relapse after rat experimental tooth movement using micro-computed tomography (micro-CT), and to identify the effects of daidzein on the expression of COL-I and MMP1 during experimental tooth movement due to the application of a force of 10 g using an immunofluorescence analysis in vivo.

Materials and methods

Animals

Breeding of the rats was managed at Nihon University School of Dentistry Matsudo Animal Center using specific-pathogen-free clean racks with a 12-h light/dark cycle at a constant temperature of 23°C. The bedding and gauges were all sterilized, and the animals were provided powder material and drinking water ad libitum. The Ethics Committee for Animal Experiments at Nihon University School of Dentistry at Matsudo approved this study protocol (approval No. AP15MD017).

Thereafter, 6-week-old male Wistar strain rats (weight: 139.8 ± 9.8 g; Sankyo Lab Service Co., Tokyo, Japan) were randomly assigned to 3 groups as follows (Fig. 2):  
1. Tooth movement (TM) + PBS injection group (n=6)  
2. TM + daidzein 50 ng/ml injection group (n=6)  
3. TM + daidzein 500 ng/ml injection group (n=6)

Experimental tooth movement and daidzein PDL injection

The animals were anesthetized, with all operations for the application of orthodontic devices carried out under general anesthesia, using an intraperitoneal injection of three mixed anesthesia agents: hydrochloric acid Medetomidine (Domitoru), Mitazoramu and butorphanol tartrate (Betorufamu) (0.15 mg/kg body weight). The rats were given PDL injections for 1 week starting at the end of tooth movement. The experimental group received daidzein injections, and the control group received PBS injections during the same period. The tooth movement method used in this study has been previously described by Asano et al. (8), with treatments performed once a day for one week (Fig. 1A,B). In brief, a closed stainless-steel (SS) coil spring (thickness: 0.005 inches, diameter: 1/12 inch, Accurate Sales Co., Chiba, Japan) was ligated to the maxillary right first molar using ligature wire, and the other side of the coil spring was also ligated to the maxillary left and right incisor (Fig. 1A). The orthodontic force applied was that described by Kawasaki et al. (9), who applied a force of 10 g without glass degeneration of weight loss and periodontal tissue. The upper right first molars were moved to the mesial side at a force of 10 g.

Fig. 1.  Experimental tooth movement and daidzein injection to the periodontal ligament.  
A: A schematic illustration of the appliance used for experimental tooth movement. The upper first molar was moved mesially by a closed-coil spring with a 10-g force.  
B: Black arrows, periodontal ligament injection points. The local administration of daidzein was delivered in the mesial, distal, buccal and palatal sides of the periodontal ligament in the area of the upper right first molar.
The 50 ng/ml and 500 ng/ml daidzein groups were injected in the PDL after tooth movement. The injection regimen was that previously reported by Hirate et al. (10) as follows: cheek side of the tooth, palatal side, mesial, around four locations of the distal side, 15 μl each, daily for 7 days, into the PDL space (Fig. 1B). The experimental tooth movement was performed for 14 days, and experimental injections were performed for 7 days (Fig. 2).

Micro-CT to quantify tooth movement

The distances of the tooth movement and the relapse were determined by measuring the distance between the contact of the first and second molars on micro-CT (Fig. 3). Measurements were conducted experimentally according to the method described by Yamaguchi et al. (11). We measured the distance between the 1st and 2nd molars as the relapse distance, using the sagittal, occlusal and coronal planes. A median palatine suture was used on the sagittal planes. The occlusal plane was the plane connecting the 2nd molar of the tension side with the 1st and 2nd molars of the non-tension side. The coronal plane was the plane from the contact point perpendicular to the occlusal plane. A quantitative imaging analysis of the amount of tooth movement was performed using an in vivo micro-CT system (Rigaku-micro-CT®, Tokyo, Japan). The first molars were scanned using micro-CT from seven days after the closed SS coil spring was removed. After the rats had been deeply anesthetized with the anesthesia mixture mentioned above (0.15 mg/kg body weight), each rat was set on the stage, and imaging was performed over a full 360° rotation. The images were displayed, and the scanning data were analyzed.

Thin-slice preparation of tissue

The animals were deeply anesthetized with the anesthesia mixture mentioned above and after which the maxilla was immediately dissected. The specimens were decalcified in 10% disodium ethylenediamine tetracetic acid (EDTA, pH 7.4) solution for 4 weeks. The decalcified specimens were then dehydrated through a graded series of ethanol washes and embedded in paraffin using the usual methods for preparation. Each sample was sliced into 4-μm-thick sections continuously in the horizontal direction and prepared for hematoxylin and eosin (H&E) staining as well as for immunohistochemical staining. In this study, we sliced and observed sagittal orientation sections using the methods of Gonzales et al. (12) as a reference. The distal buccal root was observed at 280 μm from the furcation area to the root apex after tooth movement (Fig. 4). Coronal observation of the sections were made according to the method of Kawasaki et al. (9), as a
reference, the measurements were performed in the tension areas. The periodontal tissue in the tension areas were one-quarter of the distal area facing the distal buccal root (DBR), as determined when linked with the center of the DBR and the mesial root (MR) of the first molar.

**Immunohistochemistry**

Immunohistochemical staining was performed according to the method of Hirate et al (10). First, the 4-μm-thick sections were cut from paraffin embedded skin tissue specimens, and the sections were deparaffinized. The sections were then heated in a microwave oven at 92°C for 20 min in a citrate buffer, incubated overnight at 4°C. The endogenous peroxidase activity was blocked by incubating the sections with 3% H₂O₂ in methanol for 30 min. Monoclonal anti-mouse PCNA (Dako, Glostrup, Denmark; working dilution: 1:100), polyclonal anti-rabbit COL-I (Santa Cruz Biotechnology, Inc, CA, USA; working dilution: 1:250), polyclonal anti-rabbit MMP1 (Abnova Co, CA, USA; working dilution, 1:100) for 1 h. PBS was used instead of the primary antibody as a negative control. After the primary antibody reaction, the sections were rinsed in PBS 3 times for 5 min each. The secondary antibody (horse radish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG) was then incubated at room temperature for 30 min. After washing in PBS 3 times for 5 min each, 3,3'-
diaminobenzidine-tetrahydrochloride Tris–HCl buffer (pH 7.6) was used to visualize reactivity. Finally, all sections were counterstained with hematoxylin, dehydrated and covered. COL-I, MMP1 and PCNA were stained using a Histofine Simple Stain MAX-Po (Multi) kit (Nichirei, Co., Tokyo, Japan) according to the manufacturer’s protocol. The PCNA-positive ratios were calculated using the methods of Sato (13) et al. as a reference. The positive ratio was calculated for the tension side of the distal buccal root about PCNA investigation as follows; positive ratio = positive cells/total cells × 100(%) results are presented as median (interquartile range). Both relapse distance and PCNA-positive cells were analyzed by the Mann–Whitney test as a nonparametric method. All data were confirmed by multiple clinical pathologists.

**Results**

**Distances and ratio of relapse by the administration of daidzein**

*In vivo* study, the spaces between molars decreased in all groups. The distances and ratio of relapse significantly decreased each day compared in comparison to the findings on day 0 (Fig. 5A). We made a 100% ratio basis on day 0. On day 7, the relapse ratios in the 50 ng/ml daidzein and 500 ng/ml daidzein groups were 24.7% and 24.9%, respectively, compared to the value on day 0. In contrast, the relapse ratio

![Fig. 5. Relapse distance.](image)

A: The spaces between molars decreased in all groups.
B: Quantifying the relapse distance between the maxillary right first molar and the maxillary right second molar. At the day 7, the relapse ratios in the daidzein groups (50 ng/ml, 500 ng/ml) were significantly lower than in the PBS group (*p < 0.05*).

**Statistical analyses**

A statistical analysis was performed using the JMP software (8.0.2; SAS Institute Inc., Cary, NC, USA), and the
in the control group was 59.0%. The relapse ratio in the daidzein group was significantly lower than in the PBS group (Fig. 5B).

**Histological changes in periodontal tissues during tooth movement by daidzein (H.E.)**

The concentration of daidzein injection was applied to the 50 ng/ml in H.E staining and immunohistochemistry as the results of relapse distance ratio was similar for 50 ng/ml and 500 ng/ml. The TM+daidzein 50 ng/ml group at day 7 was observed collagen fibers of comparatively clarity and showed wavelike in PDL. The TM + PBS group were observed weak collagen fiber (Fig. 6).

**Immunohistochemical findings of COL-I and MMP1**

The daidzein-treated sections showed marked positive staining of COL-I and MMP1 in the PDL around the tooth compared with the control group (Fig. 7).

**Immunohistochemical findings of PCNA**

The daidzein group, the ratio of PCNA-positive cells on day 7 was significantly higher than in the PBS group on day 7. The PCNA positive cell ratio of the daidzein group on day 7 was 31.4%, which was 1.5 times that of PBS group (19.6%...
Discussion

In this study, we examined the inhibitory effects of daidzein on relapse after rat experimental tooth movement. The results showed that the relapse distance in the daidzein group was significantly lower than in the PBS group at days 21. Han et al. (14) reported the relapse in the control group after one week to be the fastest among the groups receiving simvastatin, accounting for almost half of the total distance. The energy of relapse may have been stored in the stretched PDL collagenous fiber and then it was gradually released after the apparatus was removed. As a result, relapse occurred within one week. These reports support the findings of the present study. In contrast to the substantial relapse noted in the control group, indicating that the distances and rate of relapse were significantly lower in the daidzein group than in the control group. Taken together, these findings show that daidzein inhibited relapse after experimental tooth movement.

We also examined that inhibitory effect of daidzein on relapse through the collagen metabolism. The PDL specimens were found to be composed of relatively dense connective tissue fibers and fibroblasts at day 0 on hematoxylin and eosin (H.E.) staining. Taken together, these findings suggest that daidzein may accelerate the collagen metabolism of PDL.

The results of immunohistostaining of COL-I and MMP1 showed these proteins to be detected at high levels on day 7. The results suggest that daidzein activates the collagen metabolism in PDL cells through the release and expression of COL-I and MMP1.

Finally, we investigated the correlation between daidzein injection and PCNA in PDL after tooth movement by immunohistochemistry. The cell cycle consists of a series of steps during which chromosomes and other cell materials double to make two copies. PCNA synthesis occurs from the G1 phase through the S phase. However, it can still be detected in M phase cells, thus making it a useful marker for evaluating the proliferative activity cells.
Immunohistochemical staining showed that PCNA protein was detected in the control group, and the administration of daidzein strengthened such staining and the number of PCNA-positive cells in the daidzein group was significantly increased at day 7 compared with the PBS group. Yoshimatsu et al. (15) suggested that the remodeling of collagen in the PDL occurs mainly on the tension side during orthodontic tooth movement. Furthermore, Mabuchi et al. (16) reported that the ratios of cell proliferation are closely related to the regeneration and reconstruction of the PDL, which reflects the orthodontic force. In concordance with our results, these previous studies showed the activation of PCNA by orthodontic tooth movement. These above results suggest that daidzein inhibits relapse after orthodontic tooth movement by increasing the expression of COL-I and MMP1 in stretched PDL cells.

Considering about safety of daidzein, many studies have demonstrated that daidzein promotes improved health in humans. Smeriglio et al. (17) reported that isoflavone prevented osteoporosis and postmenopausal symptoms in women. Bao et al. (18) reported that daidzein suppressed the migration and invasion of human breast cancer cells via inhibiting hedgehog/Gli1 signaling. While, Murata et al. (19) reported that daidzein was related to the development of breast cancer via DNA damage. We therefore believe that the dose should be monitored closely, given the differences between rats and humans. The appropriate dose in humans should therefore be determined in the future. Recently, Han et al (14) reported that simvastatin, a 3-hydroxy-3-methylglutary-coenzyme A (HMG-CoA) reductase inhibitor, may also be useful for preventing relapse. It was shown to inhibit relapse by increasing alveolar bone formation via regulating the osteoprotegerine (OPG) and receptor activator of NF-kappa B ligand (RANKL) expression. Daidzein may affect not only the collagen metabolism of PDL cells, but also the alveolar bone metabolism. Further investigation is needed to confirm the effects of daidzein on bone remodeling during tooth movement.

Conclusions
1. Daidzein injection was reduced the relapse distance.
2. Daidzein injection increased the cell proliferative capacity of PDL and the expression levels of COL-I and MMP1 increased at the gene level and the protein level.

Conflict of interest
We declare that there are no conflict of interest in this paper.

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