Daidzein induces bone morphogenetic protein-2 and runt-related transcription 2 on periodontal ligament cells after experimental tooth movement

Mioko Sakurai [1)], Mami Shimizu [2)]

[1)] Department of Orthodontics, Nihon University Graduate School of Dentistry at Matsudo, Chiba, Japan

[2)] Department of Orthodontics, Nihon University School of Dentistry at Matsudo, Chiba, Japan

*Corresponding author:

Mioko Sakurai

Department of Orthodontics

Nihon University School of Dentistry at Matsudo

2-870-1, SakaeCho-Nishi, Matsudo-city, Chiba, 271-8587 Japan

Tell: +81-47-360-9412

Fax: +81-47-360-9414

Email: mami17013@g.nihon-u.ac.jp

Keywords: Daidzein, Relapse, Periodontal ligament cells

ABSTRACTS

Purpose: To analysis daidzein increases the expression of bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor 2 (Runx2) after experimental tooth movement (EXTM).

Materials and Methods: 36 Wistar rats were randomly divided into the EXTM group and EXTM with daidzein group (EXTM+DZ group). After EXTM, the rats in the EXTM+DZ group were treated with daidzein (10 mg/kg/day). The bone mineral density (BMD), ratio of bone volume to tissue volume (BV/TV), and ratio of relapse were analyzed by micro-computed tomography. Histological changes and BMP-2 and Runx2 expressions were examined by immunohistochemical staining. Human periodontal ligament cells were subjected to tension force and treated with 10 μg/mL daidzein in vitro. The effects on BMP-2 and Runx2 with daidzein were confirmed by real-time polymerase chain reaction and enzyme-linked immunosorbent assay.

Results: The EXTM+DZ group showed a significantly decreased ratio of relapse and increased BMD and BV/TV as compared to the EXTM group. Histological changes showed that the osteoblasts were observed in the EXTM+DZ group. The ratios of BMP-2 and Runx2 positive cells in the EXTM+DZ group were higher than that in the EXTM group. Moreover, daidzein upregulated the mRNA and protein expressions of BMP-2 and Runx2 in a time-dependent manner.

Conclusion: daidzein decreased the ratio of relapse and increased BMD, BV/TV and the expressions of BMP-2 and Runx2 in PDLCs. These results suggest that daidzein may promote the differentiation of PDLCs into osteoblasts and bone formation.

INTRODUCTION

The relapse of teeth after orthodontic treatment is a clinical problem that prevents long-term stability of occlusion [1]. It is a physiological reaction of the dental supporting tissue, such as the periodontal ligament and alveolar bone, to apply force. Periodontal ligament cells (PDLCs) are a mixture of mesenchymal cells that are capable of differentiate into osteoblasts or cementoblasts to respond to mechanical stresses [2]. PDLCs are exposed to tension and compression forces during orthodontic tooth movement (OTM), and these forces are stored in periodontal and transseptal fiber systems. After the orthodontic appliance is removed, these forces are released, which causes the teeth to move back toward their original positions. The relapse pressure remains until alveolar bone resorption is completed [3]. Therefore, treated dentition needs to be stabilized with retainers for a long time. Several studies showed that therapeutic agents, such as simvastatin [4], relaxin [5], and bisphosphonate [6], prevent the relapse of teeth in animal models after experimental tooth movement (EXTM). Although the molecular mechanisms are different, the extent of relapse is decreased by modifying the remodeling process of the dental supporting tissues. These findings indicate the clinical utility of safe pharmacotherapy agents to decrease relapse after orthodontic treatment.

Daidzein is a soy-derived isoflavone and its structure is similar to estrogen. It promotes osteoblastic proliferation and differentiation by stimulating the activation of bone morphogenic protein (BMP)-2/ Smads signaling [7]. BMP-2 has a strong osteoinductive capacity and promotes the differentiation of mesenchymal cells into osteoblasts by controlling the expression and function of runt-related transcription factor 2 (Runx2) [8]. Runx2 is the osteoblast-specific product of the core-binding factor 1 (Cbfa1) gene and a direct target of mechanical force signaling in osteoblasts and

periodontal osteoblastic cells [9, 10]. It has been reported that periodontal ligament fibroblasts are different from dermal fibroblasts and express genes for BMP-2 and Runx2 [11,12]. Under tension force (TF), these gene expressions mark the beginning of the osteogenic differentiation of PDLCs in a time-dependent manner [13].

Therefore, this study aimed to investigate whether daidzein promotes differentiation of PDLCs into osteoblasts and bone formation and to reveal the possibility of inhibiting relapse after EXTM.

MATERIALS AND METHODS

EXTM and treatment with daidzein in rats

The experimental protocol was approved by the Animal Experimentation Committee of Nihon University (AP17MD008-2). The animals were maintained in separate cages in 12 hours light and dark environment at a constant temperature of 23 °C. Wistar male rats (n=36, 180.0 ± 10.0 g in weight and 6 weeks old) were randomly divided into the following two groups: EXTM group (n=18) and EXTM with daidzein (EXTM+DZ) group (n=18). The rats were anesthetized using three anesthetic agents, hydrochloric acid medetomidine, mitazoramu, and butorphanol tartrate, mixed in an intraperitoneal injection (0.15 mg/kg) before each operation. The rats underwent insertion of an orthodontic elastic module (Tomy International, Inc., Tokyo, Japan) between the left maxillary first (M1) and second molars (M2) for 7 days, according to the method of Waldo et al. [14] (Fig. 1A, B). After the EXTM, the tooth was fixed with composite resin to maintain the distance between M1 and M2 from day 8 to day 14 (retention period) (Fig. 1A, C). Following this, the composite resin was removed, and relapse of the tooth was observed until day 21 (relapse period).

During the retention and relapse periods, the rats were intraperitoneally administered daidzein (10 mg/kg/day) dissolved in phosphate-buffered saline (PBS) in the EXTM+DZ group and PBS in the EXTM group (Fig. 1A). At the end of this experiment, six rats on days 7, 14 and 21 were sacrificed, and the maxillae were dissected for histological and immunohistochemical analysis.

Micro-computed tomography scanning

After being deeply anesthetized, the rats were scanned using a micro-computed tomography (micro-CT) system (R_mCT2, Rigaku, Tokyo, Japan) on days 7, 14 and 21. Micro-CT scan was performed according to the method of Bae et al. [15]. Three-dimensional (3D) images were generated for each specimen using TRI/3D-BON (RATOC System Engineering, Tokyo, Japan), as recommended by the manufacturer, for quantitative analysis. The region of interest (ROI) was defined as the area of alveolar bone on the distal side near the mesiobuccal root, with the top surface of the cube (500 μm) located at the junction of the intermediate and apical two-thirds of the root (Fig. 1D). The quantity of new bone was evaluated using the ratio of bone volume to total volume (BV/TV) and the bone mineral density (BMD).

The extent of relapse was measured in pixels using Image J (National Institutes of Health, Bethesda, USA) on two-dimensional sagittal sections displaying the least inter-molar distance between M1 and M2 were selected for this measurement (Fig. 1E).

The EXTM distance in the rats at 7 days was set to 100%, and the ratio of relapse at 21 days in both groups was calculated using the following formula:

Ratio of relapse = (EXTM distance at 7days- EXTM distance at 21 days) / EXTM at 7 days ×100

Histology and Immunohistochemistry

The rats were sacrificed on days 7,14 and 21 after EXTM. The maxillae were dissected and trimmed into small blocks. The blocks were sliced into 4 μm horizontal sections and prepared for histological and immunohistochemical staining. Periodontal tissues in the same area as the ROI were observed. The right M1 in the EXTM group, which showed no movement until day 7, were assigned for histological and immunohistochemical analysis on day 0. The de-paraffinized sections were incubated with mouse monoclonal antibody against rat BMP-2 (Abcam, Tokyo, Japan, 1:500) and rabbit polyclonal antibody against rat Runx2 (Abcam, 1:500) for 18 hours at 4 °C. BMP-2 and Runx2 were stained using the Histofine Simple Stain MAX-Po kit (Multi; Nichirei, Co., Tokyo, Japan) in accordance with the manufacturer's protocol. Positive staining was determined microscopically and counted by three independent observers.

Cell culture

Six teeth were obtained from healthy volunteers (2 males, 4 females; age, 12-20 years) during the orthodontic treatment. All volunteers signed informed consent, and the study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC18-17-020-1). Human PDLCs (hPDLCs) were collected from the premolars extracted according to a modification of the method described by Somerman et al. [16]. The hPDLC culture was performed according to the method of Takano et al. [17] and used for the experiments at passages 6-9.

Application of TF and treatment with daidzein in hPDLCs

We performed the following in vitro experiments as described by Takano et al. [15]. Briefly, hPDLCs (7×10^6 cells/well) were seeded into a 10 cm^2 STREX-chamber (STREX Inc.Osaka, Japan) and subjected to TF for 12 hours in a culture medium containing 1% fetal calf serum. The cells were then treated with daidzein (10 μg/mL) after removing TF, and incubated for up to 72 hours. The hPDLCs were divided into the following three groups: control group, cells after application of (TF) group, and TF with daidzein (TF+DZ) group.

Real-time PCR

Total RNA was extracted from hPDLCs using a RNeasy® Mini kit (Qiagen Co., Tokyo, Japan). The mRNA was reverse transcribed to cDNA using a Prime Script™ RT Reagent Kit, and Real-time PCR amplification was performed with a SYBR Premix Ex Taq™ by TP-800 Thermal Cycler Dice® (Takara Co., Shiga, Japan). The primers are listed in Table 1.

Enzyme-linked immunosorbent assays (ELISA)

The protein expressions of BMP-2 and Runx2 were detected using ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). The absorbance was measured at a wavelength of 405 nm using a microplate reader (Tecan Japan Co., Ltd, Kanagawa, Japan).

Statistical analysis

All data are expressed as mean ± standard deviation. Differences among multiple

groups were calculated using Kruskal-Wallis test, and differences between two groups were determined using the Mann-Whitney U test. The p-values <0.05 and <0.01 were considered statistically significant.

RESULTS

Micro-CT analysis of the ratio of relapse, BMD, and BV/TV in rats

Micro-CT analysis showed that the distance between M1and M2. The distance in the EXTM+DZ group was wider than that in the EXTM group on day 21 (Fig. 2A). The ratio of relapse in the EXTM+DZ group was significantly lower than that in the EXTM group (Fig. 2B). Micro-CT analysis of the ROI in both groups is shown in Figs. 2C and D. The BMD and BV/TV values were significantly higher in the EXTM+DZ group than those in the EXTM group on day 21.

Histological and immunohistochemical findings of BMP-2and Runx2

The histological findings are shown in Fig. 3A. In both the EXTM and EXTM+DZ groups, the periodontal ligament specimens consisted of relatively dense connective tissue fibers and PDLCs that ran horizontally from the root cement to the alveolar bone on day 0. The fibers became coarse and irregular, and the PDLCs were elongated among the fibers on day 7. The EXTM+DZ group showed extended the fibers and PDLCs compared to the EXTM group on day 14. The arrangement of fibers gradually returned to its previous state before the EXTM in both the EXTM and EXTM+DZ groups on day 21. The PDLCs showed larger nucleus with a rich cytoplasm and osteoblasts were observed in the EXTM+DZ group

Immunohistochemical findings for BMP-2 and Runx2 were shown in Fig. 3B, C. The

positive reactions of BMP-2 and Runx2 in the cytoplasm of PDLCs were recognized. The ratio of BMP-2 and Runx2 positive cells peaked at day 7 in the EXTM group. In contrast, the ratio of BMP-2 positive cells increased in a time-dependent manner in the EXTM+DZ group. The ratio of Runx2 positive cells peaked at day 14 in the EXTM+DZ group. Quantitative evaluations revealed that the ratios of BMP-2 and Runx2 positive cells in the EXTM+DZ group were significantly higher than that in the EXTM group on day 14 and 21 (Fig. 3D).

Gene and protein expressions of BMP-2 and Runx2 after application of TF in hPDLCs

The mRNA expressions of *BMP-2* and *Runx2* significantly increased in the TF+DZ group as compared to the TF and control groups in a time-dependent manner (Fig. 4A, B).

The protein expressions of BMP-2 and Runx2 significantly increased in the TF+DZ group in a time-dependent manner (Fig. 4C, D).

DISCUSSION

In this study, we investigated the effect of daidzein on relapse after EXTM. In the ratio of relapse, the EXTM+DZ group showed significantly decreased as compared to the EXTM group at 21 days. In addition, there was no significant difference between the EXTM and EXTM+DZ groups with respect to BMD and BV/TV from days 0 to 14. However, the BMD and BV/TV values in the EXTM+DZ group were significantly higher than those in the EXTM group on day 21. Mao et al. [18] showed that BMD and BV/TV of the alveolar bone on the tension side significantly increased after orthodontic stimulation. These results indicate that the application of TF induces bone formation, and

daidzein promote more bone formation on the tension side. Thus, daidzein has an effect on decreasing relapse to promote bone formation after the OTM.

The histological staining suggested that daidzein promoted the proliferation of PDLCs and differentiation into osteoblasts in the EXTM+DZ group. The ratio of BMP-2 positive cells in the EXTM+DZ group was significantly higher than that in the EXTM group on days 14 and 21 after EXTM. Furthermore, in vitro studies showed that daidzein significantly enhanced the mRNA and protein expressions of BMP-2 in hPDLCs after the application of TF in a time-dependent manner. The expression of BMP-2 in mesenchymal stem cells and osteoblasts promotes osteogenic differentiation of these cells and bone formation [19]. Nokhbehsaim et al. [20] have indicated that cyclic stretching increases the gene expression level of BMP-2 in PDLCs. Furthermore, daidzein stimulates osteoblastic differentiation at various stages from osteoprogenitors to terminally differentiated osteoblasts [21]. Therefore, the present results suggest that daidzein promotes bone formation through BMP-2 expression.

BMP-2 controls the expression and functions of Runx2 through Smad signaling [22]. BMP-2 acts through a complex serine-threonine kinase receptor mechanism to activate the downstream transcription factors, Smad1, 5, and 8, which are phosphorylated on serine residues [23]. Smad1, 5, and 8 form a complex with Smad4, and the complex is subsequently translocated into the nucleus to activate the transcription of Cbfa1/Runx2, which regulates bone formation [8]. Runx2 controls bone development and maturation by regulating osteoblastic differentiation and function. The expression of Runx2 is upregulated in preosteoblasts and immature osteoblasts, but is downregulated in mature osteoblasts [24]. In vivo studies showed that the ratio of Runx2 positive cells in the EXTM+DZ group was significantly higher than that in the EXTM group on days 14 and

21. However, the ratio of Runx2 positive cells in the EXTM+DZ group peaked at 14 day and decreased on day 21. The results of this study indicated that the differentiation of preosteoblasts and immature osteoblasts into mature osteoblasts were promoted by downregulation of Runx2 expression.

In vitro studies demonstrated that daidzein significantly enhanced the mRNA and protein expressions of Runx2 in hPDLCs after the application of TF in a time-dependent manner. Shen et al. [25] reported that increased Runx2 expression in a time-dependent manner under mechanical tension marked the beginning of osteogenic differentiation of PDLCs. The results of this study indicated that daidzein enhanced the early stage of the osteogenic lineage of PDLCs through the expression of Runx2.

In conclusion, daidzein decreased the ratio of relapse and increased BMD, BV/TV and the expressions of BMP-2 and Runx2 in PDLCs. These results suggest that daidzein may promote the differentiation of PDLCs into osteoblasts and bone formation.

ACKNOWLEDGEMANT

This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (16k11796).

CONFLICT OF INTEREST

The authors state that there is no conflict of interest in connection with this article.

REFERENCES

[1] Sadowsky C, Schneider BJ, BeGole EA, Tahir E. Long-term stability after orthodontic treatment: Nonextraction with prolonged retention. Am J Orthod Dentofacial Orthop

1994;106,243-249.

[2] Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004;364,149-155.

[3] Parker GR. Transseptal fibers and relapse following bodily retration of teeth: a histologic study. Am J Orthod 1972;61,331-44.

[4] Han G, Chen Y, Hou J, Liu C, Chen C, Zhuang J et al. Effects of simvastatin on relapse and remodeling of periodontal tissues after tooth movement in rats. Am J Orthod Dentofacial Orthop 2010;138,550.e1-550.e7.

[5] Hirate Y, Yamaguchi M, Kasai K. Effects of relaxin on relapse and periodontal tissue remodeling after experimental tooth movement in rats. Connect Tissue Res 2012;53:207-219.

[6] Kim TW, Yoshida Y, Yokoyama K, Sasaki T. An ultrastructural study of the effects of bisphosphonate administration on osteoclastic bone resorption during relapse of experimentally moved rat molars. Am J Orthod Dentofacial Orthop 1999;115: 645-653.

[7] Hu B, Yu B, Tang D, Li S, Wu Y. Daidzein promotes osteoblast proliferation and differentiation in OCT1 cells through stimulating the activation of BMP-2/Smads pathway. Genet Mol Res 2016;15:1-10.

[8] Liang W, Lin M, Li X, Li C, Gao B, Gan H et al. Icariin promotes bone formation via the BMP-2/Smad4 signal transduction pathway in the hFOB 1.19 human osteoblastic cell line. Int J Mol Med 2012;30:889-895.

[9] Baumert U, Golan I, Becker B, Hrala BP, Redlich M, Roos HA et al. Pressure simulation of orthodontic force in osteoblasts: a pilot study. Orthod Craniofac Res

2004;7:3-9.

[10] Ziros PG, Gil APR, Georgakopoulos T, Habeos I, Kletsas D, Basdra EK et al. The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. J Biol Chem 2002;277:23934-23941.

[11] Zhang Z, Li X, Zhao J, Jia W, Wang Z. Effect of autogenous growth factors released from platelet concentrates on the osteogenic differentiation of periodontal ligament fibroblasts: a comparative study. Peer J 2019; 7:e7984.

[12] Saito Y, Yoshizawa T, Takizawa F, Ikegami M, Ishibashi O, Okuda K et al. A cell line with characteristics of the periodontal ligament fibroblasts is negatively regulated for mineralization and Runx2/Cbfa1/Osf2 activity, part of which can be overcome by bone morphogenetic protein-2. J Cell Sci 2002;115:4191-200.

[13] Tang N, Zhao Z, Zhang L, Yu Q, Li J, Xu Z, et al. Up-regulated osteogenic transcription factors during early response of human periodontal ligament stem cells to cyclic tensile strain. Arch Med Sci 2012;8:422-430.

[14] Waldo CM. Method for the study of tissue response to tooth movement. J Dent Res 1953;32:690-691.

[15] Bae SH, Ha MH, Choi E-Y, Choi J-I, Choi IS, Kim S-J. Effects of daidzein on alveolar bone loss and internal microstructures of bone in a rat model of experimental periodontitis: a study using micro-computed tomography. J Periodontal Res 2016;51:250-256.

[16] Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. J Dent Res 1988;67: 66-70.

[17] Takano M, Yamaguchi M, Nakajima R, Fujita S, Kojima T, Kasai K. Effects of

relaxin on collagen type I released by stretched human periodontal ligament cells. Orthod Craniofac Res 2009;12:282-288.

[18] Mao Y, Wang L, Zhu Y, Liu Y, Dai H, Zhou J et al. Tension force-induced bone formation in orthodontic tooth movement via modulation of the GSK-3β/β-catenin signaling pathway. J Mol Histol 2018;49:75-84.

[19] Huang W, Rudkin GH, Carlsen B, Ishida K, Ghasri P, Anvar B et al. Overexpression of BMP-2 modulates morphology, growth, and gene expression in osteoblastic cells. Exp Cell Res 2002;274:226-234.

[20] Nokhbehsaim M, Deschner B, Winter J, Bourauel C, Rath B, Jäger A et al. Interactions of regenerative, inflammatory and biomechanical signals on bone morphogenetic protein- 2 in periodontal ligament cells. J Periodontal Res 2011;46: 374-381.

[21] Jia TL, Wang HZ, Xie LP, Wang XY, Zhang RQ. Daidzein enhances osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production. Biochem Pharmacol 2003;65:709-715.

[22] Nishimura R, Hata K, Harris SE, Ikeda F, Yoneda T. Core-binding factor alpha 1 (Cbfa1) induces osteoblastic differentiation of C2C12 cells without interactions with Smad1 and Smad5. Bone 2002;31:303-312.

[23] Wang L, Zhang X, Guo Y, Chen X, Li R, Liu L et al. Involvement of BMPs/Smad signaling pathway in mechanical response in osteoblast. Cell Physiol Biochem 2010;26:1093-1102.

[24] Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res 2010;399:189-195.

[25] Shen T, Qiu L, Chang H, Yang Y, Jian C, Xiong J et al. Cyclic tension promotes

osteogenic differentiation in human periodontal ligament stem cells. Int J Clin Exp Pathol 2014;7;7872-7880.

Table 1. DNA primers applied in Real-time PCR analysis

Gene	Primer sequences
BMP-2	Forward: 5 - GTGGTGGAAGTGGCCCACTT-3
	Reverse: 5'- CTGTTTGTGTTTGGCTTGACG-3
RUNX2	Forward: 5'- CAGATGGGACTGTGGTTACTGT-3'
	Reverse: 5 - GTGAAGACGGTTATGGTCAAGG-3
GAPDH	Forward: 5 - GCACCGTCAAGGCTGAGAAC-3
	Reverse: 5 - TGGTGAAGACGCCAGTGGA-3

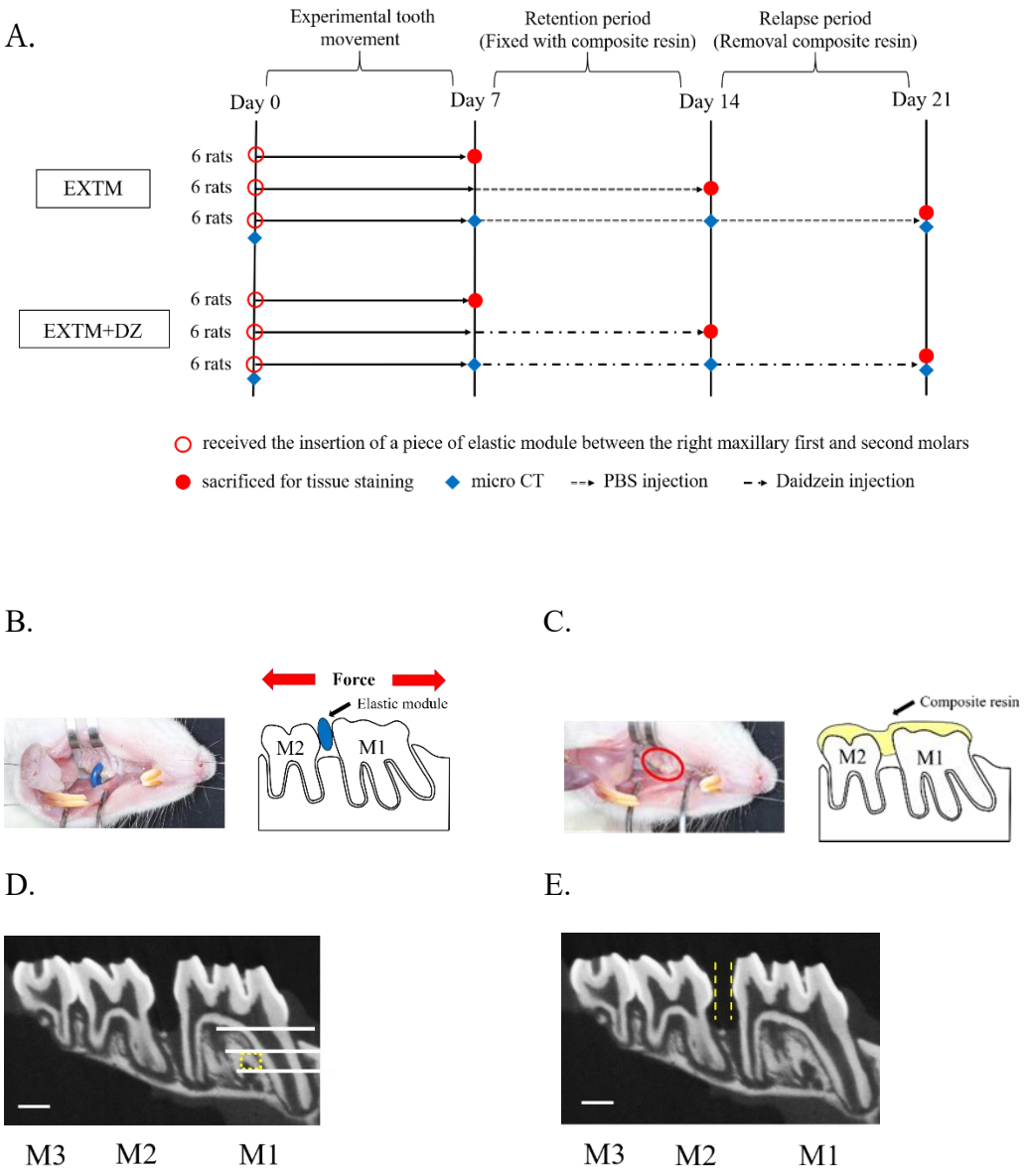


FIGURE 1. Experimental tooth movement (EXTM) in rats.

(A) Experimental timings for each group. (B) Insertion of an orthodontic elastic module between the left maxillary first (M1) and second molars (M2) of rats. (C) After the EXTM, M1 and M2 were fixed with composite resin to maintain the distance. (D) BMD and BV/TV were calculated in the ROI by micro- CT analysis (bar, 1 mm).

(E) Distance of relapse after EXTM was measured as the least intermolar distance between M1 and M2 (bar, 1 mm).

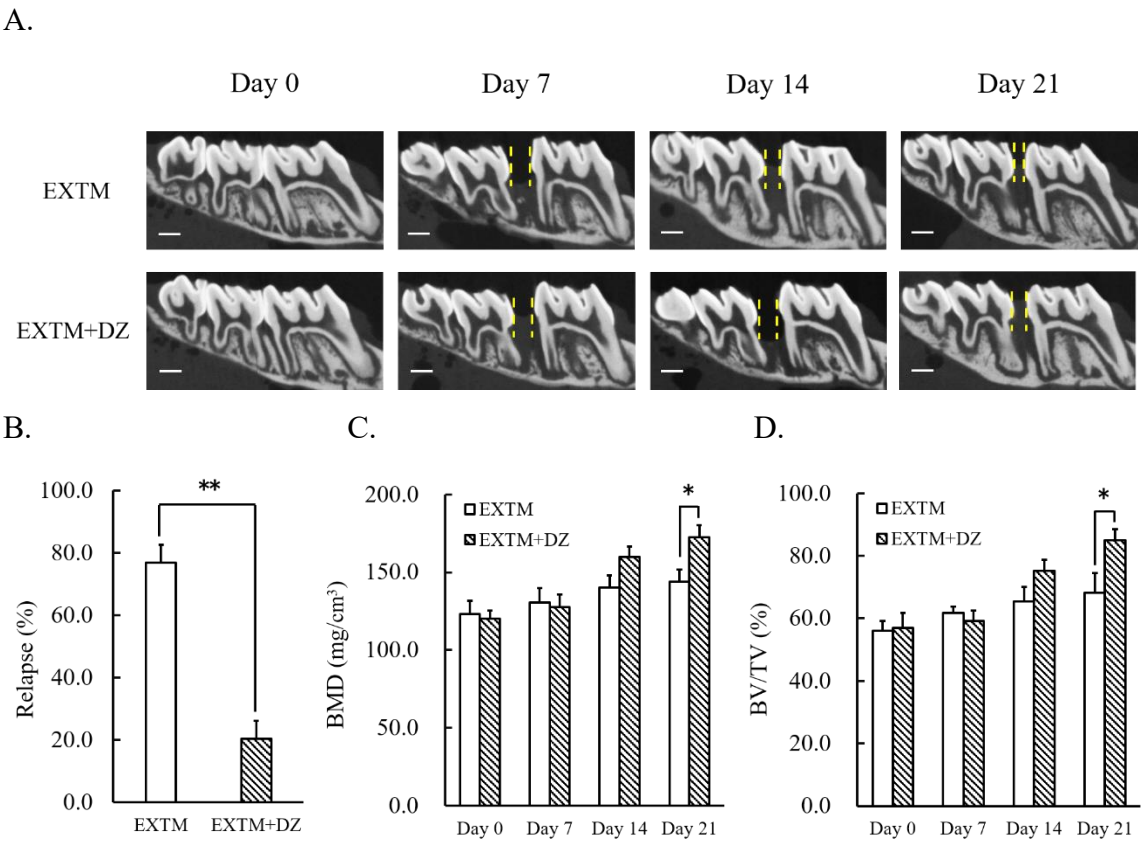
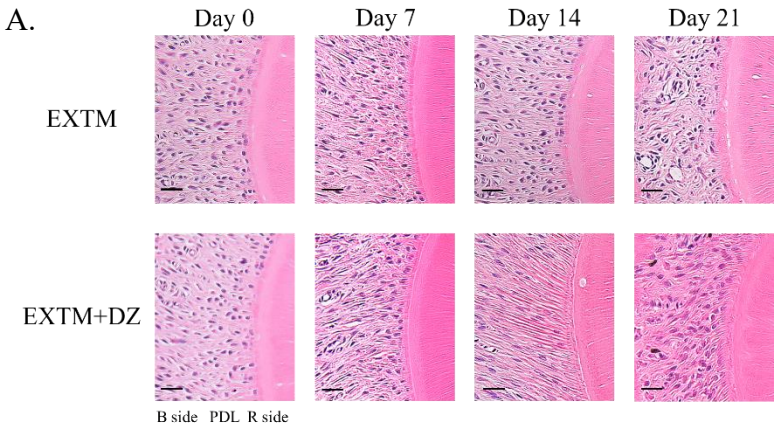
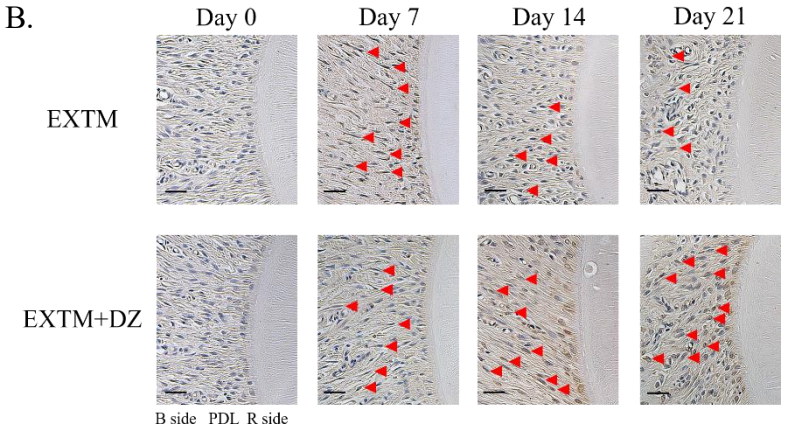
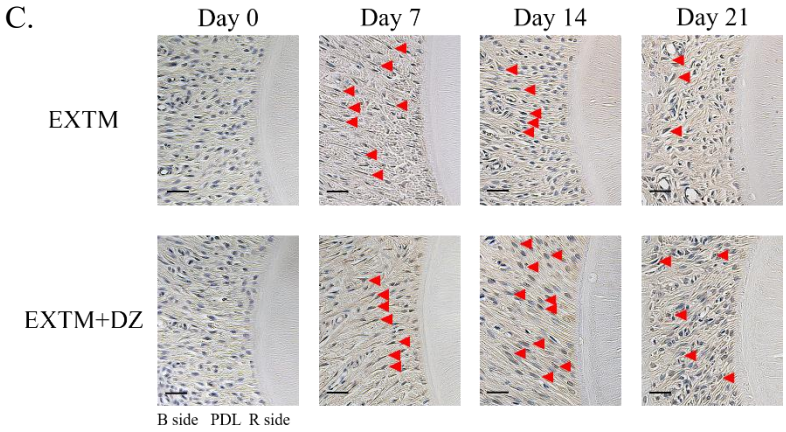


FIGURE 2. Micro-CT analysis.

(A) Micro-CT images showing the maxillary left first (M1) and second molars (M2) in the experimental tooth movement (EXTM) group and EXTM+daidzein (DZ) group (bar, 1 mm). (B) Ratio of relapse in the EXTM+DZ group was significantly lower than that of the EXTM group on day 21. (C) BMD and (D) BV/TV were significantly higher in the EXTM+DZ group as compared to the EXTM group on day 21. Data were represented as mean ± standard deviation (n = 6), and *p < 0.05; **p < 0.01 indicated the levels of statistical significance.


A.
Day 0
Day 7
Day 14
Day 21
EXTM
EXTM+DZ
B side PDL R side


B.
Day 0
Day 7
Day 14
Day 21
EXTM
EXTM+DZ
B side PDL R side


C.
Day 0
Day 7
Day 14
Day 21
EXTM
EXTM+DZ
B side PDL R side

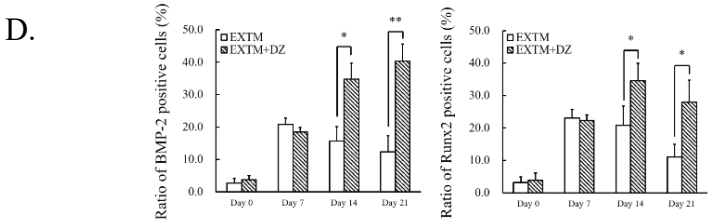

D.
Ratio of BMP-2 positive cells (%)
EXTM
EXTM+DZ
50.0
40.0
30.0
20.0
10.0
0.0
Day 0
Day 7
Day 14
Day 21
*
**
Ratio of Runx2 positive cells (%)
EXTM
EXTM+DZ
50.0
40.0
30.0
20.0
10.0
0.0
Day 0
Day 7
Day 14
Day 21
*
*

FIGURE 3. Histological staining and immunohistochemical staining for BMP-2 and Runx2.

(A) histological staining (B side, bone side; R side, root side; original magnification, ×200; bar, 50 μm). (B) Immunohistochemical staining for BMP-2 (B side, bone side; R side, root side; original magnification, ×200; bar, 50 μm; Red arrow heads indicate BMP-2-positive cells). (C) Immunohistochemical staining for Runx2 (B side, bone side; R side, root side; original magnification, ×200; bar, 50 μm; Red arrow heads indicate Runx-2-positive cells). (D) Ratios of BMP-2 and Runx2 positive cells in the experimental tooth movement (EXTM)+daidzein (DZ) group were significantly higher than that of the EXTM group on day 14 and 21. Data were represented as mean ± standard deviation (n = 6), and the levels of statistical significance were indicated as $*p < 0.05$ and $**p < 0.01$.

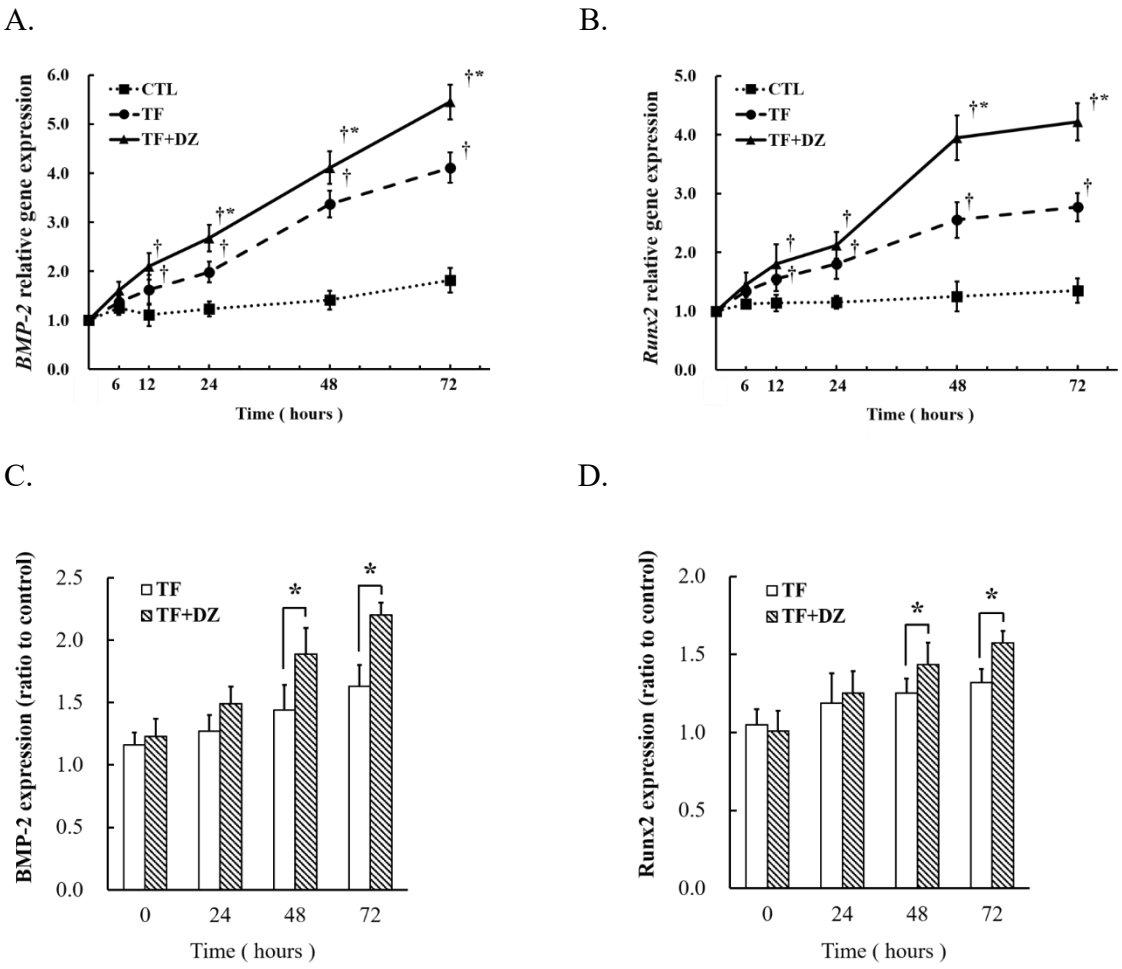


FIGURE 4. Enhanced expression of osteogenic markers in human periodontal ligament cells by daidzein (DZ).

The mRNA expressions of *BMP-2* (A) and *Runx2* (B) were detected using real-time PCR. Protein expressions of BMP-2 (C) and Runx2 (D) were detected using ELISA. The relative expressions of each protein were compared with that of the control (CTL). Data were represented as mean ± standard deviation (n = 6), and the levels of statistical significance were indicated as †$p < 0.05$ vs. CTL group and *$p < 0.05$ vs. tension force (TF) group.