

**Vitamin C and eggshell membrane facilitate orthodontic tooth movement and induce histological changes in the periodontal tissue**

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<sup>1</sup> Abbreviations: VC, vitamin C; ESM, eggshell membrane; PDL, periodontal ligament; ODS, osteogenic disorder Shionogi; HE, hematoxylin-eosin; ROIs, regions of interest

## **Abstract**

*Objectives:* Collagen remodeling of the periodontal tissue is an important mechanism that involves several biologically active substances to accelerate orthodontic tooth movement. It is known that Vitamin C (VC) enhances collagen production and induces tooth movement. Moreover, the eggshell membrane (ESM) is an integral component of various formulations used to promote wound healing. The purpose of our study was to determine the effects of combined treatment with VC and ESM on periodontal tissues during tooth movement.

*Methods:* Nine-week-old male osteogenic disorder Shionogi rats were randomized into four groups: control, VC, ESM, and VC + ESM. The control group was given tap water, and the VC, ESM, and VC + ESM groups were orally administered 0.1% VC solution, 1 wt% ESM solution, and a combination of 0.1 wt% VC and 1 wt% ESM solutions, respectively. A force of 25 or 75 g was applied for 10 days to produce orthodontic tooth movement. Distances of tooth movement were measured on days 3, 7, and 10 of treatment. Histological examination of the periodontal ligament was performed to determine the increase in type I and III collagen levels in response to treatment.

*Results:* Distances of tooth movement were significantly greater in the VC + ESM

group than in the control group. The compression area of the alveolar bone showed increased osteoclastic activity and higher levels of bone resorption in the VC + ESM group. Expression levels of type I and III collagen in the tension area of the alveolar bone were higher in the VC + ESM group than in the control group.

*Conclusions:* This study revealed that the combined administration of VC and ESM accelerated tooth movement by protecting the periodontal tissue during orthodontic treatment. The combined clinical application of VC and ESM could potentially shorten orthodontic treatment time.

**Keywords:** Orthodontic tooth movement, Vitamin C, Eggshell membrane, Periodontal tissue, Collagen

## **1. Introduction**

Orthodontic tooth movement adversely affects both the cells and the extracellular matrix of the periodontal tissue, including the periodontal ligament (PDL) and the alveolar bone [1, 2]. The PDL is a connective tissue that attaches the tooth to the alveolar bone [3] and consists of cells such as fibroblasts, osteoblasts, and osteoclasts. Fibroblasts are responsible for the synthesis and maintenance of the extracellular matrix [1]. Fibrillar collagens form the major component of the extracellular matrix of the PDL, with type I and III collagen constituting the majority. These collagens colocalize with each other in the extracellular matrix [4]. Type III collagen is expressed in the early stages of wound healing [5] and plays an important role in regulating type I collagen synthesis [4]. Both types I and III collagen contribute to periodontal tissue repair [6] and extracellular matrix metabolism [7]. Remodeling of the extracellular matrix in PDL is necessary to facilitate tooth movement [8]. The PDL plays a key role in alveolar bone remodeling associated with orthodontic treatment [9].

Several studies have been conducted to achieve remodeling of the periodontal tissue by using various biological modulators to accelerate orthodontic tooth movement [10-12]. A previous study has reported that vitamin C (VC) administration can induce orthodontic tooth movement [13]. VC is required for the hydroxylation of procollagen,

proline, and lysine. Furthermore, it enhances collagen production by upregulating osteocalcin expression [14] and promotes osteoblast differentiation by modulating the interaction between type I collagen and  $\alpha 2\beta 1$  integrin [15]. Moreover, VC is known to upregulate the expression of  $\alpha 2\beta 1$  integrin, the major receptor for type I and III collagen [15, 16]. Collagen synthesis by fibroblasts is inhibited in the absence of VC [17]. The eggshell membrane (ESM) contains type I collagen and has been used as an integral component of various formulations to enhance wound healing [18]. Moreover, ESM exerts an anti-aging effect by increasing type III collagen levels [19]. Although VC and ESM have been shown to upregulate the expression of both collagen types I and III, their combined effects on tooth movement remain unclear.

This study aimed to determine the effects of the combined administration of VC and ESM on orthodontic tooth movement. In this study, the morphological tissue changes in VC-deficient osteogenic disorder Shionogi (ODS) rats were studied by applying different orthodontic forces (25 g or 75 g).

## **2. Materials and methods**

### *2.1. Animals*

A total of 20 nine-week-old male ODS rats (CLEA Japan, Tokyo, Japan) were housed in the animal laboratory of Kanagawa Dental University, Japan. The rats were housed at a room temperature of  $22 \pm 3$  °C with a relative humidity of 50-60% under a 12-h light/dark cycle. They were given *ad libitum* access to food and water. All experimental procedures with animals were performed in accordance with the Animal Experiment Guidelines of Kanagawa Dental University. The approval numbers are 16-025, 17-004, and 18-048.

### *2.2. Experimental Tooth Movement*

ODS rats were randomized into four groups: control, VC (Itoh Kanpo Pharmaceutical Co. Ltd., Osaka, Japan); ESM (Kewpie Corporation, Tokyo, Japan); and VC + ESM. All groups were orally administered 0.1 wt% VC solution for 1 week before this examination. The weight of the rats ranged from 204 g to 276 g after 1 week. While the control group was given tap water, the VC, ESM, and VC + ESM groups were orally administered 0.1% VC solution, 1 wt% ESM solution, and a combination of 0.1 wt% VC and 1 wt% ESM solutions, respectively. Simultaneously, orthodontic tooth

movement was initiated and observed in all rats (shown in Fig. 1A, B). A groove was created in the maxillary left incisor by the elevation of the interdental papilla. A wire ligature was attached to the maxillary left first molar through one end of a nickel-titanium closed coil spring (1.5 N). Another wire ligature was attached to the groove of the maxillary left incisor through the other end of the spring. Wire ligatures were affixed with a dental composite material. A constant force of 0.75 N per tooth was applied [20].

A mesial force of 75 g was applied to the maxillary left first molar. A nickel-titanium closed coil spring (0.5 N) was similarly attached to the maxillary right first molar, and a mesial force of 25 g was applied [21, 22].

The distance of tooth movement was measured for 10 days [23]. On days 3, 7, and 10, the interproximal distance between the first and second molar was measured using a combination of 30-, 50-, and 110- $\mu\text{m}$ -thick contact gauges.

### *2.3. Histological Observations*

The maxillae were collected and fixed in 10% neutral buffered formalin after 10 days of observation. The samples were decalcified with 10% HCl for 1 week and embedded in paraffin to prepare 5- $\mu\text{m}$ -thick horizontal sections. The observation site was at the upper



one third of the area between the root furcation and the root apex. Subsequently, hematoxylin-eosin (HE) stained and immunostained sections were observed under an optical microscope.

For immunofluorescence, antigen retrieval was performed with 0.1% Triton X (Pfizer Inc., NY, USA)/PBS, and the tissues were blocked with 10% goat serum (Sigma-Aldrich Inc., St. Louis, MO, USA). The following primary antibodies were used at 1:100 dilution: polyclonal anti-rabbit collagen type I (Bioss Antibodies Inc., Woburn, MA, USA) and monoclonal anti-mouse collagen type III (Novus Biologicals, Centennial, CO, USA). Monoclonal goat anti-mouse IgG (Cell Signaling Technology Japan, Tokyo, Japan) was used at 1:1000 dilution as the secondary antibody. The slides were visualized under a Zeiss Axioimager microscope (Carl Zeiss, Oberkochen, Germany). Digital images were acquired using AxioVision 4.8.2 (Carl Zeiss), and the type I and III collagen-stained areas were quantified. Osteoclast regions of interest (ROIs) were enumerated on the experimental and control sides. The ROIs consisted of the mesial PDL, mesial bone, distal PDL, and distal bone (Fig. 1C) [24]. Areas of immunostained collagen in the ROIs were measured using Image J (vol. 48, NIH) [25].

#### *2.4. India ink vascular casting*

Four specimens from each group were fixed by perfusion. Following India ink perfusion, they were immersed in 4% paraformaldehyde for 24 h. The samples were decalcified with 10% HCl for 1 week, embedded in paraffin, and cut into 50- $\mu$ m-thick horizontal sections. Subsequently, the sections were stained with HE and observed under an optical microscope.

### *2.5. Quantitative Analysis*

The number of osteoclasts and immunostained areas were analyzed using ImageJ. Data are expressed as the mean  $\pm$  standard deviation of the mean. The experimental data were analyzed by ANOVA followed by Student's *t*-test and Tukey's test using GraphPad Prism (v. 6.05.; GraphPad Software Inc., San Diego, CA, USA). The significance level was set at  $p < 0.05$ .

### 3. Results

#### 3.1. Tooth Movement

Distances of tooth movement in four groups are shown in Fig. 2. At both mesial forces, the distance of tooth movement at each time point after spring insertion in the VC + ESM group was significantly greater than that in other groups ( $p < 0.05$ ). At a mesial force of 75 g, the distances of tooth movement at each time point in the VC and ESM groups were significantly greater than in the control group ( $p < 0.05$ ). No significant difference in the distance of tooth movement was observed between the VC and ESM groups on days 7 and 10. At a mesial force of 25 g, the distances of tooth movement at each time point in the VC and ESM groups were significantly greater than in the control group ( $p < 0.05$ ). Moreover, the distance of tooth movement in the ESM group was significantly greater than that in the control group ( $p < 0.05$ ) at each time point. Furthermore, there was no significant difference in the distance of tooth movement between the control and VC groups on days 3 and 7. These results indicate that the combined administration of VC and ESM facilitated tooth movement. Thus, further analysis was performed in the control and VC + ESM groups.

### *3.2. HE staining*

To determine the histological changes in the periodontal tissue induced by tooth movement, we examined the HE staining pattern in each group.

At both mesial forces, PDL width on the tension side after 10 days of tooth movement was greater in the VC + ESM group than that in the control group (Fig. 3A, B, E, F). A remarkable neonatal bone formation on the tension side was observed in the VC + ESM group. PDL width on the compression side was lesser in the VC + ESM group than that in the control group (Fig. 3C, D, G, H). The compression side showed numerous osteoclasts in Howship's lacunae along the bone surface. These results indicated that the combined administration of VC and ESM accelerated tooth movement and remodeling of the periodontal tissue.

Mature osteoclasts in Howship's lacunae along the bone surface were enumerated in the control and the VC + ESM group at each force (Fig. 4). At both mesial forces, the number of osteoclasts in the VC + ESM group was significantly higher than that in the control group ( $p < 0.05$ ).

### *3.3 Collagen immunohistochemistry in PDL*

The immunohistochemical staining of type I and III collagen in PDL is shown in Figs. 5 and 6. At both mesial forces, the expression of type I and III collagen in the VC + ESM group was higher than that in the control group on the tension side. Neither type I nor type III collagen was detected in the negative control samples (slides not treated with primary or secondary antibody) (data not shown).

#### *3.4. India ink vascular casting*

Blood vessels in the PDL were observed by injecting India ink. At both mesial forces, thick and stretched blood vessels were observed in the PDL of the VC + ESM group on the tension side (Fig. 7A, B, E, F). A vascular loop in the PDL was observed in the VC + ESM group, and this loop was more pronounced at the mesial force of 75 g than 25 g. A few blood vessels in the PDL were observed in both groups on the compression side.

## **4. Discussion**

To our knowledge, this is the first study to show that the combined administration of VC and ESM accelerates tooth movement. Previous studies have demonstrated that the administration of VC promotes tooth movement [13]. Moreover, it is well established that VC and ESM promote collagen synthesis [14, 19]. The findings of the present study indicated that a combination of VC and ESM had an additive effect on tooth movement. Our results showed that the distances of tooth movement in the VC + ESM group were significantly greater than the control group. VC plays an essential role in collagen synthesis and bone remodeling in the periodontal tissue [15]. Moreover, VC deficiency during orthodontic tooth movement can result in tissue degradation [26]. VC promotes not only the differentiation of osteoblasts but also upregulates tartrate-resistant acid phosphatase expression in osteoclast precursors [27]. Consistent with these findings, we observed a significantly high number of osteoclasts in the VC + ESM group. Furthermore, osteoclasts in Howship's lacunae were observed on the bone surface. However, a single administration of VC or ESM did not induce a significant increase in tooth movement over the control. These results raise concerns about the effective

concentration of VC and ESM. A previous study has shown that daily administration of 500 mg of VC is more effective for fracture healing than 200 or 1500 mg [28]. ODS rats are unable to synthesize VC [29] and require a minimum dose of 1000 mg/kg of VC [30]. A 0.1 % VC solution was used in this study. Moreover, a 1% ESM solution was administered to the ESM and VC + ESM groups. A previous study has reported that a 1% ESM solution can significantly activate NF- $\kappa$ B [31]. In the present study, we found that the administration of appropriate concentrations of VC and ESM induced tooth movement at an early stage.

At orthodontic forces of 25 g and 75 g, the VC + ESM group showed more tooth movement than other groups. Interestingly, the distances of tooth movement in the VC + ESM group were greater at a 75-g force than a 25-g force. Previous studies have demonstrated that a force of 25 g is ideal for orthodontic molar tooth movement [21, 32, 33]. An *et al.* demonstrated that a 25-g force maintains the morphological features of periodontal tissues during tooth movement [21]. Our results showed that the distance of tooth movement in the VC + ESM group was greater with a force of 75-g than with 25-g. Moreover, tooth movement at a force of 75 g increased the number of osteoclasts on the compression side and upregulated collagen expression on the tension side of the periodontal ligament. Thus, our findings suggest that the administration of VC and ESM

can potentially protect the periodontal tissues from force-related damage during orthodontic treatment.

In this study, the expression of type I and III collagen in the VC + ESM group was markedly high on the tension side. Type III collagen is expressed before type I collagen during wound healing, and the upregulation of type I collagen expression promotes periodontal tissue healing [4, 5]. On the compression side, the expression of type III collagen was significantly higher in the VC + ESM group than in the control group. The collagenous extracellular matrix acted as a scaffold for osteoclasts [34]. The results of HE staining showed that the number of osteoclasts in the VC + ESM group was significantly higher than that in the control group, suggesting that bone resorption on the compression side was promoted by the increased number of osteoclasts. The expression of extracellular matrix proteins, including type III collagen, matrix metalloproteinase-2, and decorin increased after the fibroblasts were treated with ESM proteins [35]. The results of India ink vascular casting revealed the formation of a vascular loop in the PDL of the VC + ESM group. Another study has shown that angiogenesis significantly accelerates alveolar bone formation [36]. The administration of VC and ESM increased collagen expression and enhanced bone remodeling, thereby suggesting a potential role in promoting tooth movement and protecting periodontal



tissues. Future studies should examine the effects of VC and ESM on alkaline phosphatase activity and the expression of  $\alpha 2\beta 1$  receptors, matrix metalloproteinase-2, and decorin.

This study has several limitations. No appreciable difference in the distance of tooth movement was observed between the control and the VC or ESM group at forces of 25 and 75 g, compared with the VC + ESM group. The effects of VC and ESM were apparent from the immunohistochemical observation of the VC + ESM group, compared with the control group. It has been reported that VC and ESM upregulate collagen expression. Although the concentration of VC and ESM in our study was based on previous studies, the single administration of VC or ESM did not sufficiently accelerate tooth movement. It is well-known that VC overdose can cause adverse effects. Further studies may be needed to evaluate the effects of different concentrations of VC to determine its potential benefits for clinical use. Moreover, the present study did not monitor the water intake of the animals. The water bottles provided to the VC or ESM group were regularly replaced by the experimenters. Further studies using a precise measurement instrument should confirm the effect of water intake.

## **5. Conclusions**

Our study provides evidence that the administration of VC and ESM accelerates tooth movement. Importantly, the combined administration of VC and ESM promoted periodontal tissue recovery in the early stages of wound healing during orthodontic treatment. Therefore, the clinical application of VC and ESM may shorten orthodontic treatment time.

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## **Conflict of interest**

The authors declare that they have no conflict of interest.

## References

- [1] Krishnan V, Davidovitch Z. On a path to unfolding the biological mechanisms of orthodontic tooth movement. *J Dent Res.* 2009;88:597-608.
- [2] Kanzaki H, Nakamura Y. Orthodontic tooth movement and HMGB1. *J Oral Biosci.* 2018;60:49-53.
- [3] Dorow C, Krstin N, Sander FG. Determination of the mechanical properties of the periodontal ligament in a uniaxial tensional experiment. *J Orofac Orthop.* 2003;64:100-7.
- [4] Liu X, Wu H, Byrne M, Krane S, Jaenisch R. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci U S A.* 1997;94:1852-6.
- [5] Haruka M, Kazuya T, Tsurayuki T, Akio T, Makoto U. Comparison of the influence of a new synthetic peptide and enamel matrix derivative in the early wound healing process of artificial periodontal tissue defects in rats. *Jpn J Conserv Dent.* 2018;61:17-29.
- [6] Maehata Y, Lee M-C-i, Hata R-I. Roles of collagen molecules in growth and differentiation of human osteoblasts. *J Oral Biosci.* 2009;51:72-80.
- [7] Chen X, Li N, LeleYang, Liu J, Chen J, Liu H. Expression of collagen I, collagen III and MMP-1 on the tension side of distracted tooth using periodontal ligament

distraction osteogenesis in beagle dogs. Arch Oral Biol. 2014;59:1217-25.

[8] Yoshida Y, Sasaki T, Yokoya K, Hiraide T, Shibasaki Y. Cellular roles in relapse processes of experimentally-moved rat molars. J Electron Microsc (Tokyo). 1999;48:147-57.

[9] McCormack SW, Witzel U, Watson PJ, Fagan MJ, Groning F. The biomechanical function of periodontal ligament fibres in orthodontic tooth movement. PLoS One. 2014;9:e102387.

[10] An S, Zhang Y, Chen Q, Xiong B, Hao J, Zheng Y, Zhou X, Wang J. Effect of systemic delivery of substance P on experimental tooth movement in rats. Am J Orthod Dentofacial Orthop. 2019;155:642-9.

[11] Zou Y, Xu L, Lin H. Stress overload-induced periodontal remodelling coupled with changes in high mobility group protein B1 during tooth movement: An in-vivo study. Eur J Oral Sci. 2019;127:396-407.

[12] Chang JH, Chen PJ, Arul MR, Dutra EH, Nanda R, Kumbar SG, Yadav S. Injectable RANKL sustained release formulations to accelerate orthodontic tooth movement. Eur J Orthod. 2019;*in press*.

[13] Makrygiannakis MA, Kaklamanos EG, Athanasiou AE. Does common prescription medication affect the rate of orthodontic tooth movement? A systematic review. Eur J

Orthod. 2018;40:649-59.

[14] Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res.* 1992;7:235-46.

[15] Ishikawa S, Iwasaki K, Komaki M, Ishikawa I. Role of ascorbic acid in periodontal ligament cell differentiation. *J Periodontol.* 2004;75:709-16.

[16] Kim JK, Xu Y, Xu X, Keene DR, Gurusiddappa S, Liang X, Wary KK, Hook M. A novel binding site in collagen type III for integrins alpha1beta1 and alpha2beta1. *J Biol Chem.* 2005;280:32512-20.

[17] Ten Cate AR, Deporter DA, Freeman E. The role of fibroblasts in the remodeling of periodontal ligament during physiologic tooth movement. *Am J Orthod.* 1976;69:155-68.

[18] Yamauchi K, Manabe N, Matsumoto Y, Yamauchi KE. Increased collagen accumulation in eggshell membrane after feeding with dietary wood charcoal powder and vinegar. *Connect Tissue Res.* 2013;54:416-25.

[19] Ohto-Fujita E, Shimizu M, Sano S, Kurimoto M, Yamazawa K, Atomi T, Sakurai T, Murakami Y, Takami T, Murakami T, Yoshimura K, Hasebe Y, Atomi Y. Solubilized eggshell membrane supplies a type III collagen-rich elastic dermal papilla. *Cell Tissue Res.* 2019;376:123-35.

[20] Kirschneck C, Meier M, Bauer K, Proff P, Fanghanel J. Meloxicam medication reduces orthodontically induced dental root resorption and tooth movement velocity: A combined in vivo and in vitro study of dental-periodontal cells and tissue. *Cell Tissue Res.* 2017;368:61-78.

[21] An J, Li Y, Liu Z, Wang R, Zhang B. A micro-CT study of microstructure change of alveolar bone during orthodontic tooth movement under different force magnitudes in rats. *Exp Ther Med.* 2017;13:1793-8.

[22] Novaes AP, da Rocha MJ, Leite-Panissi CR. Tooth movement activates the central amygdala and the lateral hypothalamus by the magnitude of the force applied. *Angle Orthod.* 2010;80:111-5.

[23] Hashimoto F, Kobayashi Y, Matakai S, Kobayashi K, Kato Y, Sakai H. Administration of osteocalcin accelerates orthodontic tooth movement induced by a closed coil spring in rats. *Eur J Orthod.* 2001;23:535-45.

[24] Nilforoushan D, Manolson MF. Expression of nitric oxide synthases in orthodontic tooth movement. *Angle Orthod.* 2009;79:502-8.

[25] Tejiram S, Zhang J, Travis TE, Carney BC, Alkhalil A, Moffatt LT, Johnson LS, Shupp JW. Compression therapy affects collagen type balance in hypertrophic scar. *J Surg Res.* 2016;201:299-305.

- [26] Litton SF. Orthodontic tooth movement during an ascorbic acid deficiency. *Am J Orthod.* 1974;65:290-302.
- [27] Ragab AA, Lavish SA, Banks MA, Goldberg VM, Greenfield EM. Osteoclast differentiation requires ascorbic acid. *J Bone Miner Res.* 1998;13:970-7.
- [28] Aim F, Klouche S, Frison A, Bauer T, Hardy P. Efficacy of vitamin C in preventing complex regional pain syndrome after wrist fracture: A systematic review and meta-analysis. *Orthop Traumatol Surg Res.* 2017;103:465-70.
- [29] Mizushima Y, Harauchi T, Yoshizaki T, Makino S. A rat mutant unable to synthesize vitamin C. *Experientia.* 1984;40:359-61.
- [30] Horio F, Ozaki K, Kohmura M, Yoshida A, Makino S, Hayashi Y. Ascorbic acid requirement for the induction of microsomal drug-metabolizing enzymes in a rat mutant unable to synthesize ascorbic acid. *J Nutr.* 1986;116:2278-89.
- [31] Ruff KJ, Durham PL, O'Reilly A, Long FD. Eggshell membrane hydrolyzates activate NF-kappaB in vitro: Possible implications for in vivo efficacy. *J Inflamm Res.* 2015;8:49-57.
- [32] Ren Y, Maltha JC, Kuijpers-Jagtman AM. The rat as a model for orthodontic tooth movement--a critical review and a proposed solution. *Eur J Orthod.* 2004;26:483-90.
- [33] Waldo CM, Rothblatt JM. Histologic response to tooth movement in the laboratory



rat; procedure and preliminary observations. *J Dent Res.* 1954;33:481-6.

[34] Alford AI, Kozloff KM, Hankenson KD. Extracellular matrix networks in bone remodeling. *Int J Biochem Cell Biol.* 2015;65:20-31.

[35] Ohto-Fujita E, Konno T, Shimizu M, Ishihara K, Sugitate T, Miyake J, Yoshimura K, Taniwaki K, Sakurai T, Hasebe Y, Atomi Y. Hydrolyzed eggshell membrane immobilized on phosphorylcholine polymer supplies extracellular matrix environment for human dermal fibroblasts. *Cell Tissue Res.* 2011;345:177-90.

[36] To M, Su CY, Hidaka K, Okudera T, Matsuo M. Effect of advanced platelet-rich fibrin on accelerating alveolar bone formation in dogs: A histological and immunofluorescence evaluation. *Anat Sci Int.* 2019;94:238-44.

## **Figure legends**

### **Fig. 1. Experimental procedures and analysis method**

Strong ( $75 \text{ g/mm}^2$ ) and weak ( $25 \text{ g/mm}^2$ ) orthodontic forces were applied within the physiological range (A). A closed coil spring was attached between the first molar and an incisor. The distance between the first and second molar was measured using a contact gauge (B). Regions of interest (ROI) are areas measuring  $200 \times 100\text{-}\mu\text{m}^2$  surrounding the periodontal ligament (C).

### **Fig. 2. Changes in the distance of tooth movement ( $\mu\text{m}$ ) with time**

The experimental animals were divided into four groups (control, VC, ESM, and VC + ESM), and the experimental period following force application was 3, 7, and 10 days.

At both 75 g (A) and 25 g (B) forces, the distance of tooth movement in the VC + ESM group was significantly greater than that in other groups ( $p < 0.05$ ). The VC + ESM and control groups were compared in this experiment.

### **Fig. 3. Histological analysis (HE staining)**

At both forces, the width of the periodontal ligament increased on the tension side and decreased on the compression side in the VC + ESM group. The phenomenon was more

pronounced in the 75-g group (B, D).

The number of dilated blood vessels ( $\rightarrow$ ) along the alveolar bone wall to root surface (B, F) on the tension side in the VC + ESM group was higher than that in the control group (A, E).

Osteoclasts in Howship's lacunae ( $\leftarrow$ ) on the alveolar bone surface were observed on the compression side (D, H). HE staining results revealed significant remodeling of the periodontal tissue in the VC + ESM group. Bar = 50  $\mu$ m.

#### **Fig. 4. HE analysis of osteoclasts**

The number of osteoclasts in Howship's lacunae along a bone surface on the compression side in ROI was calculated from HE sections ( $n = 5$ ). At both orthodontic forces of 75 g or 25 g, the number of osteoclasts in the VC + ESM group was significantly higher than that in the control group ( $p < 0.05$ ). Numerous osteoclasts were detected at the application of 75-g force.

#### **Fig. 5. Immunostaining analysis for type I and III collagen**

Immunostained sections for type I and III collagen in the control and the VC + ESM groups on the tension and compression sides at mesial forces of 25 g and 75 g are

shown. Type I collagen is stained in green and type III collagen in red. A merged image was acquired by superposition. Type III collagen (I, J, K, L) colocalized with type I collagen (E, F, G, H), indicating that type I collagen is expressed using type III collagen as a scaffold. Bar = 50  $\mu$ m.

### **Fig. 6. Immunostaining analysis of collagen fibers**

The immunostained areas for type I and III collagen on the tension side and compression side in ROIs at 75 g and 25 g mesial forces were measured. At both orthodontic forces of 75 g and 25 g, the expression of type I and III collagen was higher in the VC + ESM group than that in the control group on the tension side;  $*p < 0.05$ .

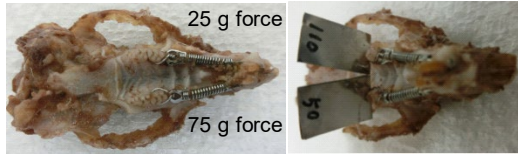
### **Fig. 7. Analysis of vasculature and periodontal ligament fiber (India ink injection, H-E staining)**

The India ink injected into the periodontal ligament blood vessel is shown as a black line. On the tension side, the blood vessel appears as a long hairpin-like loop along the stretched fiber. The blood vessels in the 75-g group (B) were denser and thicker than those in the 25-g group (F). The control group (A, C) showed less vascular density and a smaller diameter than the VC + ESM group.

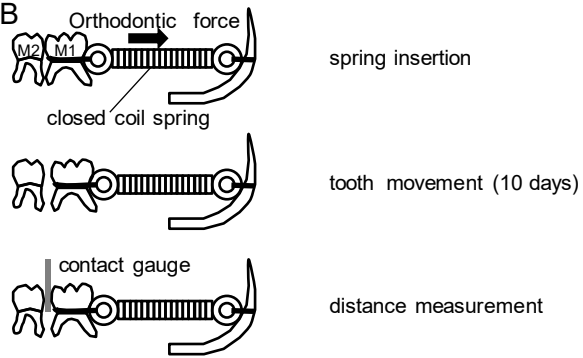
A concentrated vascular layer was observed in the compressed fibers on the compression side of the VC + ESM group. The density and thickness were remarkably high in the 75-g group. Vascular morphology in the control group was rougher than that in the VC + ESM group. Scale bar = 50  $\mu\text{m}$ .

Fig.1

A



B



C

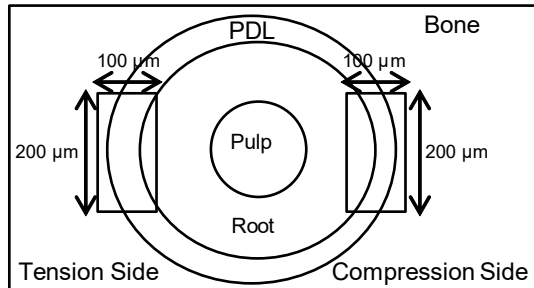


Fig.2

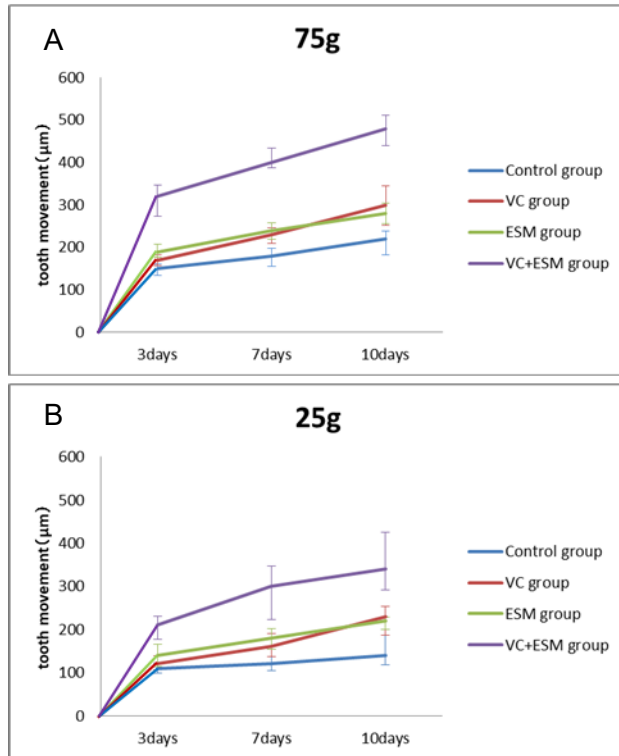


Fig.3

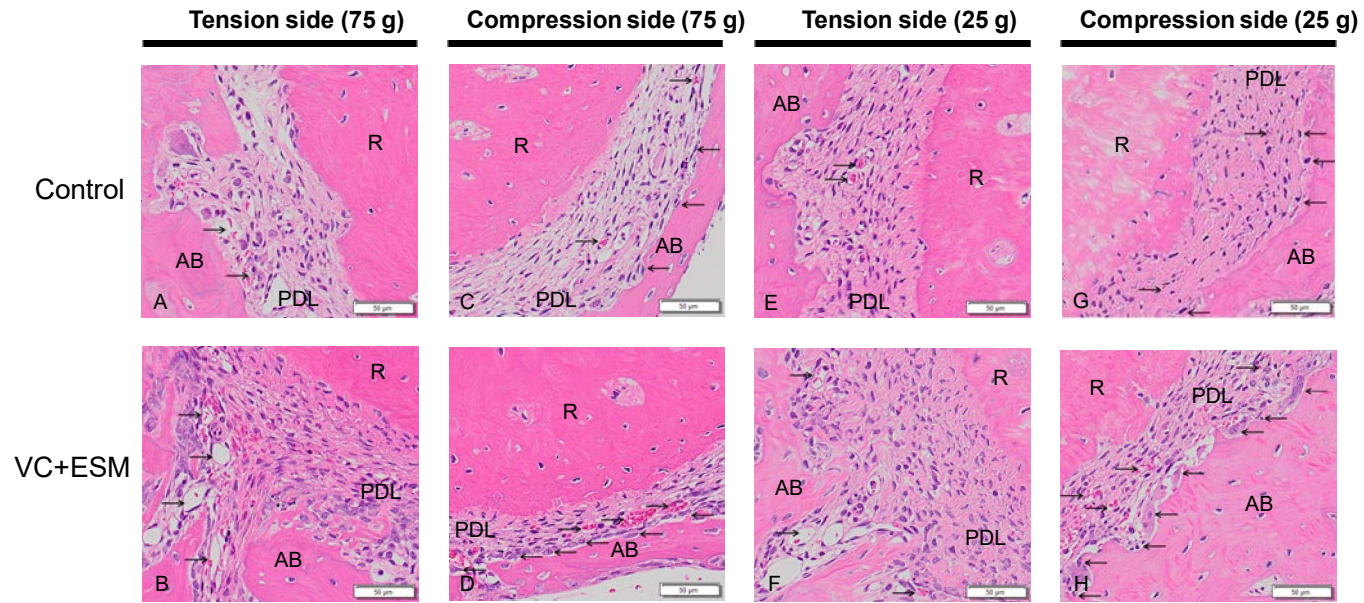




Fig.4

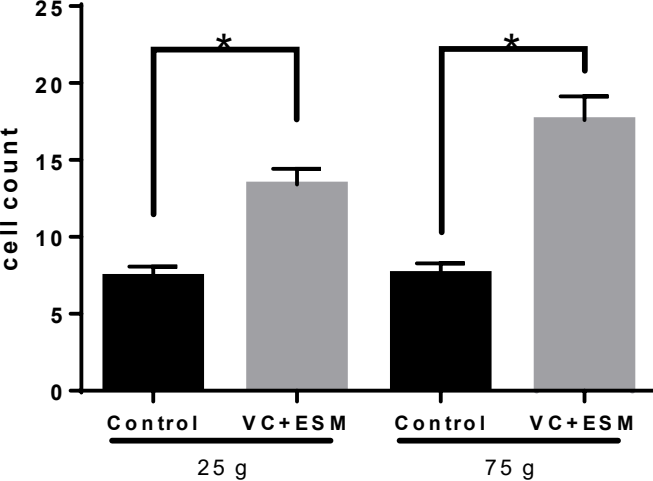


Fig.5

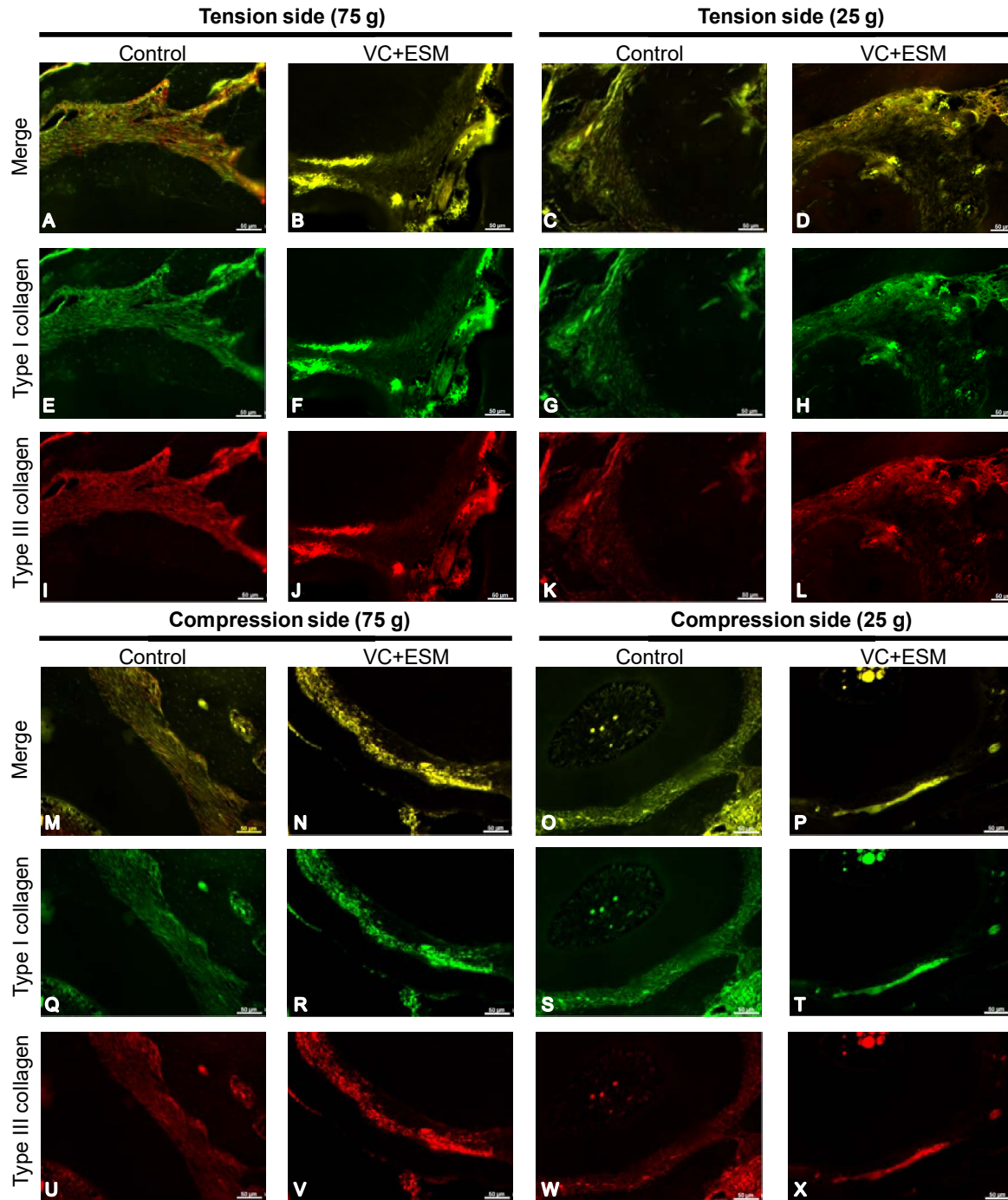


Fig.6

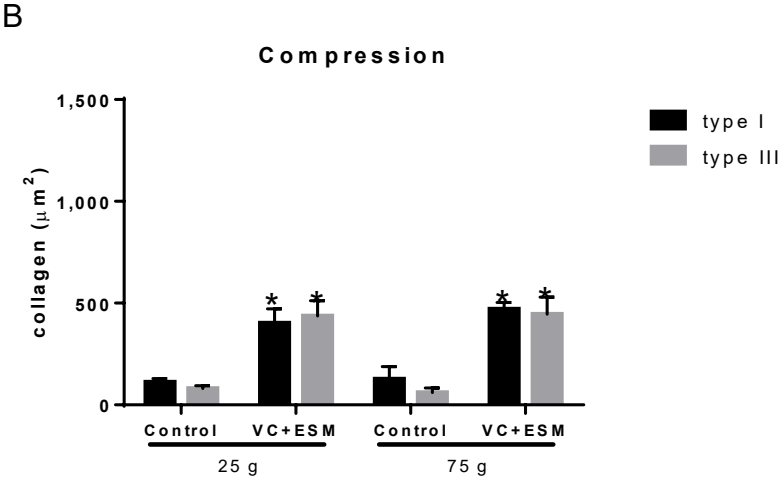
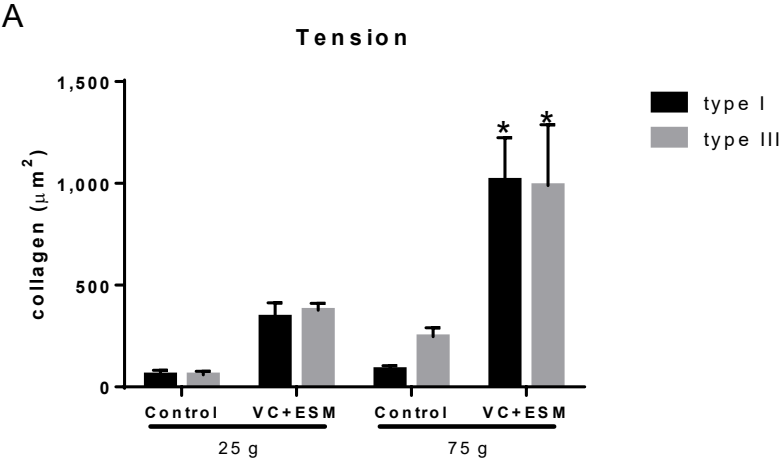


Fig.7

