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Stem cells derived from human exfoliated deciduous teeth-based media in a rat root resorption model

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ABSTRACT

Objective: Root resorption may occur during orthodontic treatment. Herein, we investigated the effect of a culture supernatant of stem cells derived from human exfoliated deciduous teeth on root resorption.
Design: Twelve 8-week-old male Sprague–Dawley rats were used, and their maxillary first molars were pulled with excessive orthodontic force to induce root resorption. On days 1 and 7 after traction initiation, stem cells derived from human exfoliated deciduous teeth and alpha minimum essential medium (control group) were administered. After 14 days, the maxillary bone was evaluated for tooth movement. The expression of osteoprotegerin, receptor activator of nuclear factor κB ligand, tumor necrosis factor α, interleukin 1β, interleukin 6, and interleukin 17 was evaluated on the compression side and tension side.
Results: No significant difference in tooth movement was observed between the two groups. Root resorption decreased in the group administered the culture supernatant compared with in the control. Immunohistochemical staining revealed increased osteoprotegerin expression and decreased receptor activators for nuclear factor κB ligand, tumor necrosis factor α, interleukin 1β, interleukin 6, and interleukin 17 on the compression side and tension side.
Conclusions: Administration of stem cells derived from human exfoliated deciduous teeth affected the expression of osteoprotegerin, receptor activator of nuclear factor κB ligand, tumor necrosis factor α, interleukin 1β, interleukin 6 and interleukin 17; hence, these stem cells may inhibit root resorption by regulating their expression.

1. Introduction

Orthodontic treatment is a medical treatment that aims to obtain good maxillofacial morphology and normal oral functions by applying orthodontic forces to the tooth roots, periodontal tissues, and maxillofacial surfaces. Tooth movement is achieved through bone remodeling by artificially applying loads to the teeth and alveolar bone, resulting in bone resorption by osteoclasts on the compression side and bone formation by osteoblasts on the traction side. Severe root resorption can occur when the load applied to the tooth root greatly exceeds the repair capacity of the cementoblasts (Abass et al., 2008). Among the medicalogenic disorders that may occur during the orthodontic treatment process (such as pain, root resorption, caries, and temporomandibular joint disorder), root resorption is irreversible and difficult to predict, prevent, or repair. It is an asymptomatic disorder and can only be radiographically and histologically diagnosed. Approximately 48–66 % of orthodontically treated teeth show root resorption < 2 mm, and histological root resorption occurs in > 90 % of cases (Wishney, 2017). Anterior teeth are particularly susceptible to root resorption, with 1–5 % of the teeth reported to have resorption ≥ 4 mm (Weltman et al., 2010). Nevertheless, the clinical and biological factors that cause root resorption remain unclear (Sameshima & Iglesias-Linares, 2021).

Root resorption is caused by multinucleated giant cells that appear in the vitreous-like degenerative tissue formed in the periodontal ligament on the pressure side during tooth movement. Odontoclasts are multinucleated giant cells that are structurally and functionally similar to osteoclasts; they reside on the surface of the resorbed root (Sahara et al., 1996) and are involved in hard tissue resorption. Osteoclasts are part of the normal bone structure, whereas odontoclasts are rarely found on normal cementum or tooth root surfaces (Abass et al., 2008).

Receptor activator of nuclear factor κB ligand (RANKL), a cytokine belonging to the tumor necrosis factor (TNF) family, is essential for the

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induction of osteoclastogenesis. RANKL is produced by osteoblasts and binds to the receptor activator of nuclear factor κB (RANK) receptors localized on the surface of osteoclast progenitor cells to promote differentiation into osteoclasts via signal transduction (Suda et al., 1999). Osteoprotegerin (OPG), a member of the TNF receptor family produced by osteoblasts, acts as a decoy receptor for RANKL and inhibits progenitor cell differentiation into osteoclasts by binding to RANKL, with a higher affinity than that of RANK, the true receptor of RANKL (Udagawa et al., 2021). Thus, the RANKL/RANK/OPG system in periodontal tissues is an important determinant that regulates orthodontic tooth movement, bone remodeling, and root resorption (Matsuda et al., 2017).

TNF-α and interleukin (IL)− 1β are proinflammatory cytokines involved in bone remodeling and are highly expressed at inflammatory sites. TNF-α induces the production of IL-1β (Dinarello et al., 1987; Kaushansky et al., 1988). Increased expressions of TNF-α and IL-1β have been detected in the gingival sulci of patients undergoing orthodontic treatment (Uematsu et al., 1996). The expression of IL-17, produced by helper T cells, is induced by excessive corrective force (Hayashi et al., 2012) and subsequently induces the production of IL-6 (Shimizu et al., 2013). These inflammatory cytokines are important mediators involved in root resorption that upregulate the production of RANKL, an osteoclast activator, and activate osteoclast progenitor cells through synergistic action with the RANKL/RANK/OPG system (Sato et al., 2006).

Stem cells from human exfoliated deciduous teeth (SHED) were first isolated by Miura et al. (2003) and are noninvasively available. SHED can multiply into osteoblasts, adipocytes, chondrocytes, and neurons; hence, they have recently attracted attention in the field of regenerative medicine. Nakajima et al. (2018) transplanted SHED, human permanent dental pulp-derived stem cells, and human bone marrow-derived stem cells into induced skull defects in immunocompromised mice and observed good bone regeneration at 12 weeks after transplantation, suggesting that SHED may be an excellent cell source for bone regeneration.

The paracrine action of cytokines secreted by mesenchymal stem cells (MSCs) has hitherto attracted attention; growth factors and cytokines secreted by MSCs may accumulate in culture supernatant (CM) by paracrine action, and many studies have reported tissue regeneration-inducing effects using MSCs-CM (Ando et al., 2014; Chen et al., 2018, 2020; Katagiri et al., 2013, 2017, 2021; Kawai et al., 2015; Kim et al., 2020; Ogasawara et al., 2020; Ogata et al., 2015; Tsai et al., 2018; Yamaguchi et al., 2015). Hiraki et al. (2020) reported that treatment of bone defects in the parietal bones of immunocompromised mice with SHED-CM and SHED grafts significantly increased regenerated bone mass in both groups than that of the control group. Furthermore, quantitative analysis revealed that SHED-CM contains a large amount of angiogenic (e.g., vascular endothelial growth factor [VEGF]), bone metabolic (e.g., OPG and bone morphogenetic protein [BMP]−2,4), and neurotrophic (e.g., brain-derived neurotrophic) factors. Furthermore, SHED-CM reportedly suppresses the expression of inflammatory cytokines (Shimojima et al., 2016). Ogasawara et al. (2020) reported that SHED-CM administration via the tail vein to mice with temporomandibular joint osteoarthritis markedly suppressed inflammation and triggered temporomandibular repair and regeneration.

Based on these findings, the paracrine action of proteins contained in SHED-CM is expected to enhance the ability of periodontal tissues to induce metabolic activity, thereby affecting tooth movement-induced root resorption during orthodontic treatment. However, to our knowledge, no study has reported on the effects of SHED-CM on root resorption during tooth movement. Therefore, this study aimed to investigate the effect of SHED-CM administration on root resorption and periodontal tissue during experimental tooth movement.

2. Materials and methods

2.1. Isolation and culture of SHED

Stem cells were isolated and cultured from human deciduous tooth pulp according to the method described by Gronthos et al. (2000). The deciduous tooth was a clinically healthy upper right deciduous canine obtained from an 11-year-old male patient who required orthodontic treatment. Informed consent was obtained from the parents of the donor. Human dental pulp tissue harvest was approved by the Preliminary Review Board of the Epidemiological Research Committee of Hiroshima University (approval number: E-20-2). The extracted deciduous teeth were immersed in phosphate buffered saline (PBS, LSI Medience, Tokyo, Japan) containing 100 mM amphotericin (MP Biomedicals, Strasbourg, France) and transferred to a clean bench. The periodontal ligament was disinfected with isodine (Maruishi-pharma, Osaka, Japan) and hibitene (Sumitomo-pharma, Osaka, Japan) and removed using a scalpel. The pulp tissue was disinfected again and divided at the cemento-enamel junction using bone forceps (Natsume Seisakujo, Tokyo, Japan). The pulp tissue was shredded using a scalpel and dissolved in alpha minimum essential medium (α-MEM) (Sigma-Aldrich, St. Louis, MO, USA) with 4 mg/mL collagenase (ThermoFisher Scientific, Waltham, MA, USA) and 3 mg/mL dispase (Godo-Sake, Tokyo, Japan). Cell aggregates were removed using a 7-μm cell strainer (Corning Inc., Corning, NY, USA). The filtered solution was diluted with medium and centrifuged at 1800 rpm for 5 min. The supernatant was aspirated and mixed with 20 % fetal bovine serum (FBS) (Daiichi Kagaku, Tokyo, Japan), 0.24 μg/mL kanamycin (Meiji Seika Pharma, Tokyo, Japan), 0.5 μg/mL penicillin (Meiji Seika Pharma), 0.5 μg/mL penicillin (Meiji Seika Pharma), and 1 μg/mL amphotericin in α-MEM; the mixture was seeded in a petri dish for cell culture (Corning) under 37 ˚C and 5 % CO_2 conditions. When the number of colony-forming units reached approximately 200, the cells were detached using 0.25 % trypsin (Nacalai Tesque, Kyoto, Japan) and PBS containing 1 mM EDTA (Wako Pure Chemicals Co., Osaka, Japan), collected, and passaged. After the first passage (P1), the cells were cultured in α-MEM containing 10 % FBS and the aforementioned antibiotics at 37 ˚C and 5 % CO_2. In this study, P6–P10 cells were used, which was stored at − 80 ˚C or − 190 ˚C (in liquid nitrogen) until use.

The International Society for Cellular Therapy has established the following criteria for defining human MSCs: (1) the attachment of cells to plastic containers under standardized culture conditions, (2) cells must be positive for CD73, CD90, and CD105 and negative for CD11b or CD14, CD19 or CD79α, CD34, CD45, and HLA-DR, and (3) the capacity of cells to differentiate into osteoblasts, chondrocytes, and adipocytes. Our previous studies have demonstrated and elucidated that these cells meet the aforementioned criteria (Kunimatsu et al., 2022, 2023; Nakajima et al., 2018). In the present study, the cells were isolated and cultured in an identical manner; therefore, they were classified as SHEDs.

2.2. SHED-CM

SHED was incubated; after reaching 70–80 % confluency, the medium was replaced with serum-free α-MEM supplemented with 0.5 μg/mL penicillin (Meiji Seika Pharma), 0.24 μg/mL kanamycin (Meiji Seika Pharma), and 1 μg/mL amphotericin (MP Biomedicals). After 48 h of incubation, the CM was centrifuged at 1500 rpm for 5 min and then at 3000 rpm for 3 min 10 kDa Millipore (Millipore EMD, Burlington, MA, USA) and concentrated 40-fold for experiments. It was stored at − 80 ˚C until the time of use.

2.3. Animals

Twelve 8-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were used in this study. The rats were reared at Kasumi Animal Experiment Facility, Life Science Research Section, Natural Science and Research Support Center, Hiroshima University. The cages were maintained at 22–24 ˚C and 50–60 % humidity, and all rats were fed ad libitum with powdered feed (CE-2, Japan CLEA, Tokyo, Japan) and drinking water. The lighting of the facility was automatically controlled in a 12-h light/dark cycle. Animals were pre-fed and acclimatized for 1 week prior to the study. This study was approved by the Ethics Committee of the Hiroshima University Animal Research Institute (approval number: A21-24).

2.4. Preparation of root resorption model in rats

A nickel-titanium closed coil spring (Centalloy, I.D. 0.9 mm, wire diameter 0.15 mm; Tomy International, Tokyo, Japan) was ligated to the maxillary left first molar of the rat using an orthodontic ligature wire (Tomy International). The other end of the wire was attached to the ipsilateral incisor and fixed with light-cured composite resin (Unifil Loflow®; GC, Tokyo, Japan) to prevent the appliance from detaching. The orthodontic force was set at 50 g, which is an excessive force that induces root resorption (Gonzales et al., 2008). Light-cured composite resin was placed on the occlusal surfaces of the maxillary left second and third molars and the maxillary right first, second, and third molars to eliminate occlusal interference (Fig. 1a, b). The device was affixed to the rats for 14 days, and the maxillary left-side first molar was moved proximally; on day 14, the rats were euthanized under deep anesthesia. All procedures were performed under general anesthesia with inhalation anesthesia using isoflurane (Mylan Pharma, Tokyo, Japan), followed by subcutaneous injection of a triple anesthetic mixture of dexmedetomidine hydrochloride (0.15 mg/kg, Domitor®, Zenoaq, Fukushima, Japan), midazolam (2.0 mg/kg, midazolam injection 10 mg, Sandoz, Tokyo, Japan), and butorphanol tartrate (2.5 mg/kg, butorphanol 5 mg, Meiji Seika Pharma).

2.5. Study groups and SHED-CM administration procedure

The animals were randomly and equally assigned into two groups (six animals each): the SHED-CM-treated and control groups. On days 1 and 7, SHED-CM (250 μL per dose) was administered via the tail vein with the rats being under general anesthesia. In the control group, serum-free α-MEM supplemented with 0.5 μg/mL penicillin (Meiji Seika Pharma), 0.24 μg/mL kanamycin (Meiji Seika Pharma), and 1 μg/mL amphotericin (MP Biomedicals) was administered in the same manner.

2.6. Measurement of travel distance during experimental tooth movement

The distance of first molar migration was measured via high-resolution micro-computed tomography (CT) (SkyScan1176; Bruker micro CT, Kontich, Belgium) on day 14 post-attachment of the device to the maxillary bone of rats, and the shortest distance between the crowns of the first and second molars on the CT image was measured using the analysis software Data Viewer (Bruker micro CT) (Fig. 1c).

2.7. Histological evaluation during experimental tooth movement

Based on the report by Gonzales et al. (2008), the distal buccal root, which has the most pronounced root resorption, was set as the target for evaluation. Based on the protocol described by Aghili et al. (2013), three equally spaced sections were obtained three times from the cement-enamel junction to the root apex of the centric buccal root. This method was used in this study because of its excellent reproducibility and the capability to assess identical regions in different individual. At 14 days after device attachment, the rats were euthanized under deep anesthesia, followed by perfusion fixation; the maxillary bone was removed enbloc, immersed, and fixed in 4 % neutral buffered paraformaldehyde solution (Wako Pure Chemicals Co.) for 24 h at 4 ˚C and then demineralized in 14 % tetrasodium ethylene diamine tetra acetic acid solution (Sigma–Aldrich) for 7 weeks at 4 ˚C. Subsequently, paraffin embedding was performed using Excelsior AS tissue processor

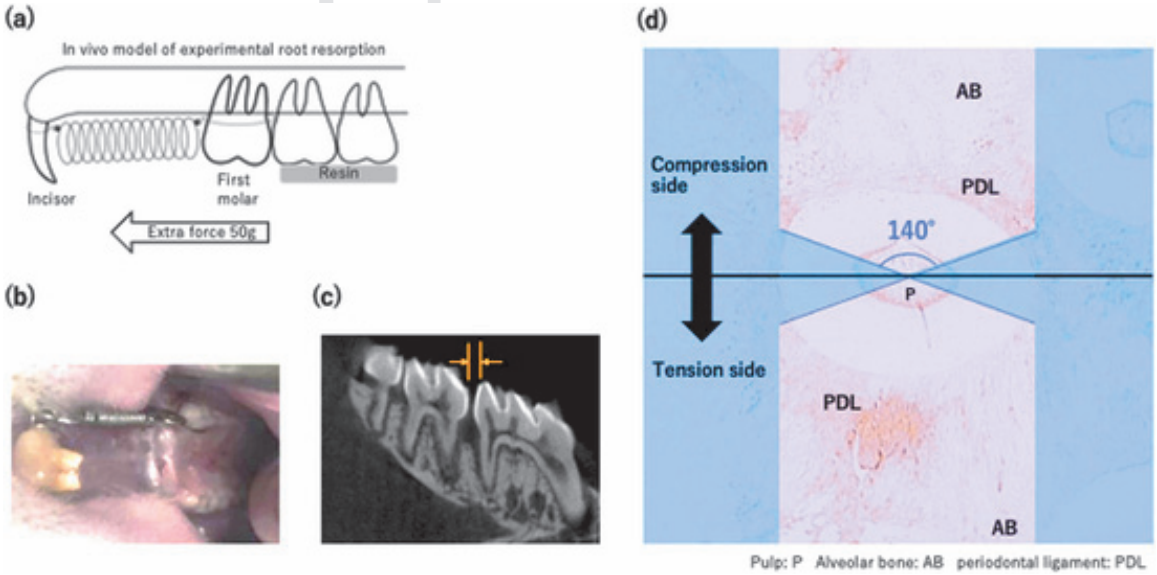

Fig. 1. (a) Lateral schematic view of the rat root resorption model. A nickel-titanium closed coil spring fabricated to achieve an orthodontic force of 50 g is ligated to the maxillary left-side first molar of the rat with an orthodontic ligature wire, and the other end is attached to the same lateral incisor and fixed with light-curing composite resin. Light-cured composite resin is placed on the occlusal surfaces of the maxillary left second and third molars as well as the maxillary right first, second, and third molars. The arrow indicates the direction of traction. (b) Intraoral photographs of a rat with the device attached. (c) The shortest distance between the distal surface of the maxillary first molar and the proximal surface of the second molar on the computed tomographic image is measured as the tooth movement distance. (d) Regions of interest of Immunohistochemical stain positive area percentage. The periodontal ligament region from the dentin to the alveolar bone are included within the range of 140˚ from the middle of the pulp, as depicted on the images. Regions shown in blue were excluded. The upper portion is the compression side and lower is the tension side.

(Thermo Fisher Scientific), a sealed automated embedding device; thereafter, the specimens were placed in a rotary microtome (Microtom 315; CralZeiss, Oberkoshen, Germany) to prepare 7-μm-thick horizontal serial sections. After deparaffinization and dehydration of the tissue sections, staining was performed following the standard protocol.

2.7.1. Hematoxylin and eosin (HE) staining

The cells were stained with HE staining solution (Muto Pure Chemical Co., Ltd., Tokyo, Japan), dehydrated, permeabilized, and sealed using Mount-Quick (Daido Sangyo, Saitama, Japan). Tissue sections were observed using an all-in-one microscope BZ-X800 (Keyence, Osaka, Japan). For the aforementioned evaluation sites, the absorption fossa area was measured using an image analysis software (ImageJ; National Institute of Health, Bethesda, MD, USA).

2.7.2. Tartrate-resistant acid phosphatase (TRAP) staining

Staining was performed using a TRAP/ALP staining kit (Wako Pure Chemicals Co.). The cells were observed with an all-in-one microscope BZ-X800 (Keyence), and the number of TRAP-positive cells was analyzed using an image analysis software BZ-H4A (Keyence). Multinucleated giant cells in contact with the cementum surface on the compression side were counted and quantified as TRAP-positive cells.

2.7.3. Immunohistochemical staining

Each section was reacted with Dako Protein block (Dako, Carpinteria, CA, USA) for 20 min to prevent adsorption of nonspecific antibodies and then reacted with anti-OPG (ab73400; Abcam, Cambridge, UK) diluted to 1/500 concentration, anti-RANKL (NB100-56512; Novus Biologicals, St. Louis, MO, USA) diluted to 1/25 concentration, anti-TNF-α antibody (ab220210; Abcam) diluted to 1/1000 concentration, anti-IL-1β (ab9722; Abcam) diluted to 1/250 concentration, anti-IL-6 (ab9324; Abcam) diluted to 1/2100 concentration, and anti-IL-17 (ab214588; Abcam) diluted to 1/400 concentration as primary antibody for 12 h at 4 °C. Primary antibodies were diluted in sterile PBS, and after washing with PBS, each section was reacted with secondary antibodies, anti-rabbit IgG Histofine Simple Stain MAX-PO(R) (Nichirei Bioscience, Tokyo, Japan), anti-mouse IgG Histofine Simple Stain MAX-PO(M) (Nichirei Bioscience), and anti-goat IgG Histofine Simple Stain MAX-PO (G) (Nichirei Bioscience) for 2 h at room temperature. The samples were then chromogenized by Histofine DAB Substrate Kit (Nichirei Bioscience). After spontaneous drying, the tubes were sealed using Mount-Quick (Daido Sangyo). Tissue sections were observed using an all-in-one microscope BZ-X800 (Keyence), and positive cells for each antibody were quantified using an image analysis software BZ-H4A (Keyence). Positive cells were subjected to automatic calculation of positive area percentage using a hybrid cell count application (BZ-H4C, Keyence) in the BZ-X Analyzer software (BZ-H4A, Keyence). The immunohistochemical staining analysis focused on the periodontal ligament region, encompassing the dentin to the alveolar bone, within the range of 140° from the middle of the pulp, as depicted in the acquired image (Fig. 1d).

2.8. Statistical analysis

The results are presented as means ± standard deviations. One-way analysis of variance (ANOVA) was used to examine the differences between each group. Significance level was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Weight change of rats during the experiment

Changes in body weight of rats during the experimental period were measured over time. Rats in both the control and SHED-CM-treated groups showed weight gain over time; however, there was no significant between-group difference (Fig. 2a).

3.2. Effect of SHED-CM administration on tooth movement distance

After 14 days of experimental tooth movement, the distance of the first molar migration was measured. No significant differences were observed between the control and SHED-CM-treated groups (Fig. 2b).

3.3. Histological changes after SHED-CM administration

3.3.1. HE staining

Tooth movement with excessive orthodontic force resulted in the formation of resorption fossae from the root surface to the dentin layer in both the control and SHED-CM-treated groups (Fig. 3a). After 14 days of experimental tooth movement, the root resorption area of the

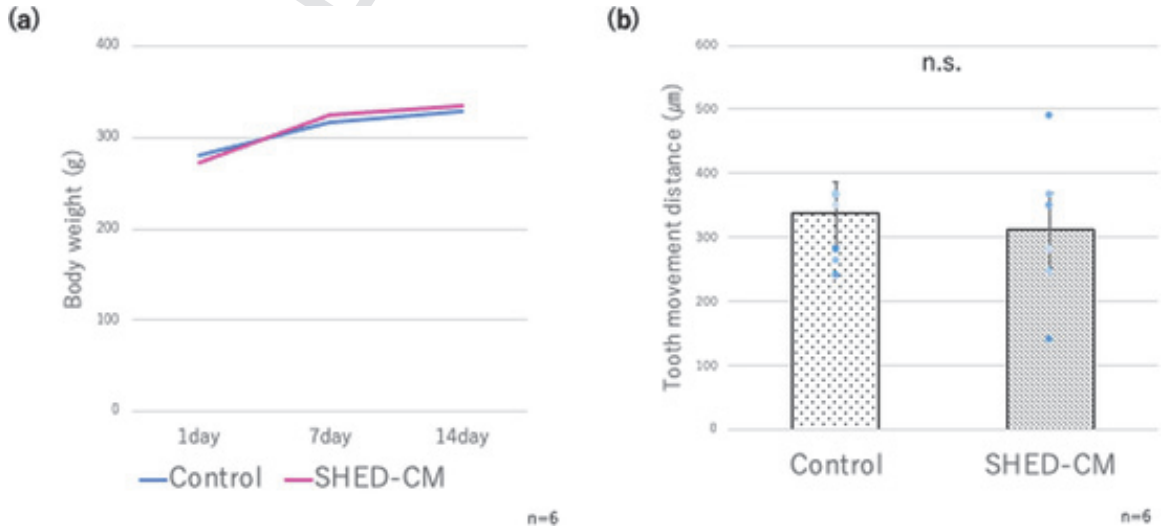

Fig. 2. (a) Weight change of rats during the experimental period. Compared with the control group, no differences are observed in the SHED-CM-treated group in terms of rat body weight changes (n = 6 per group). (b) Comparison of the distance of movement of the rat first molars during the experimental period. The distance travelled by the rat first molars is 336.9 ± 50.3 μm in the control group and 310.7 ± 57.7 μm in the group treated with SHED-CM. There is no significant difference in tooth movement distance between the control and SHED-CM-treated groups (n = 6 per group, $P < 0.9882$, ANOVA). SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth.

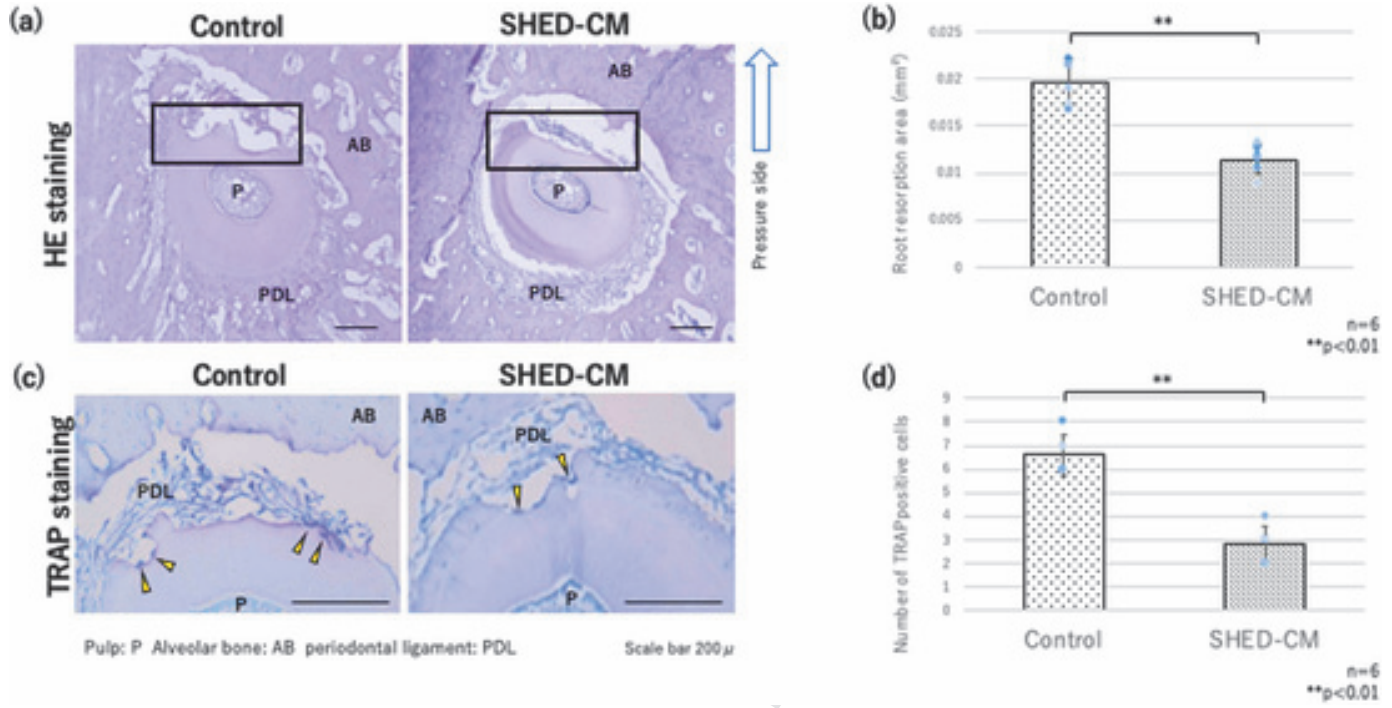

Fig. 3. (a) HE-stained images of the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The area marked with a square indicates the area of root resorption. Traction was applied in the direction of the arrow in the figure, and hence the upper part of each root is the compression side. Resorption foci are observed from the root surface to the dentin layer. Scale bar = 200 μm. (b) Root resorption area on day 14 of device placement. A significant reduction in root resorption area was observed in the SHED-CM-treated group compared with the control group (n = 6 per group, $P < 0.0001$, ANOVA). (c) TRAP-stained images of control and SHED-CM-treated group specimens on the compression side obtained on day 14 of device attachment. Yellow arrowheads in the figure indicate TRAP-positive cells. The upper side is the compression side. Scale bar = 200 μm. (d) TRAP-positive cell counts. Quantitative evaluation shows a significant reduction in the number of TRAP-positive cells in the SHED-CM-treated group compared with that in the control group (n = 6 per group, $P < 0.0001$, ANOVA). HE, hematoxylin-eosin; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth; TRAP, tartrate-resistant acid phosphatase.

centobuccal root of the first molar on the compression side, was measured, and the root resorption area was significantly reduced in the SHED-CM-treated group compared with in the control group (Fig. 3b).

3.3.2. *TRAP staining*

On the compression side, TRAP expression was evaluated on the root and alveolar bone surfaces of multinucleated osteoclasts (Fig. 3c). Compared with in the control group, the number of osteoclasts was reduced in the SHED-CM-treated group (Fig. 3d).

3.3.3. *Immunohistochemical staining*

On the compression side, the expression of OPG, RANKL, TNF-α, IL-1β, IL-6, and IL-17 was measured in the periodontal ligament, alveolar bone, and root surface (Figs. 4a, 5a, 6a). A quantitative evaluation of the positive area ratio (Figs. 4b, 5b, 6b) revealed more than 10-fold increase in OPG expression in the SHED-CM-treated group than that of the control group. In contrast, the expression of RANKL, TNF-α, IL-1β, IL-6 and IL-17 were all suppressed by more than 2-fold in the SHED-CM group.

On the tension side, the expression of OPG, RANKL, TNF-α, IL-1β, IL-6, and IL-17 was also measured in the periodontal ligament, alveolar bone, and root surface (Figs. 7a, 8a, 9a). Consequently, the quantitative evaluation of the positive area ratio yielded similar results as observed on the compression side (Figs. 7b, 8b, 9b). Compared to the control group, the SHED-CM group exhibited approximately 8-fold increase in OPG expression. In contrast, the expression of RANKL, TNF-α, IL-1β, IL-6, and IL-17 were all significantly suppressed in the SHED-CM group.

4. Discussion

In this study, we investigated the effect of SHED-CM, administered systemically via the tail vein of the rats, on root resorption. Osugi et al. (2012) administered MSC-CM via the tail vein and confirmed its distribution to the head in a rat model of parietal bone defects; hence, we believe that our experiment produced a similar distribution of SHED-CM to the head. No difference in body weight change was observed between the control and SHED-CM-treated groups during the experimental period, indicating that SHED-CM administration did not affect the general condition of the rats.

Numerous studies have used a model in which a nickel-titanium closed coil spring was placed in the oral cavity of rats to perform proximal movement of the first molar (Gunji et al., 2018; Kunimatsu et al., 2020; Nakatani et al., 2022; Tsuka et al., 2019). Although the SHED-CM-treated group showed slightly smaller values, there was no significant difference in the movement of rat first molars between the control and SHED-CM-treated groups during the experimental period, suggesting that SHED-CM administration had no effect on rat tooth movement. Gonzales et al. (2008) reported that when an excessive orthodontic force of 50 g was applied to rat maxillary first molars, a larger root resorption area was observed on day 14 of movement compared with that when an optimal orthodontic force of 10 g was applied. In our study, rat maxillary first molars were pulled with an orthodontic force of 50 g for 14 days, and HE staining revealed the formation of a resorption fossa from the root surface to the dentin layer, showing a significantly smaller resorption area in the SHED-CM-treated group than in the control group.

Immunohistochemical staining showed increased OPG expression and decreased RANKL expression on the compression side of rats in the SHED-CM-treated group compared with that in the control group.

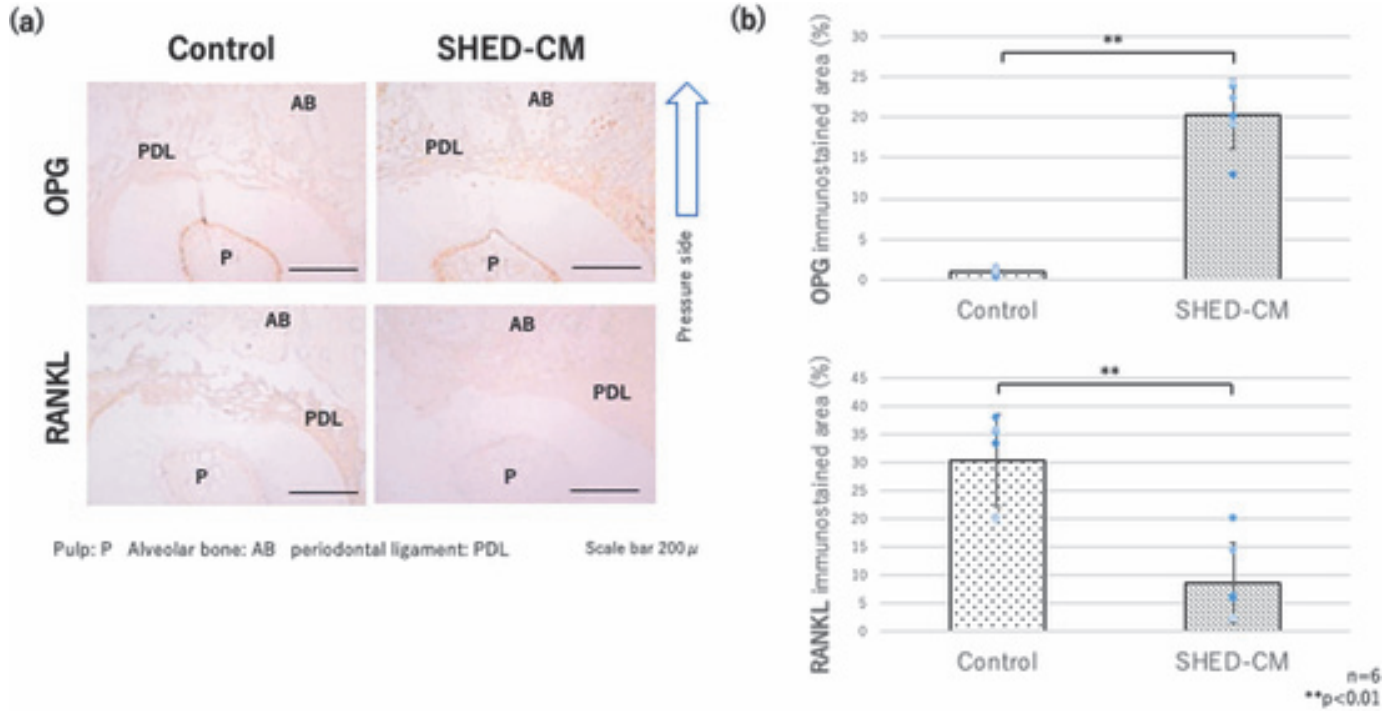

Fig. 4. (a) Immunohistochemical staining images of OPG and RANKL on the compression side in the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The upper side is the compression side. Scale bar = 200 μm. (b) Quantitative evaluation show OPG upregulation in the SHED-CM-treated group compared with the control group (n = 6 per group, $P < 0.0001$, ANOVA); however, RANKL is downregulated in the SHED-CM-treated group compared with the control group (n = 6 per group, $P < 0.0006$, ANOVA). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth.

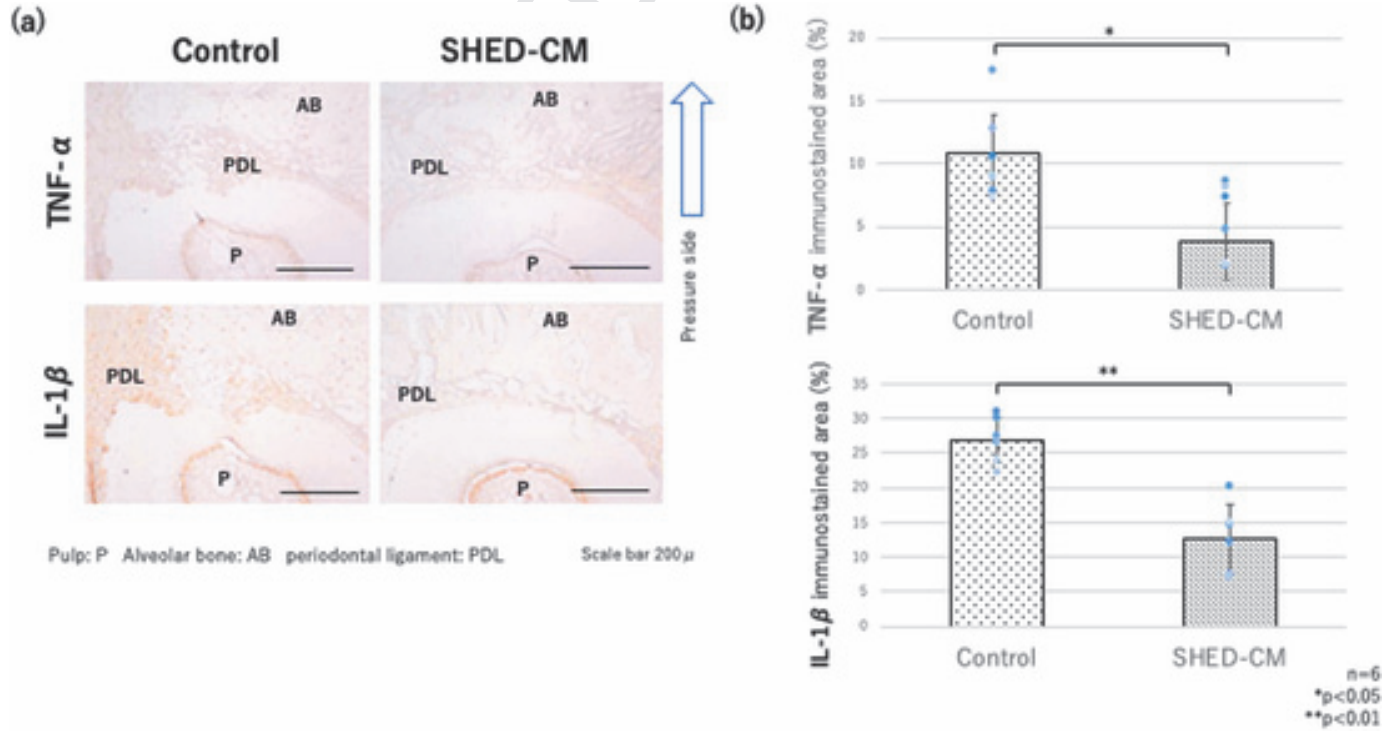

Fig. 5. (a) Immunohistochemical staining images of TNF-α and IL-1β on the compression side in the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The upper side is the compression side. Scale bar = 200 μm. (b) Quantitative evaluation show TNF-α (n = 6 per group, $P < 0.0231$, ANOVA) and IL-1β (n = 6 per group, $P < 0.0002$, ANOVA) are downregulated in the SHED-CM-treated group compared with the control group. IL, interleukin; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth; TNF, tumor necrosis factor.

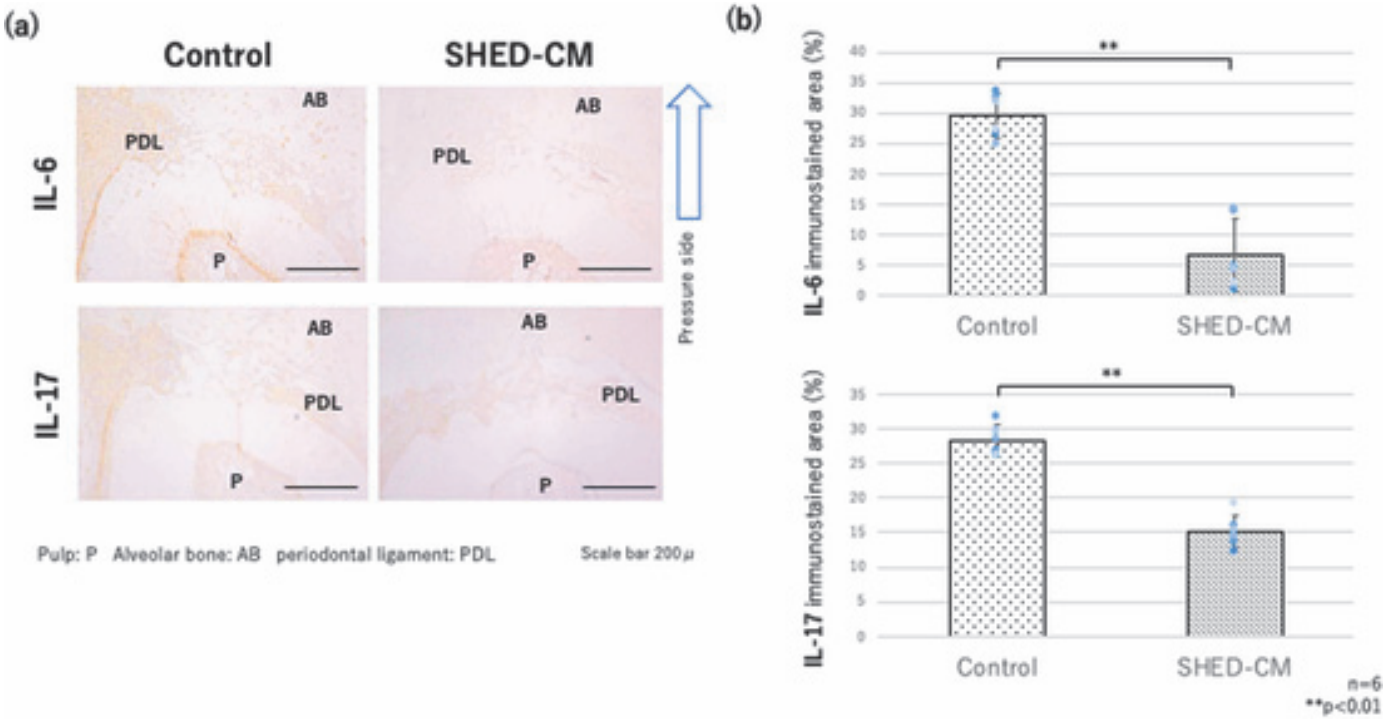

Fig. 6. (a) Immunohistochemical staining images of IL-6 and IL-17 on the compression side in the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The upper side is the compression side. Scale bar = 200 μm. (b) Quantitative evaluation show IL-6 (n = 6 per group, $P < 0.0001$, ANOVA) and IL-17 (n = 6 per group, $P < 0.0001$, ANOVA) are downregulated in the SHED-CM-treated group compared with the control group. IL, interleukin; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth.

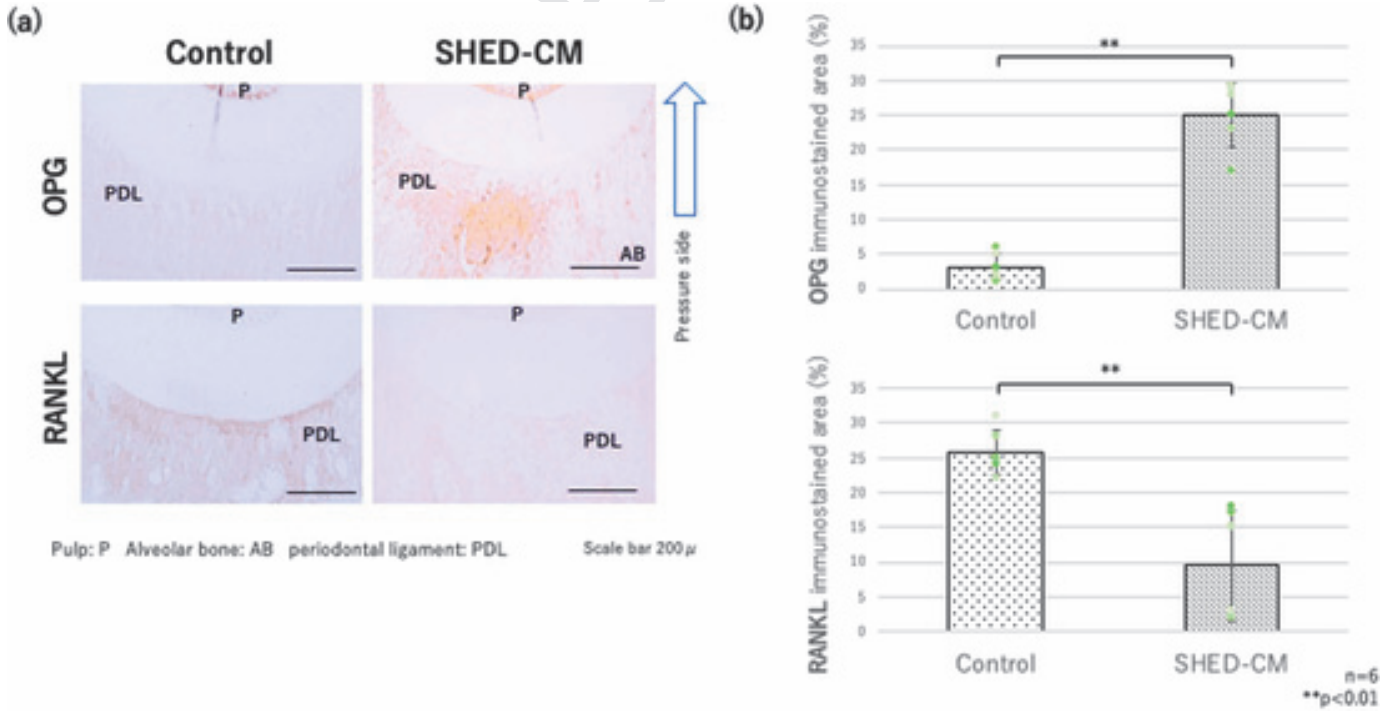

Fig. 7. (a) Immunohistochemical staining images of OPG and RANKL on the tension side in the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The lower side is the tension side. Scale bar = 200 μm. (b) Quantitative evaluation show OPG upregulation in the SHED-CM-treated group compared with the control group (n = 6 per group, $P < 0.0001$, ANOVA); however, RANKL is downregulated in the SHED-CM-treated group compared with the control group (n = 6 per group, $P < 0.0023$, ANOVA). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth.

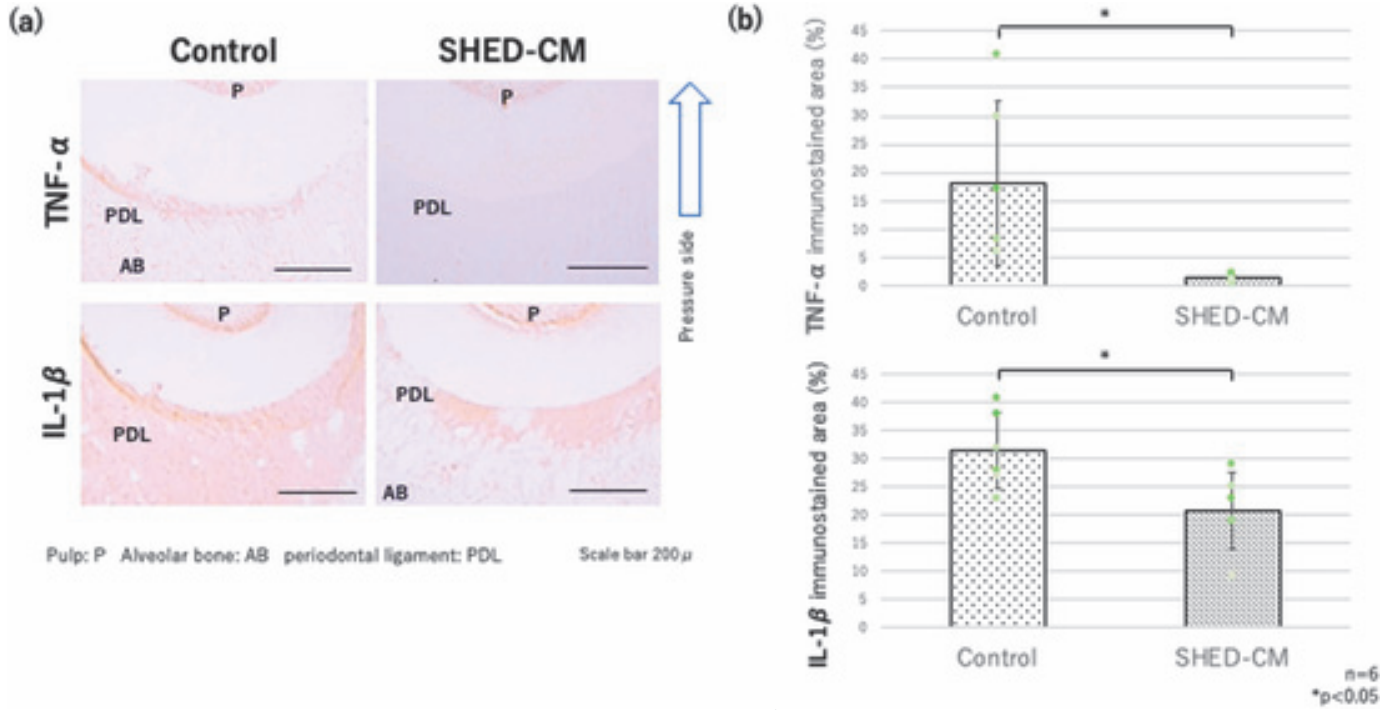

Fig. 8. (a) Immunohistochemical staining images of TNF-α and IL-1β on the tension side in the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The lower side is the tension side. Scale bar = 200 μm. (b) Quantitative evaluation show TNF-α (n = 6 per group, $P < 0.0184$, ANOVA) and IL-1β (n = 6 per group, $P < 0.0212$, ANOVA) are downregulated in the SHED-CM-treated group compared with the control group. IL, interleukin; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth; TNF, tumor necrosis factor.

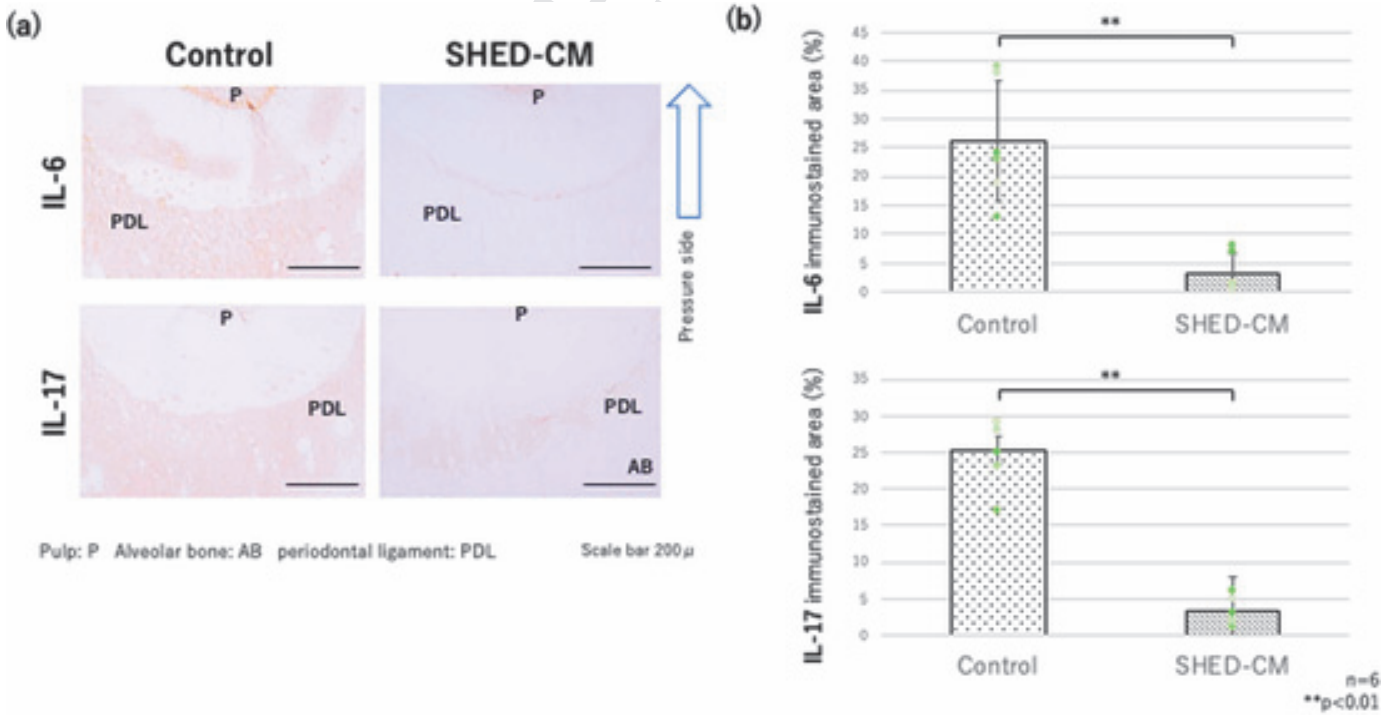

Fig. 9. (a) Immunohistochemical staining images of IL-6 and IL-17 on the tension side in the control and SHED-CM-treated group specimens obtained on day 14 of device placement. The lower side is the tension side. Scale bar = 200 μm. (b) Quantitative evaluation show IL-6 (n = 6 per group, $P < 0.0005$, ANOVA) and IL-17 (n = 6 per group, $P < 0.0001$, ANOVA) are downregulated in the SHED-CM-treated group compared with the control group. IL, interleukin; SHED-CM, culture supernatant of stem cells derived from human exfoliated deciduous teeth.

Hiraki et al. (2020) analyzed the protein expression of SHED-CM and found that it contained high levels of bone metabolism- (OPG, OPN, BMP-2, and BMP-4) and angiogenesis-related factors (M-CSF, MCP-1, ANG, bFGF, HGF, VEGF-C, and VEGF-A). Ando et al. (2014) also reported a high OPG content in SHED-CM. In our study, TRAP staining showed a decrease in the number of TRAP-positive cells on the compression side of rats in the SHED-CM-treated group than in those in the control group, suggesting that OPG, which is abundant in SHED-CM, inhibits the binding of RANKL to the RANK receptor and differentiation into osteoclasts and that SHED-CM may be involved in the regulation of the RANK/RANKL/OPG system.

Orthodontic movement is correlated with inflammatory processes, coordinated with the mechanical response of periodontal and oral tissues, and is essential for achieving tooth movement (Di Domenico et al., 2012). Many changes in the periodontal tissue result in the synthesis and release of cytokines, growth factors, colony-stimulating factors, and neurotransmitters (Krishnan and Davidovitch, 2006). Inflammatory cytokines, such as TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-17, RANKL, and M-CSF, have been detected in gingival exudates from patients undergoing orthodontic treatment (Takano-Yamamoto, 2016), and these factors affect osteoclastogenesis. Specifically, TNF-α and IL-1β have been implicated in bone remodeling (Bertolini et al., 1986). RANKL is highly expressed by stimulation of inflammatory cytokines, such as TNF-α and IL-1β (Takayanagi, 2005). In vitro, RANKL expression was induced by IL-1β stimulation of cementoblasts (Diercke et al., 2012). TNF-α and IL-1β activate osteoclast surface receptors, enhance osteoclastogenesis in vitro, and stimulate bone resorption in vivo (Boyle et al., 2003). IL-6 is a multifunctional cytokine and plays an important role in osteoclastic bone resorption (Ohsaki et al., 1992). Kurihara et al. (1990) reported that IL-6 stimulates the formation of early osteoclast precursors by inducing the release of IL-1β. Furthermore, Adebanjo et al. (1998) reported that IL-6 activates mature osteoclasts. IL-17 is an inflammatory cytokine that is produced exclusively by the activated T-helper (Th)17 cell subset of $CD4^+$ T lymphocytes (Yao et al., 1995), stimulates RANKL expression in osteoblasts, and induces osteoclast differentiation (Lubberts et al., 2003). Kotake et al. (1999) reported that IL-17-induced differentiation was completely inhibited by OPG, a decoy receptor for RANKL. Moreover, many studies have reported that IL-17 indirectly promotes osteoclast differentiation by inducing the expression of inflammatory cytokines TNF-α, IL-1β, and IL-6 (Chabaud et al., 1998; Fossiez et al., 1996; Hayashi et al., 2012; Jovanovic et al., 1998; Kotake et al., 1999; Lubberts et al., 2003). In this study, immunohistochemical staining showed decreased expression of TNF-α, IL-1β, IL-6 and IL-17 on the compression side of rats in the SHED-CM-treated group compared with that in the control group, suggesting that SHED-CM plays an important role in suppressing the production of inflammatory cytokines induced by orthodontic forces and preventing root resorption. In addition, on the traction side, increased expression of OPG and decreased expression of TNF-α, IL-1β, IL-6 and IL-17 were observed in the SHED-CM administration group, which were comparable to those observed on the compression side. To our knowledge, this is the first report on the effect of the administration of SHED-CM on root resorption during orthodontic tooth movement. However, the mechanisms by which SHED-CM influences bone metabolism during tooth movement are not fully understood. Although the results of this study revealed that root resorption was suppressed by SHED-CM administration, some degree of resorption was observed in the SHED-CM group, which is an issue that requires further attention and investigation. Further in vivo and in vitro studies are required to elucidate the exact mechanisms of SHED-CM activity in periodontal tissues.

This in vivo trial had some limitations. First, in the present study, SHED-CM was purified following previous protocols (Hiraki et al., 2020; Kunimastu et al., 2022). When purifying SHED-CM, the antibiotics were not excluded from the culture medium to prevent contamination at the time of culture. In addition, no culture medium specialized for transplantation is used. Our study's results should be interpreted with caution, including the possible effects of the inclusion of antibiotics, etc. In order to confirm the efficacy and safety of SHED-CM in living donor transplantation, future studies should use serum-free, growth-fac tor-reduce d media with stable growth in MSCs. Second, it has been suggested that MSCs-CM differ in the components they contain depending on their tissue origin. For instance, Ogasawara et al. compared proteins present in SHED-CM and fibroblast-derived CM and observed that SHED-CM contained more than 10-fold increased levels of insulin-like growth factor (IGF) and hepatocyte growth factor (HGF) compared with fibroblast-derived CM (Ogasawara et al., 2020). Our previous quantitative analysis of the proteins included in SHED-CM identified various factors, included bone-turnover (such as OPG, BMP-2,4), neurotrophic (such as BDNF), and angiogenic (such as VEGF) (Hiraki et al., 2020) factors. These reports suggest that SHED-CM may differ in nature from other MSCs-CM and fibroblast-derived CM. However, few reports have compared different active ingredients or efficacies of different origins of MSCs-CM and fibroblast-derived CM. A comparative study on MSCs-CM and fibroblast-derived CM of distinct origins is warranted in the future. Third, the present study focused on root resorption by applying an excessive force system (50 g) in an experimental tooth migration model, aiming to reproduce root resorption and evaluate the effects of the anti-inflammatory and bone resorption factors. However, studies that used the optimal force system (10 g) for experimental teeth migration modeling and assessments of osteogenic factors have not been investigated. In the future, it is necessary to examine the effect of the detailed SHED-CM using another model, and to verify the in vivo effects of SHED-CM repeatedly.

Fourth, with the recent advancements and widespread adoption of tissue transparency technology, we can now assess and analyze biological tissue with three-dimensional structure in their natureal three-dime nsional state. In thus study, IHC analysis using DAB was conducted, highlighting the importance of incorporating tissue transparency technology for periodontium assessments. It is crucial to employ this technology to appraise fluorescence immunostaining in three dimensions, paving the way for more comprehensive and detailed elucidation in future investigations.

In conclusion, SHED-CM administration during experimental tooth movement in rats inhibited root resorption without affecting the tooth movement distance. In addition, OPG expression increased, whereas that of RANKL, TNF-α, IL-1β, IL-6, and IL-17 decreased. Thus, SHED-CM may reduce tooth remodeling in patients undergoing orthodontic treatment, and SHED-CM administration may be a potential preventive measure in orthodontic treatment for patients at a high risk of root resorption.

Ethics approval statement

Human dental pulp tissue harvest was approved by the Preliminary Review Board of the Epidemiological Research Committee of Hiroshima University (Approval number E-20-2). This study was approved by the Ethics Committee of the Hiroshima University Animal Research Institute (Approval number A21-24) and conducted in accordance with the principles of the 1964 Declaration of Helsinki and its subsequent amendments or equivalent ethical standards.

Patient consent statement

Informed consent was obtained from all specimen donors.

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CRediT authorship contribution statement

Ayaka Odo : Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Funding acquisition. **Ryo Kunimatsu :** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Project administration. **Takaharu Abe :** Visualization, Supervision, Funding acquisition, Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review and editing, Visualization, Supervision, Funding acquisition. **Shuzo Sakata :** Validation, Formal analysis, Resources, Writing – review and editing. **Ayaka Nakatani :** Validation, Formal analysis, Resources, Writing – review and editing. **Kodai Rikitake :** Validation, Formal analysis, Resources, Writing – review and editing. **Yuma Koizumi :** Writing – review and editing. **Izumi Tanabe :** Validation, Formal analysis, Writing – review and editing. **Naonobu Okimura :** Validation, Formal analysis, Writing – review and editing. **Yuki Yoshimi :** Conceptualization, Methodology, Software, Writing – review and editing, Supervision, Funding acquisition. **Kotaro Tanimoto :** Conceptualization, Methodology, Software, Resources, Writing – review and editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Not applicable.

Acknowledgements

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