EXPRESSION OF SUPEROXIDE DISMUTASE 3 (SOD3) DURING ORTHODONTIC TOOTH MOVEMENT IN RATS

จุฬาลงกรณ์มหาวิทยาลัย
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ภาวะพร่องทางออกซิเจนระหว่างการเคลื่อนฟันด้วยแรงจัดฟัน กระตุ้นการสร้างรีแอคทีฟออกซิเจนสปีชีส์ (ROS) ในเนื้อเยื่อปริทันต์ (PDL) ได้ และซุปเปอร์ออกไซด์ดิสมิวเทส 3 (SOD3) เป็นเอนไซม์ที่ป้องกันเซลล์จากผลเสียจาก ROS ได้ การศึกษานี้ต้องการสืบหาการแสดงออกและหน้าที่ของ SOD3 ในเนื้อเยื่อปริทันต์ระหว่างการเคลื่อนฟันด้วยแรงจัดฟันในหนูและในเซลล์ PDL ที่อยู่ภายใต้ภาวะพร่องทางออกซิเจน โดยทดลองในหนู Sprague-Dawley อายุ 8 สัปดาห์ (6 ตัวต่อกลุ่ม) ดึงฟันกรามบนซี่ที่ 1 ด้วยสปริงเป็นเวลา 1 และ 14 วัน เนื้อเยื่อรากฟันถูกนำมาย้อมเพื่อดูการแสดงออกของโปรตีน SOD3 และ Hypoxia-inducible factor 1-alpha (HIF-1α) การศึกษาในเซลล์ PDL ศึกษาผลของ SOD3 ต่อการอยู่รอดของเซลล์ การสร้าง ROS และระดับ mRNA ของ *Hif-1α*, *Rankl* และ *Opg* ในเซลล์ PDL ของหนู และการสร้างของ Osteoclast ภายใต้ภาวะออกซิเจนปกติและภาวะพร่องทางออกซิเจน พบว่าการแสดงออกของ SOD3 ใน PDL รอบรากฟันลดลงแค่ในด้านที่ถูกกดทับใน 1 วันและลดลงทั้งสองด้านใน 14 วันหลังฟันได้รับแรง ส่วนในเซลล์ PDL ที่ถูกระงับการแสดงออกของ SOD3 พบว่าการอยู่รอดของเซลล์ และระดับ *Opg* mRNA ลดลง แต่มีการเพิ่มขึ้นของ ROS ระดับ mRNA ของ *Hif-1α* และ *Rankl* และการสร้างของ Osteoclast ในขณะที่ภาวะพร่องทางออกซิเจนทำให้ระดับ mRNA ของ *Sod3* และ *Opg* ลดลง แต่ ROS ระดับ *Rankl* mRNA และ osteoclast เพิ่มขึ้น โดยการเพิ่มระดับการแสดงออกของ SOD3 ในเซลล์สามารถช่วยลดความรุนแรงเหล่านี้ลงมาได้ ดังนั้น SOD3 เกี่ยวข้องกับการทำงานของ PDL ระหว่างการเคลื่อนฟันทางทันตกรรมจัดฟัน ผ่านการทำงานของ ROS, HIF-1α และ RANKL/OPG และการเพิ่มระดับของ SOD3 ช่วยลดความรุนแรงของภาวะพร่องออกซิเจนต่อเซลล์ลงมาได้

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Hypoxia induces reactive oxygen species (ROS) production in periodontal tissues. Superoxide dismutase 3 (SOD3) is an enzyme protecting cells from ROS. This study investigated SOD3 expression and function during rat orthodontic tooth movement (OTM) and in hypoxia-exposed rat PDL cells. OTM of right maxillary first molars were performed in Sprague-Dawley rats for 1 and 14 days (n = 6 per group). SOD3 and HIF-1α expression were evaluated by immunohistochemistry. SOD3 effects on cell viability and proliferation, ROS production, and mRNA expression of *Hif-1α, Rankl,* and *Opg* in PDL cells and osteoclast differentiation were investigated under normoxia and hypoxia. SOD3 expression in PDL tissues decreased on compression side on day 1 and on both sides on day 14. HIF-1α levels significantly increased on compression side on day 14. Cell viability, cell proliferation, and *Opg* mRNA expression decreased, whereas ROS production and *Hif-1α* and *Rankl* mRNA expression increased in SOD3-silenced PDL cells. Hypoxia reduced *Sod3* and *Opg* mRNA expression and increased ROS, *Rankl* mRNA expression, and osteoclast formation; SOD3 treatment attenuated these effects. Therefore, SOD3 plays a role in periodontal remodelling during OTM and in hypoxia-exposed PDL cells through ROS, HIF-1α, and RANKL/OPG pathways.

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TABLE OF CONTENTS

Page

.... iii
ABSTRACT (THAI) iii
.... iv
ABSTRACT (ENGLISH) iv
ACKNOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF FIGURES viii
CHAPTER I INTRODUCTION 1
1.1 Background and Rationale 1
1.2 Research Questions 2
1.3 Research Objectives 2
1.4 Research Hypotheses 2
1.5 Benefits of this Study 3
1.6 Limitation of this study 3
1.7 Conceptual Framework 4
CHAPTER II REVIEW LITERATURE 1
2.1 Orthodontic tooth movement (OTM) 1
2.2 Hypoxia 3
2.3 Superoxide dismutase 3 (SOD3) 4
CHAPTER III MATERIALS AND METHODS 1
3.1 *In vivo* sample characterization 1

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2 Immunohistochemistry 2

3.3 Rat primary PDL cell culture 3

3.4 Measurement of the ROS production 4

3.5 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis 4

3.6 Rat osteoclast culture and tartrate-resistant acid phosphatase (TRAP) assay...... 5

3.7 Statistical analysis 5

CHAPTER IV RESULTS 1

4.1 Expression of SOD3 and HIF-1α after 1 day and 14 days of OTM 1

4.2 Effects of SOD3 on PDL cell viability and cell proliferation 4

4.3 Effect of SOD3 on total ROS production in PDL cells 5

4.4 Effects of hypoxia and SOD3 on *Sod3, Hif-1α, Rankl,* and *Opg* mRNA expression in PDL cells 6

4.5 Effect of SOD3 on osteoclastogenesis *in vitro* 7

CHAPTER V DISCUSSION 1

CHAPTER VI CONCLUSION 1

REFERENCES 2

VITA 12

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Page

Figure 1 Common mechanism of SOD against O_2^- 4

Figure 2 Experimental orthodontic appliance using 25-g force Ni-Ti closed coil spring for providing OTM between M1 and incisors. 2

Figure 3 Three-dimensional reconstructed and two-dimensional micro-CT images after 14 days of OTM. 2

Figure 4 Morphological and histological observations of rat M1 after 1 and 14 days of OTM 1

Figure 5 SOD3 and HIF-1α expression in rat PDL tissues after 1 and 14 days of OTM 3

Figure 6 Sod3 mRNA expression in periodontal ligament tissues around rat after 14 days of OTM. 3

Figure 7 Gene expression profile of rat periodontal ligament (PDL) cells. 4

Figure 8 Effects of SOD3 on rat PDL cell viability and cell proliferation under normoxia and hypoxia 5

Figure 9 Effect of SOD3 on ROS production in rat PDL cells under normoxia and hypoxia 6

Figure 10 The mRNA expression of (A) Sod3, (B) Hif-1α, (C) Rankl, and (D) Opg in rat PDL cells in vitro. 7

Figure 11 Effects of SOD3 on osteoclastogenesis in vitro 8

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CHAPTER I INTRODUCTION

1.1 Background and Rationale

Orthodontic tooth movement (OTM) involves a series of biological events that results in alveolar bone and periodontal tissue remodelling. Orthodontic force causes compression of the periodontal blood vessels and reduces blood flow, causing hypoxia on the pressure side of the tooth (1). Reactive oxygen species (ROS), including superoxide anions, are signal mediators involved in various cellular processes and are generally redoxed by antioxidants (2). Low ROS levels stimulate proliferation and differentiation of the periodontal ligament (PDL) cells; however, when ROS overpowers the antioxidant defence systems, oxidative stress occurs, resulting in cellular damage and periodontal destruction (3). Both pressure and hypoxia from orthodontic forces independently stimulate ROS production in the periodontal tissues (4), which enhances osteoclastogenesis and bone resorption (5).

Superoxide dismutases (SODs) are major antioxidant enzymes that scavenge superoxide anion into hydrogen peroxide, which is further metabolised into water. In mammalian cells, SODs have three different isoforms, each of which performs the same dismutation function but at different subcellular locations: SOD1 in cytosol and nucleus, SOD2 in mitochondria, and SOD3 in extracellular matrix and on cell surfaces.

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Unlike the other isoforms, SOD3, which mainly redoxes extracellular ROS, is produced by smooth muscle cells and fibroblasts in vascular tissues and is highly expressed in specific tissues, such as blood vessels and lungs (6). SOD3 also exerts anti-inflammatory effects by preventing ROS-mediated tissue damage and inflammation (7), and SOD3 deficiency causes vascular dysfunction, including cardiovascular diseases and lung injury (6). Exendin-4 (E4), a glucagon-like peptide-1 receptor agonist that is commonly used to treat type 2 diabetes by lowering blood glucose levels (8), attenuate elevated ROS in various cell types, including PDL stem cells (9), and upregulate *Sod3* mRNA in endothelial cells (10). Although SOD3 has been extensively studied in various tissues (6, 7), little is known about its role in periodontal tissues. Recently, SOD3 expression has been discovered in PDL around intact dental roots in both humans and animals

(11). However, SOD3 expression and function during OTM have not yet been elucidated.

1.2 Research Questions

In vivo

1. Does orthodontic force have an effect on SOD3 expression in rat periodontium?

In vitro

1. How does hypoxia affect SOD3, RANKL, and OPG expression in rat PDL cells?
2. How does different SOD3 levels affect cell viability, cell proliferation, total ROS production, and mRNA expression of RANKL and OPG in rat PDL cells under normal and hypoxic condition?

1.3 Research Objectives

To investigate the expression and function of SOD3 in rat periodontal tissues during OTM and in hypoxia-exposed rat PDL cells.

1.4 Research Hypotheses

Null hypotheses:

1. Orthodontic force has no effect on SOD3 expression in rat periodontium.
2. Hypoxia has no effect on SOD3, RANKL, and OPG expression in rat PDL cells.
3. Different SOD3 levels has no effect on cell viability, cell proliferation, total ROS production, and mRNA expression of RANKL and OPG in rat PDL cells under normal and hypoxic condition.

Alternative hypotheses:

1. Orthodontic force has the effect on SOD3 expression in rat periodontium.
2. Hypoxia has the effect on SOD3, RANKL and OPG expression in rat PDL cells.
3. Different SOD3 levels has the effect on cell viability, cell proliferation, total ROS production, and mRNA expression of RANKL and OPG in rat PDL cells under normal and hypoxic condition.

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1.5 Benefits of this Study

Orthodontic force initiates mechanical stress- and hypoxia-induced ROS formation which can be regulated by SOD3 as a redox signaling. Knowing the role of SOD3 in periodontium during OTM and its associated signaling pathways will be beneficial for prevention and/or reduction of the adverse effect from orthodontic force or using as the biomarker indicating the optimal orthodontic force for the effective orthodontic tooth displacement.

1.6 Limitation of this study

The limitations of this study are a short time-course *in vivo* and a lack of time-course *in vitro* studies. A previous *in vitro* study that stimulated OTM was found to have both mechanical orthodontic strain and a hypoxic cell culture condition (40); however, the *in vitro* model used in this study only provides a hypoxic cell culture condition, which may only partially relate the *in vitro* data to our *in vivo* findings. Moreover, even though it has recently been discovered that SOD3 expression is epigenetically regulated in various cell types (41-43), the epigenetic regulation of SOD3 expression and the associated pathways in PDL are still poorly understood. Further research is required to clarify the molecular mechanisms of SOD3 during OTM, which could lead to the development of novel therapeutic modality in the future.

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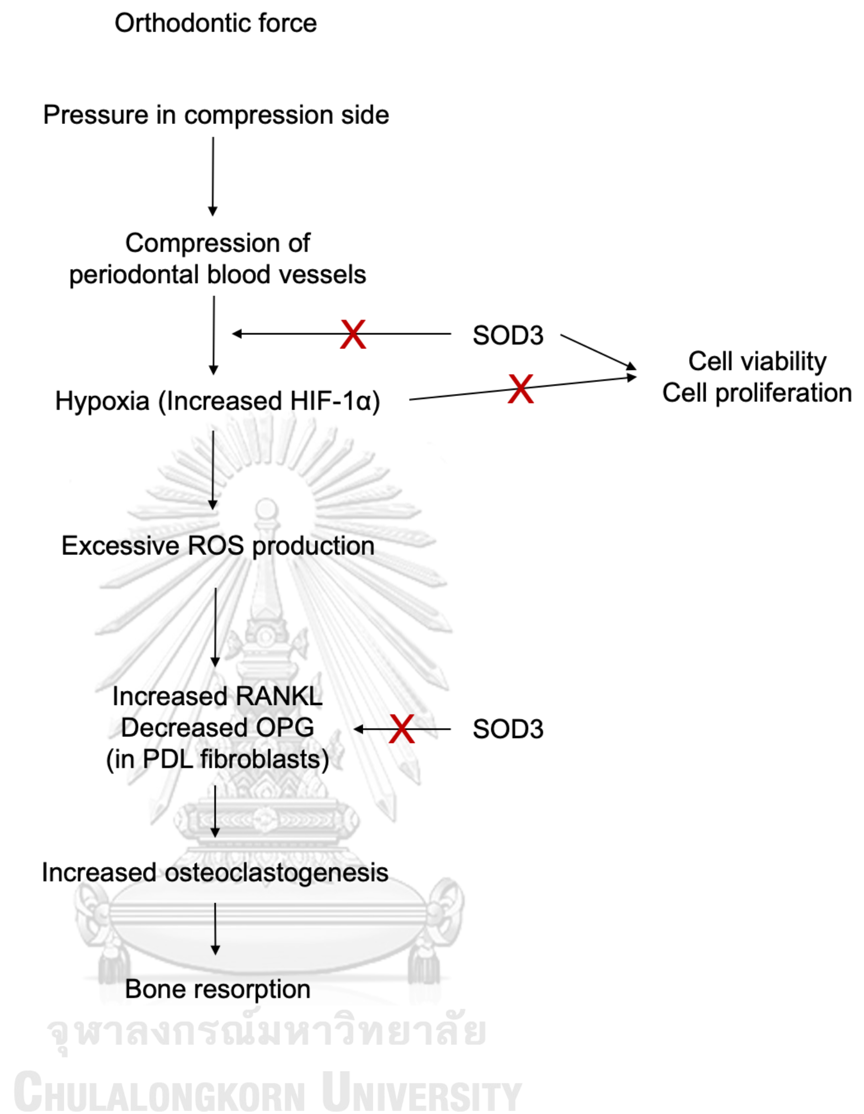
1.7 Conceptual Framework



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CHAPTER II REVIEW LITERATURE

2.1 Orthodontic tooth movement (OTM)

Pressure-tension hypothesis is the classic and well-known theory for describing orthodontic tooth movement (OTM). Teeth can be displaced by orthodontic force after the surrounding tissues are remodeled, from the coordination of resorption and formation of bone and periodontal tissues, and these remodeling processes involve in various biological events (12, 13). Receptor activator of nuclear factor-kappa ligand (RANKL) is a key cytokine for osteoclastogenesis and bone resorption which is secreted by osteoblasts and periodontal ligament (PDL) cells, and it has two main receptors, receptor activator of nuclear factor-kappa (RANK) and osteoprotegerin (OPG). RANK is the receptor on osteoclast precursor cells that can differentiate into mature osteoclasts after binding to RANKL, and RANK-RANKL binding leads to bone resorption. While, OPG is the decoy receptor for RANKL produced by osteoblasts, which plays roles in blocking and competing with RANK for binding to RANKL, and further inhibits osteoclastogenesis and stimulates bone formation. Therefore, the balance between RANKL and OPG indicates the occurrence of osteoclastogenesis and bone resorption during OTM (14, 15, 16). Moreover, RANKL and OPG express differently in compression and tension side during OTM. RANKL is upregulated and enhances osteoclastogenesis in compression side, while the expression of RANKL is decreased with the increased OPG expression in tension side due to tensile strain. Although the expression of RANKL and the formation of OPG are not dependent on each other, the relative expression of RANKL and OPG affects bone remodeling (17). Patients undergoing orthodontic treatment have shown a higher level of RANKL and decreased OPG concentration in gingival crevicular fluid (GCF) at the compression side of the displaced tooth (18). The similar changes of RANKL and OPG expression are found in human PDL cells treated with compressive force in time- and force-magnitude-dependent manner (18, 19). RANKL expression in periodontal tissues and tartrate-resistant acid phosphatase (TRAP) activity are also higher in compression side of OTM group, compared to the non-force controls in rats, indicating the occurrence of bone resorption during OTM (20).

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Root resorption is a common unpredictable and undesired event from orthodontic treatment. Teeth experiencing orthodontic force have a higher rate of root resorption than non-treated teeth (21), and orthodontically-induced root resorption (OIRR) is irreversible if it goes beyond cementum into dentin (22). The systematic reviews have reported that the severity of OIRR positively correlates with the magnitude of force (21, 23). A clinical trial by Barbagallo et al. found that human premolars under light and heavy force could produce 5-fold and 9-fold more extensive root resorption than control, respectively (24). In rat experiments, King et al. have suggested that the effective force for the mesial tooth movement of rat molars in 10 days has been ranged from 20 to 40 g, and the force more than 40 g does not accelerate tooth movement (25). Furthermore, the lighter force can provide higher tooth movement and less root resorption area in rats. Gonzales et al. found that amount of tooth movement in 14 days after force application was not significantly different among 10, 25, and 50 g of force but these 3 force magnitudes could produce more significant tooth movement of rat molars than 100-g force. However, only the 10-g force could move the greatest tooth movement in 28 days. Due to root resorption correlating with force magnitude and increasing over time, 10-g force created the least root resorption among the other forces in both 14 and 28 days after force initiation and the most resorbed root in rats' maxillary first molars was distobuccal root followed by distopalatal, middle-buccal, middle-palatal and mesial root, respectively, which was identified by scanning electron microscope (SEM) (26). Role of RANK/RANKL/OPG system in root resorption is similar to bone remodeling. During physiological root resorption, RANKL dose-dependently stimulates resorptive activity in odontoclasts and OPG suppresses the process (17). RANKL and OPG also involve in OIRR as their mRNA can be detected in the area of root resorption under heavy orthodontic force (27). Heavy force induces the expression of RANK and RANKL in root resorption area and the RANKL/RANK activity in odontoclasts and PDL fibroblasts in rats (28). The increased RANKL level and decreased OPG level in PDL cells can lead to OIRR and the greater expression of RANKL contributes to the more severe OIRR (29). Thus, the RANK/RANKL/OPG signaling pathway plays an important role not only in bone remodeling but also in root resorption process (15, 17, 27, 28, 29).

2.2 Hypoxia

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydrogen radical (HO•), are generated endogenously from two main resources; biological processes and cellular responses to xenobiotics, cytokines, and bacteria (2, 30). In biological processes, ROS are released as byproduct mainly from mitochondrial oxidative metabolism, but ROS in the cellular responses are deliberately produced either as a part of signal transduction pathway or a part of cell defense mechanism (2). ROS also associate with the pathogenesis of many cardiovascular diseases, including hypertension, diabetes, atherosclerosis, and heart failure (6, 30). Moreover, excessive ROS level or inadequate cellular antioxidant capacity for scavenging ROS can cause an imbalance between ROS and cellular antioxidants, and when ROS defeats the antioxidant defense system, oxidative stress occurs (30). This oxidative stress can affect cell proliferation, cell survival, and cell apoptosis of osteoblasts, osteoclasts, PDL cells, and cementoblasts (1, 31, 32).

During OTM, occlusion of periodontal vessels and reduction of blood supply lead to hypoxia in compression side of dental root (1, 33). Khouw and Goldhaber demonstrated the OTM in adult rhesus monkeys and adult German shepherd dogs and investigated the vascular changes in PDL after force application for 1, 3, and 7 days. Periodontal vessels in compression side were partially or completely occluded on 1 day after force application, then the vessels almost completely occluded on day 3, and both surface and undermining resorption of alveolar bone occurred on day 7 of OTM (33). Similarly, orthodontic force also induces hypoxic conditions in rat dental pulp tissues and hypoxia-treated dental pulp fibroblasts significantly enhance the ROS formation (34).

Either mechanical compression or hypoxia is able to enhance ROS production. In *in vitro* studies of human PDL cells, ROS can be generated under mechanical compression (4, 35, 36). Chae et al. found that PDL cells under 0.5 to 3 g/cm^2 of compressive force for 4 hours could stimulate ROS production and force of 3 g/cm^2 produced the highest ROS level (4). Six hours under 1 g/cm^2 of mechanical compression, PDL cells can induce ROS production and ROS levels are further

increased at 24 hours after force stimulation (35). Hypoxia alone also can induce ROS formation in human PDL cells. A significant increase of ROS production was found in a time-dependent manner from 1 to 4 hours under hypoxia (37), and in a dose-dependent manner under $CoCl_2$-induced hypoxia which further initiated apoptosis of the cells (38). Moreover, both compression and hypoxia independently enhance ROS production in PDL cells (4, 5), and ROS are able to increase osteoclastogenesis and osteoclastic resorption through RANKL (39, 40, 41). Hypoxia regulates osteoclasts by increasing cell proliferation, cell differentiation, and osteoclastic resorption, and also regulates osteoblasts by stimulating RANKL and inhibiting OPG (42). Human PDL cells under less than 2% hypoxic conditions were shown to express the increased RANKL and decreased OPG (43, 44), and hypoxia enhances RANKL expression through hypoxia inducible factor-1α (HIF-1α) (43). Thus, both hypoxia and mechanical compression play role in initiating osteoclastogenesis in human PDL cells and may synergistically affect osteoclast formation and bone resorption (5).

2.3 Superoxide dismutase 3 (SOD3)

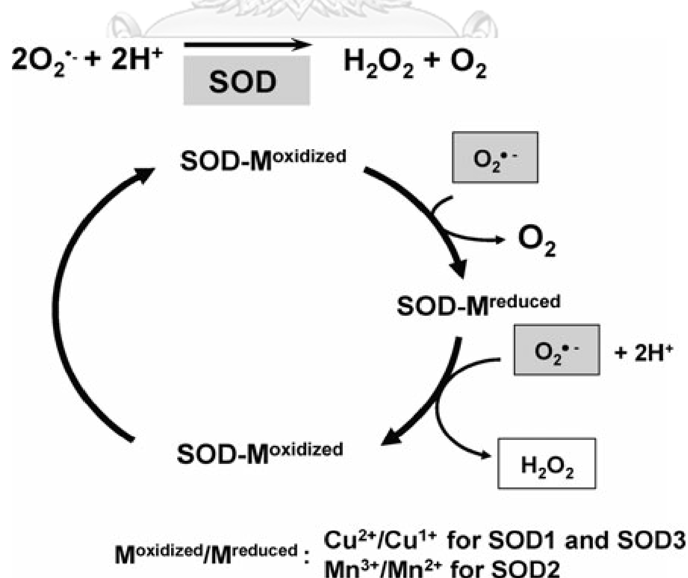

Figure 1 Common mechanism of SOD against O_2^-

(Fukai et al. Antioxid Redox Signal. 2011)

Superoxide dismutases (SODs) are the first line of defense against ROS, by converting O2- from mitochondrial oxidative mechanism into H_2O_2, which catalase and glutathione peroxidase further metabolize into water and oxygen (Figure 1) (2, 6, 45). SODs have three isoforms with different subcellular locations; cytoplasmic copper/zinc-SOD (Cu/ZnSOD, SOD1), mitochondrial manganese SOD (MnSOD, SOD2) and extracellular Cu/ZnSOD (EC-SOD, SOD3), however, each isoform has the same mechanism of dismutation. Catalytic metal is always required in the activation of each SOD into an active form. Different from intracellular SOD1 and SOD2, SOD3 is the only isoform that locates in extracellular matrix and on endothelial cell surfaces in plasma and extracellular fluids. Although SOD3 distributes in various body tissues, only in specific tissues, particularly in blood vessels, lungs, kidneys, uterus, and heart, have high expression of SOD3. In vascular tissues, smooth muscle cells and fibroblasts are the primary cells that produced SOD3 (6), and if the vascular damages occur, SOD3 expression can be reduced (46). Due to its therapeutic effect in various tissue injuries, SOD3 is an important antioxidative enzyme with anti-inflammatory nature for tissue recovery and also has an anti-apoptotic ability which essential for survival. SOD3 overexpression by gene transfer in hind limb ischemic rats can reduce tissue damages and enhance injured tissue recovery by reducing the expression of superoxide anion and increasing cell proliferation (46, 47).

Antioxidants, including SOD, have an association with human periodontal conditions. Many studies have found that patients with periodontal disease, the result of oxidative stress-induced periodontal destruction, have a significant decrease of antioxidant capacity in serum, saliva, and GCF compared with periodontally healthy patients (48, 49, 50). Immediately after non-surgical periodontal therapy, the decreased SOD activity in saliva occurs and remains for 1 month then SOD activity increases in 3 months later and patients with periodontal diseases have lower SOD activity in all time points, either before or after the treatment. Thus, scaling and root planing can change salivary SOD activity (49). On the other hand, SOD activity in severely inflamed gingiva is increased and higher than in healthy gingiva (51, 52). Interestingly, Jacob and Davis found that periodontal tissues from human tooth roots were associated with SOD activity (53). Golz et al. reported hypoxia-induced oxidative stress has occurred in both

periodontal tissue and PDL fibroblasts, and the activities of redox systems, including SOD and catalase, were affected by inflammatory gingival condition. This oxidative stress in periodontium can lead to inflammation and bone resorption. Suggesting that maintenance of PDL cell and tissue homeostasis requires the balance between ROS formation and redox system capacity (37). The increase of osteoclast-mediated ROS formation and bone resorption may be found after inhibiting SOD activity (54), and in contrast, SOD can inhibit osteoclastogenesis in bone marrow cells (55). Additionally, osteoblasts treated with oxidative stress show higher ROS formation and higher cell apoptosis, while the SOD3 expression and gene involving in bone formation are reduced. However, all of these effects on osteoblastic cells are attenuated by antioxidants (56). Another study in the rats feeding with a high-fat diet to induce the systemic oxidative stress showed the higher oxidative stress level in gingival tissues, higher bone resorption amount, and lower SOD3 expression, which could be reduced by antioxidants (57). Altogether, it may assume that SOD3 involves in some part of ROS-induced osteoclastogenesis and bone resorption processes.

In addition, SOD3 has been suggested to play a survival-supporting role in the lung and cardiovascular tissue environments (58). Mice lacking SOD3 had lower survival rate and higher mortality, in both normal and hypoxic conditions, due to severe lung damages (59, 60), and pulmonary hypertension and ventricular hypertrophy, which can lead to heart failure and death, were worsened in SOD3 knockout mice exposed to chronic hypoxia, compared to the knockout mice in normoxic condition (61). While E4, a glucagon-like peptide-1 (GLP-1) receptor agonist, is commonly used in the treatment of type 2 diabetes by lowering blood glucose levels (8), and has been shown to upregulate *Sod3* mRNA in various cell types (9, 10).

Recently, Salmon et al. have identified that SOD3 is a specific biologic marker for dental cementum by proteomic analysis. SOD3 is found in all dental cementum and PDL tissues samples of mouse, pig, and human teeth. In mice, cellular cementum of molars and acellular cementum of incisors are the location of SOD3 expression, but SOD3 is also lightly expressed in PDL tissues and alveolar bone. Strong expression of SOD3 is detected in pig cementoblasts and cementocytes of dental cementum, though it is also found in adjacent PDL and bone cells. Similar to the pattern in pig molars,

SOD3 in human teeth strongly expresses in cementoblasts and nearby PDL at the cervical area of the root and is also localized in PDL, cementocytes of dental cementum at the apical part, odontoblasts, and dentinal tubules. Due to its antioxidative potential, SOD3 may take an important part in PDL remodeling and protecting cementum from ROS-mediated oxidative stress during forming and maintaining the dental root (11). Although the role of SOD3 during OTM has not yet been studied by this time and the associated mechanism has not been defined, SOD3 may help to reduce ROS production from orthodontically-induced mechanical stress and hypoxia which may affect cell proliferation and/or cell apoptosis of PDL cells and further result in reduction or prevention of the adverse effects from orthodontic force. However, this statement still needs to be proved.

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CHAPTER III MATERIALS AND METHODS

3.1 *In vivo* sample characterization

Sample size was calculated using the mean and standard deviations from a previous study that examined the amount of tooth movement (62). According to the calculation performed using G*Power software version 3.1, a minimum of three rats was required in each group. The number of analyses in our study determined the sample size for the *in vivo* study. Six rats were, thus, selected for each group and each experimental period.

Eight-week-old male Sprague-Dawley rats (*n* = 6 per group; Charles River Laboratories Japan, Yokohama, Japan) were anaesthetised before placing a 25-g nickel-titanium closed-coil spring (TOMY International, Tokyo, Japan) between the right maxillary first molar (M1) and maxillary incisors to induce mesial tooth movement of M1 for 1 and 14 days. The left M1 served as a control (Figure 2).

Amount of OTM was measured using micro-computed tomography (Figure 3). The maxilla was scanned through a 0.1-mm filtered brass plate using a micro-focus X-ray CT system (SMX-100CT; Shimadzu, Kyoto, Japan) at 30 kV, 30 μA, and 0.05 mm/voxel. Three-dimensional micro-CT images were reconstructed using an image analysis software (TRI/3D-BON; Ratoc System, Osaka, Japan), and the distance between the distal crown surface of rat maxillary first molar (M1) and the mesial crown surface of maxillary second molar (M2) was measured at least three times to determine the average amount of tooth movement in each group.

This animal experiment was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (A2019-034C).

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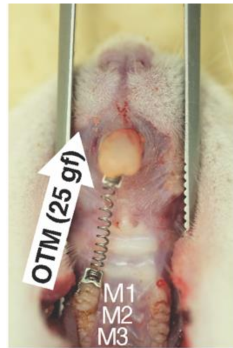

Figure 2 Experimental orthodontic appliance using 25-g force Ni-Ti closed coil spring for providing OTM between M1 and incisors.

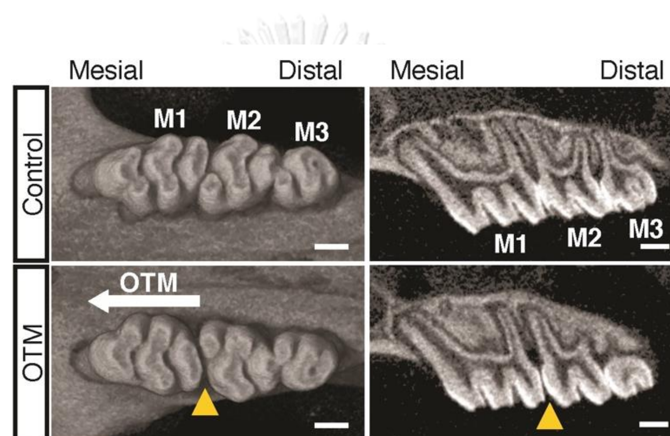

Figure 3 Three-dimensional reconstructed and two-dimensional micro-CT images after 14 days of OTM.

Space between M1 and maxillary second molars (M2) was observed only on the OTM side after 14 days, and the average OTM was 118.89 ± 3.55 µm ($p = 0.002$); Mann–Whitney U test. Scale bar, 1 mm.

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3.2 Immunohistochemistry

The dissected maxillae, including the upper molars, were fixed in 4% paraformaldehyde, demineralized in 18% ethylenediaminetetraacetic acid (EDTA) (G-Chelate Mild; GenoStaff, Tokyo, Japan) for 8 weeks at 4°C, and embedded in paraffin.

Transverse sections of 7 µm were stained with haematoxylin and eosin (HE) to examine root morphology. Tartrate-resistant alkaline phosphatase (TRAP)-positive cells were evaluated using a TRAP/ALP Stain Kit (Wako Pure Chemical, Osaka, Japan) and counterstained with methyl green. Stained sections were visualised and captured using

an inverted optical microscope (Axiovert 200M; Carl Zeiss, Jena, Germany) and AxioVision software (version 4.8).

Tissue sections were obtained and incubated overnight at 4°C with rabbit anti-SOD3 polyclonal antibody (1:200, MBS2025997, MyBioSource, San Diego, CA, USA) or rabbit anti-hypoxia-inducible factor 1-alpha (HIF-1α) polyclonal antibody (1:200, NB100-134, Novus Biologicals, Centennial, CO, USA). The sections were subsequently incubated with ImmPRESS® HRP anti-rabbit IgG (Vector Laboratories, Newark, CA, USA) for 1 h before colorimetric detection using ImmPACT® DAB (Vector Laboratories). Relative optical density of immunoreactive areas in the PDL of distobuccal roots were quantified using ImageJ software (NIH, Bethesda, MD, USA).

3.3 Rat primary PDL cell culture

Rat PDL cells were isolated from the PDL tissues around intact rat molars according to a previously modified method (12). Briefly, after molar extraction, the PDL tissues were washed with sterilised phosphate-buffered saline four times and gently scraped from all the roots using 23-gauge needles under the stereomicroscope (Leica MZ12.5, Leica Microsystems GmbH, Wetzlar, Germany). The PDL tissues were placed in 35 mm culture dishes and grown in α-modified Eagle's medium (FUJIFILM Wako Chemicals, Tokyo, Japan) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 humidified incubator. The medium was changed every 48 h. The PDL cells were grown and trypsinized after reaching 70% confluence until 3 to 5 passages before conducting the *in vitro* experiments. E4 (Abcam, Cambridge, UK) and SOD3 siRNA (si-SOD3; Thermo Fisher Scientific, Waltham, MA, USA) were used as SOD3 accelerator and SOD3 gene silencer, respectively. PDL cells were seeded in 6-well plates at a density of 5×10^5 cells/well and were grown to 70% confluence before treatment with 100 nM E4 or transfection of si-SOD3 to the cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) at a final concentration of 10 μM/well. After 24 h of E4 treatment or si-SOD3 transfection, the cells were placed in normal incubation chamber or transferred to BIONIX-3 hypoxic chamber (SUGIYAMA-GEN, Tokyo, Japan) to mimic OTM-induced

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hypoxia (5% O_2) for another 24 h before cell viability and proliferation, total ROS production, and mRNA expression were evaluated under each cell condition. All *in vitro* experiments of each sample were independently reproduced three times.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. Briefly, 1×10^4 cells/well in a 96-well plate were treated with E4 or transfected with si-SOD3 and incubated in normoxic or hypoxic conditions, before adding 15 µL MTT solution/well and incubating at 37°C for 4 h. The reaction was stopped by adding 100 µL dimethyl sulfoxide/well, and the absorbance of final product was measured at 570 nm using a microplate reader (Spectra Max Plus; Molecular Devices, San Jose, CA, USA). Cell proliferation was determined by immunostaining with 5-bromo-2-deoxyuridine (BrdU). PDL cells in 6-well plates were labelled with 10 µL of 1 mM/mL BrdU solution in culture medium for 1 h before cell fixation. BrdU-labelled cells were detected using BrdU In-Situ Detection kit (BD biosciences, Franklin Lakes, NJ, USA) according to manufacturer's protocol, and the percentage of BrdU-positive cells in total cells was quantified.

3.4 Measurement of the ROS production

Total ROS production was measured in treated PDL cells at a density of 5×10^5 cells/well in a 35-mm glass bottom dish using ROS-ID Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA). Fluorescence intensity of the visualised cells was quantified using ImageJ software.

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3.5 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Expression of *Sod3* mRNA in PDL tissues of control and OTM groups and *Sod3, Hif-1α,* receptor activator of nuclear factor kappa-β ligand (*Rankl*), and osteoprotegerin (*Opg*) mRNA in PDL cells in each cell condition was evaluated using RT-qPCR. Total RNA was extracted from PDL tissues and cultured PDL cells using ISOGEN II (Nippon Gene, Tokyo, Japan). In our study, the quality of total RNA was evaluated using a spectrophotometer (DeNovix DS-11, DeNovix Inc., Wilmington, DE, USA), and an A260/A280 ratio of 1.8 to 2.0 was considered acceptable for all samples. cDNA was

synthesised using PrimeScript™ RT reagent Kit (Perfect Real Time, RR037A; Takara Bio, Shiga, Japan), and qPCR was performed using TaqPath™ qPCR Master Mix CG (Thermo Fisher Scientific) with TaqMan Gene Expression Assays (Thermo Fisher Scientific), as shown in Table 1. All *in vivo* and *in vitro* samples were analysed in triplicate, and relative mRNA expression was calculated using relative standard curve method normalised to *Gapdh* expression.

Gene name	Assay ID
Sod3	Rn00563570_m1
Hif-1a	Rn01472831_m1
Rankl	Rn00589289_m1
Opg	Rn00563499_m1
Gapdh	Rn01775763_g1

Table 1 Real-time reverse transcriptase-PCR Gene Expression Assay ID

3.6 Rat osteoclast culture and tartrate-resistant acid phosphatase (TRAP) assay

Rat osteoclasts (OSC12C; Cosmo Bio, Tokyo, Japan) were cultured in an osteoclast differentiation medium (OSCMR; Cosmo Bio) containing 15 ng/mL RANKL and 50 ng/mL macrophage colony-stimulating factor. In 24-well plates, osteoclasts at a density of 5×10^4 cells/well were treated with or without recombinant rat SOD3 protein (LSBio, Seattle, WA, USA) for 24 h before incubation under normoxia or hypoxia. TRAP staining (Cosmo Bio) was performed after 6 days of culture. Osteoclasts were defined as TRAP-positive multinucleated cells with 3 or more nuclei.

3.7 Statistical analysis

All the data are expressed as mean and standard deviation. Shapiro–Wilk test was used to assess the normality of the data distribution. Differences between the groups were analysed by the one-way analysis of variance with Tukey's post-hoc test for normally distributed data or by the Mann–Whitney U test for non-normally

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distributed data using SPSS v.28 software (IBM, New York, USA). A *p*-value less than 0.05 was considered statistically significant.

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CHAPTER IV RESULTS

4.1 Expression of SOD3 and HIF-1α after 1 day and 14 days of OTM

Morphological and histological changes of rat M1 after 1 and 14 days of OTM are shown in Figure 4. HE staining shows uniform arrangement of dense connective tissue fibers and PDL fibroblasts along the intact root surfaces in control group on both day 1 and 14, whereas PDL arrangement became coarser and more irregular in the OTM group. Root resorption lacunae only appears on the pressure side of the OTM group on day 14 and not on the tension side (Figure 4A). TRAP staining shows that none of the TRAP-positive cells were detected on both sides of both control and OTM groups on day 1. On day 14, osteoclasts are clearly visible on the bone resorbed surfaces on the pressure side of the OTM group, while the control remained unchanged (Figure 4B).

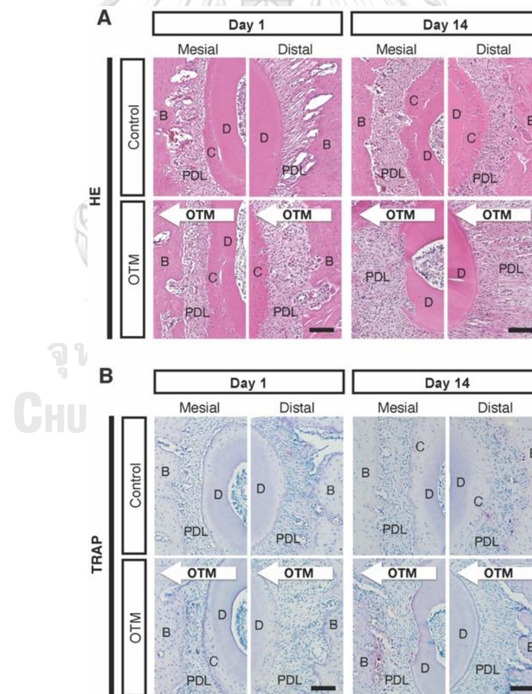

Figure 4 Morphological and histological observations of rat M1 after 1 and 14 days of OTM

(A) HE staining (B) TRAP staining. B, bone; PDL, periodontal ligament; C, cementum; D, dentin; OTM, orthodontic tooth movement; HE, hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase Scale bar, 100 µm.

SOD3 expression was observed abundantly in both the mesial and distal side of the periodontal tissue in the control group (Figure 5A). On day 1, the compression side of the OTM had significantly lower SOD3 expression than the mesial side of the control group and tension side of the OTM group (p = 0.010 and p < 0.001, respectively). While the lower expression on the distal side of the control group had no significant difference from the mesial side, there was a significant difference in the tension in the OTM group (p = 0.047). On day 14, the SOD3 expression in the OTM group was decreased on both the sides, and the expression in the OTM group on both the compression and tension sides were significantly lower than the mesial and distal sides of the control group (p < 0.001 and p = 0.030, respectively), with no significant difference between the sides of the OTM groups (Figure 1A and B). Additionally, *Sod3* mRNA expression was significantly decreased in the PDL tissues of the OTM group on day 14 (p < 0.001; Figure 6).

HIF-1α expression on both compression and tension sides was significantly higher in the OTM group than in the mesial (p = 0.017 and p = 0.005, respectively) and distal (p = 0.009 and p = 0.003, respectively) sides of the control group on day 1. On day 14, HIF-1α expression in the OTM group on compression sides was significantly higher than the mesial (p = 0.041) and distal (p = 0.033) sides of the control group; the compression side had slightly higher expression than the tension side without a statistically significant difference (Figure 5C and D).

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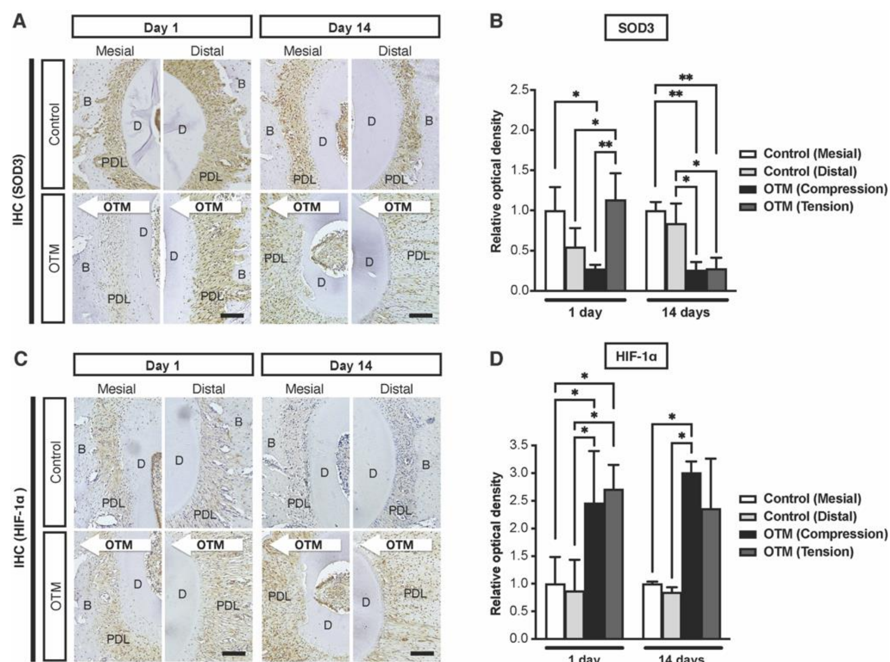

Figure 5 SOD3 and HIF-1α expression in rat PDL tissues after 1 and 14 days of OTM

*Immunohistochemical staining and relative optical density of (A, B) SOD3 and (C, D) HIF-1α expression in the PDL tissues of rat M1. SOD3, Superoxide dismutase 3; M1, right maxillary first molar; HIF-1α, hypoxia-inducible factor 1-alpha; B, bone; OTM, orthodontic tooth movement; PDL, periodontal ligament; D, dentin; Scale bar, 100 μm. *, p < 0.05; **, p < 0.001.*

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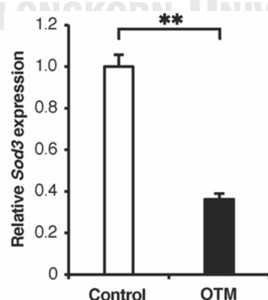

Figure 6 Sod3 mRNA expression in periodontal ligament tissues around rat after 14 days of OTM.

*Data are expressed as mean ± standard deviation and n = 4 for each group. Statistical analysis was performed using Mann–Whitney U test. **, p < 0.001.*

4.2 Effects of SOD3 on PDL cell viability and cell proliferation

The PDL cells used in our *in vitro* study had spindle-shaped morphology with the characteristic shown in Figure 7. E4 concentrations ranging from 50 to 200 nM significantly increased cell viability compared with the controls ($p < 0.001$; Figure 8A). PDL cell viability was significantly reduced by hypoxia; however, E4 could restore it to the same level as normoxic controls (Figure 8B). Cell viability of normoxic si-SOD3 group showed a significant reduction when compared to normoxic controls ($p < 0.001$; Figure 8B). Hypoxia tended to reduce cell proliferation in all groups, but the only significant difference observed was between the E4 groups under different oxygen conditions ($p < 0.001$). E4 significantly increased cell proliferation in normoxia ($p < 0.001$) and attenuated hypoxia-induced reduction of cell proliferation ($p < 0.001$), whereas si-SOD3 had no effect under either oxygen conditions (Figure 8C).

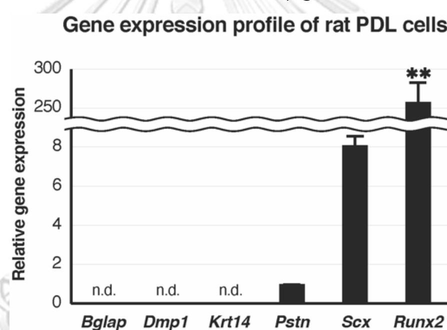

Figure 7 Gene expression profile of rat periodontal ligament (PDL) cells.

*Rat PDL cells expressed positive expression for the markers of periodontal ligament (Pstn and Scx) and immature osteoblasts (Runx2) with undetectable expression for markers related to mature osteoblasts (Bglap), osteocytes (Dmp1), and epithelial cells (Krt14). Bglap, Bone gamma-carboxyglutamate protein; Dmp1, Dentin matrix protein 1; Krt14, Keratin 14; Pstn, Periostin; Scx, Scleraxis; Runx2, Runt-related transcription factor 2; n.d.; not detected. **, p < 0.01.*

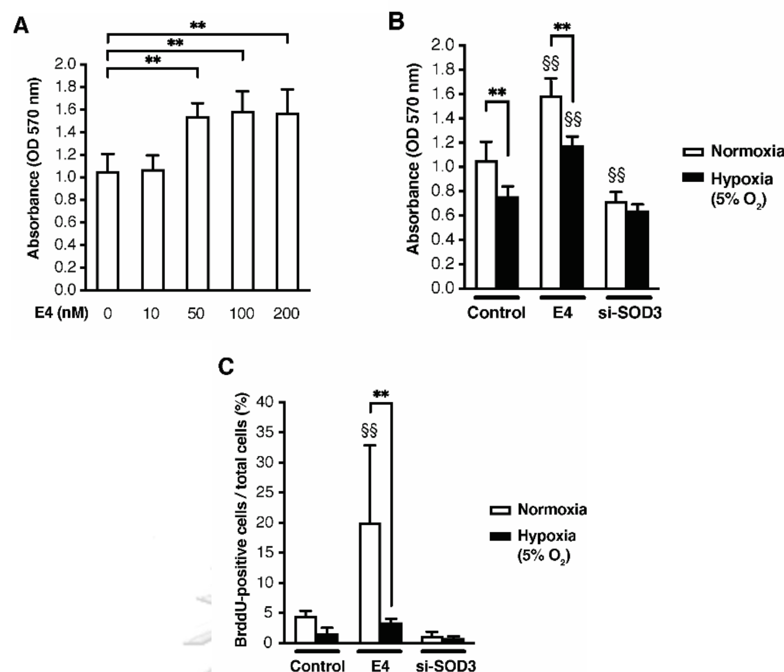

Figure 8 Effects of SOD3 on rat PDL cell viability and cell proliferation under normoxia and hypoxia

*(A) Cell viability of PDL cells determined by MTT assay after 24 h of Exendin-4 (E4) treatment at concentrations ranging from 0 to 200 nM. (B) Cell viability and (C) cell proliferation of E4-treated or si-SOD3-transfected PDL cells under different oxygen conditions. *, $p < 0.05$; **, $p < 0.001$; §, $p < 0.05$, §§, $p < 0.001$. *, compared between normoxia and hypoxia; §, compared with the matched oxygen level controls.*

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4.3 Effect of SOD3 on total ROS production in PDL cells

Only a small amount of total ROS was produced in the control group, and E4 had no effect on total ROS production under normoxia (Figure 9A). Hypoxia significantly increased ROS (p = 0.001); however, E4 reduced hypoxia-induced ROS production to a normal level ($p < 0.001$ compared to hypoxic controls; Figure 9A). While si-SOD3 significantly increased the total ROS in normoxia (p = 0.021), there was statistically no significant difference between the si-SOD3 groups under the different oxygen conditions. Additionally, total ROS production was significantly and synergistically increased when si-SOD3 and hypoxia were combined ($p < 0.001$ compared to normoxic controls; Figure 9A and 9B).

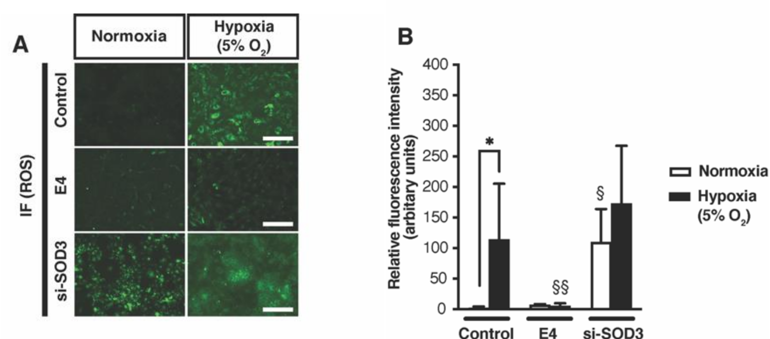

Figure 9 Effect of SOD3 on ROS production in rat PDL cells under normoxia and hypoxia

(A) Represented fluorescence images of total ROS production (green) in each cultured condition. Scale bar, 30 μm. (B) Relative fluorescence intensity of total ROS in each cultured condition. *, $p < 0.05$ compared between normoxia and hypoxia; §, $p < 0.05$ compared with the matched oxygen level controls. SOD3, Superoxide dismutase 3; ROS, reactive oxygen species; PDL, periodontal ligament.

4.4 Effects of hypoxia and SOD3 on *Sod3, Hif-1α, Rankl,* and *Opg* mRNA expression in PDL cells

Hypoxia significantly reduced the mRNA expression of *Sod3* (p = 0.022) and *Opg* (p < 0.001) while significantly increasing that of *Hif-1α* (p < 0.001) and *Rankl* (p < 0.001; Figure 10A-10D). E4 was effectively increased *Sod3* mRNA in normoxia and hypoxia (p < 0.001), which were both significantly higher than normoxic controls (p < 0.001; Figure 10A). The expression of *Hif-1α* mRNA after E4 treatment was similar to that in the controls under both oxygen conditions (Figure 10B). Even though *Hif-1α* expression was significantly lower in E4-treated cells under hypoxia compared to hypoxic controls (p < 0.001), it was still significantly higher than that in normoxic E4 group (p < 0.001; Figure 10B). E4 had no effect on *Rankl* expression in normoxia but was able to significantly decrease *Rankl* mRNA under hypoxia (p = 0.004) to nearly the same level as that in normoxic control (Figure 10C), whereas E4 significantly elevated *Opg* mRNA in both oxygen conditions (p < 0.001), without significant difference within E4-treated groups (Figure 10D). With si-SOD3, *Hif-1α* mRNA expression remained unchanged in

normoxia but significantly increased under hypoxia ($p < 0.001$; Figure 10B). *Rankl* expression significantly increased after si-SOD3 transfection alone ($p < 0.001$), and when combined with hypoxia, the increase was significantly higher ($p < 0.001$; Figure 10C). si-SOD3-transfected cells had significantly lower *Opg* mRNA levels under normoxia ($p < 0.001$), which were further suppressed under hypoxia ($p = 0.015$; Figure 10D).

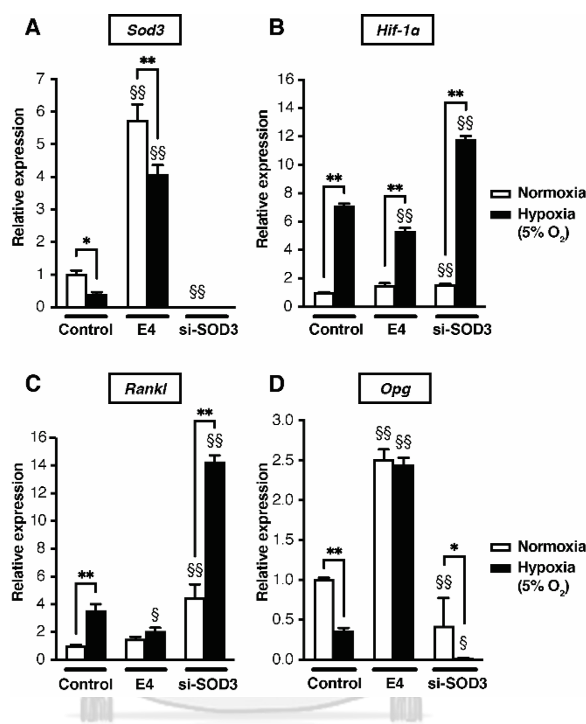

Figure 10 The mRNA expression of (A) Sod3, (B) Hif-1α, (C) Rankl, and (D) Opg in rat PDL cells in vitro.

**, p < 0.05; **, p < 0.001; §, p < 0.05, §§, p < 0.001. *, compared between normoxia and hypoxia; §, compared with the matched oxygen level controls. SOD3, Superoxide dismutase 3; PDL, periodontal ligament; HIF-1α, hypoxia-inducible factor 1-alpha; Rankl, receptor activator of nuclear factor kappa-B ligand; Opg, osteoprotegerin.*

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4.5 Effect of SOD3 on osteoclastogenesis *in vitro*

TRAP-positive osteoclasts were significantly reduced by recombinant rat SOD3 protein at concentrations of 100 and 150 ng/mL ($p < 0.001$; Figure 11A). Administration of 125 ng/mL recombinant SOD3 protein significantly decreased the number of TRAP-

positive osteoclasts under normoxia (p = 0.037) and tended to reduce osteoclasts under hypoxic conditions (Figure 11B and 11C).

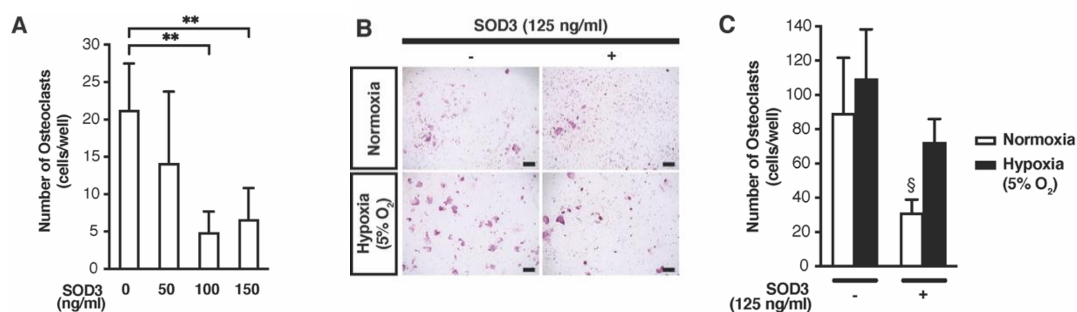

Figure 11 Effects of SOD3 on osteoclastogenesis in vitro

*(A) Number of rat osteoclasts after 6 days of administration of rat SOD3 protein at concentrations ranging from 0 to 150 ng/mL under normoxia. **, p < 0.001. (B) Represented images of TRAP-positive osteoclasts cultured with or without 125 ng/mL SOD3 under normoxia or hypoxia. Scale bar, 500 µm. (C) Number of rat osteoclasts in each cultured condition. §, p < 0.05 compared with the matched oxygen level controls. SOD3, Superoxide dismutase 3; TRAP, tartrate-resistant acid phosphatase.*

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CHAPTER V DISCUSSION

OTM proceeds by remodelling of the periodontal tissues when exposed to mechanical loading and induces local hypoxia on the compression side, which is involved in extensive cellular and molecular responses. Orthodontic force causes reversible minor tissue injuries; however, a force exceeding tissue threshold can cause permanent adverse effects (63).

HIF-1α is a key transcription regulator for hypoxic response and is relatively stable at low oxygen levels (64); thus, the increased HIF-1α expression was used as a positive control for hypoxia in this study. HIF-1α expression was found to be increased in rat PDL tissues after 1 and 14 days of OTM, with a markedly higher expression on the compression side on day 14, which was consistent with a previous report that found significantly higher HIF-1α protein expression on compression side than on the tension side on day 14 but not on day 1 in the rats (65). The increased *Hif-1α* mRNA level also confirmed the hypoxic culture conditions in our *in vitro* study, which is consistent with previous findings in hypoxia-exposed PDL cells (66, 67).

Compression and hypoxia both increase ROS production in human PDL cells (4), and either elevated ROS or hypoxia suppresses PDL cell viability (66, 68). In our study, hypoxia-exposed PDL cells also had increased total ROS production and decreased cell viability and proliferation. Increased ROS and hypoxia are known to promote osteoclast differentiation and bone resorption (69, 70), and our findings showed elevated *Rankl* and reduced *Opg* expression in hypoxia-exposed PDL cells with increased osteoclast formation. Accordingly, excessive hypoxia-induced ROS production reduces the viability and proliferation of PDL cells while promoting osteoclastogenesis.

In human dental tissues, SOD3 is exclusively found in the PDL, dentin, and cementum, but not in the alveolar bone (11). Similar expression patterns are found in mouse and pig molars, although with SOD3 localization in alveolar bone (11). Previous animal studies have reported that OTM increased ROS-mediated oxidative stress in rat periodontal tissues (71), and rats with high gingival oxidative stress levels have lower

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SOD3 expression in gingival tissues with increased alveolar bone resorption (57). Increased ROS and decreased SOD3 expression in rat peri-implant connective tissues have also been observed following dental implant placement (72). Furthermore, excessive ROS in osteoblasts suppresses cell viability, SOD3 expression, and bone formation-related genes (56). Our findings showed SOD3 expression on both sides of intact PDL, and we demonstrated for the first time that OTM causes a significant reduction in SOD3 protein level on the compression side after 1 day of OTM and on both compression and tension sides, with the increase in HIF-1α expression, after 14 days of OTM in rats. We also showed that 24-h of hypoxia significantly decreased *Sod3* mRNA in rat cultured PDL cells. Therefore, hypoxia from OTM affected SOD3 expression only on the compression side of PDL in the early stages of tooth movement, before affecting the entire PDL at a later stage.

Since ROS and SOD3 both regulate inflammatory processes, the imbalance between them leads to various tissue damages (7, 73), and SOD3 supplementation has a therapeutic effect in reducing tissue damage and improving the healing process (6, 7). SOD3-deficient mice produced more ROS and inflammatory cytokines, with lower blood SOD activity and SOD3 protein levels, leading to systemic inflammation through the increased total white blood cells (74). SOD3 has also been suggested to play a survival-supporting role in lung and cardiovascular tissues. Mice lacking SOD3 were found to have lower survival rates and higher mortality in both normal and hypoxic conditions because of severe lung damage (59, 60). Pulmonary hypertension and ventricular hypertrophy, which lead to heart failure and death, were worse in chronic hypoxia-exposed SOD3-knockout mice than in normoxic SOD3-knockout mice (61). According to our *in vitro* findings, PDL cells with si-SOD3 showed a significant reduction in cell viability under normoxia but had no effect on cell survival when exposed to hypoxia. E4 treatment increased PDL cell viability and proliferation under normoxia, while also protecting the cells from hypoxic effects, suggesting that SOD3 partially affected rat PDL cell survival.

SOD3 treatment significantly reduced HIF-1α expression in hypoxia-exposed PDL cells, which is consistent with previous findings that SOD3 overexpression reduced

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

HIF-1α protein levels in human liver cells treated with ROS induction or hypoxia (75), as well as in pancreatic cancer cells (76). Conversely, SOD3-deficient PDL cells showed a significant increase in HIF-1α expression under hypoxia. Erythropoietin, an important hormone for HIF-1α stabilisation, was significantly increased in hypoxia-exposed mice, with a greater increase in hypoxia-exposed mice with SOD3 deletion (75). However, while renal erythropoietin expression was higher in wild-type mice exposed to hypoxia for 6 h, nuclear HIF-1α accumulation in hypoxia-exposed kidney tissues was similar in SOD3-knockout and wild-type mice (77). Therefore, based on our *in vivo* immunohistochemistry findings that SOD3 expression reduced following the change in HIF-1α level after OTM and our *in vitro* data that both SOD3 deficiency and administration affected *Hif-1α* mRNA expression, it may be concluded that SOD3 plays a role in HIF-1α pathway in PDL cells.

Biomarkers in gingival crevicular fluid (GCF) have been widely used as periodontal status indicators, and ROS have been implicated in periodontitis pathogenesis (3). Patients with chronic periodontitis have elevated ROS, RANKL, and HIF-1α levels and reduced OPG concentrations in GCF (78, 79). While SOD activity in GCF was reduced in periodontitis patients, it was restored following nonsurgical periodontal therapy (80). However, to date, there has been no research on SOD3 levels in GCF. Our *in vitro* study revealed that SOD3-deficient PDL cells showed an increase in total ROS formation under both oxygen conditions. The significantly increased *Rankl* and decreased *Opg* mRNA levels were found in SOD3-deficient PDL cells under normoxia, similar to increased bone resorption in periodontitis patients (81), and the effect on *Rankl* and *Opg* mRNA expression was synergistic when SOD3-deficient PDL cells were exposed to hypoxia. Furthermore, SOD3 treatment decreased the increase in *Rankl* expression in hypoxia and in osteoclast formation in both normoxia and hypoxia, while exceeding *Opg* expression under both oxygen conditions. In rats with systemic inflammation, E4 reduced osteoclastogenesis and bone resorption (82), and ovariectomized rats with a compromised bone antioxidant system, including high oxidative stress and low SOD activity (83), had a lower RANKL/OPG ratio after E4 treatment, resulting in less bone resorption and more bone formation (84). In PDL stem

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Chulalongkorn University

cells, E4 suppresses the reduction of bone formation markers and osteoblastic formation caused by ROS induction (9). These findings suggest that periodontitis-induced SOD3 deficiency may promote ROS production and be involved in RANKL/OPG signalling pathway. Thus, periodontitis patients undergoing orthodontic treatment are at the greatest risk of experiencing an increase in oxidative stress and bone resorption, while SOD3 treatment under ROS-related hypoxia has a therapeutic effect on osteoclastogenesis.

The limitations of this study are a short time-course *in vivo* and a lack of time-course *in vitro* studies. A previous *in vitro* study that stimulated OTM was found to have both mechanical orthodontic strain and a hypoxic cell culture condition (85); however, the *in vitro* model used in this study only provides a hypoxic cell culture condition, which may only partially relate the *in vitro* data to our *in vivo* findings. Moreover, even though it has recently been discovered that SOD3 expression is epigenetically regulated in various cell types (86, 87, 88), the epigenetic regulation of SOD3 expression and the associated pathways in PDL are still poorly understood. Further research is required to clarify the molecular mechanisms of SOD3 during OTM, which could lead to the development of novel therapeutic modality in the future.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER VI CONCLUSION

To the best of our knowledge, this is the first study conducted on the effect of OTM on SOD3 expression, which showed that SOD3 expression, which is highly expressed in intact rat PDL tissues, was reduced by OTM-induced hypoxia only on the compression side in the early stage of OTM and subsequently affected the entire PDL in the later stage in the rat tooth movement model. The negative effects of hypoxia in rat PDL cells, including reduction of cell survival, increase in oxidative stress, and osteoclastogenesis, can be exacerbated by SOD3 deficiency and attenuated by SOD3 treatment. Therefore, SOD3 could play an important role in PDL tissue remodelling during OTM and in hypoxia-exposed PDL cells through ROS, HIF-1α, and RANKL/OPG signalling pathways, and SOD3 treatment may prevent the adverse effects of hypoxia on PDL cells.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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