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Effect of endothelin-1 on cyclooxygenase-2 gene expression and prostaglandin E2
synthesis in human periodontal fibroblasts



Mr. Supachai Unmanatakoon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Orthodontics

Department of Orthodontics

FACULTY OF DENTISTRY

Chulalongkorn University

Academic Year 2020

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ผลของเอนโดทีลิน-1 ต่อการแสดงออกของยีนไซโคลออกซีจีเนส-2 และการสังเคราะห์
โปร스타แกลนดินอี-2 ในเซลล์สร้างเส้นใยจากเนื้อเยื่อปริทันต์มนุษย์



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพันธุกรรมจัดฟัน ภาควิชาพันธุกรรมจัดฟัน
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ปีการศึกษา 2563
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	Effect of endothelin-1 on cyclooxygenase-2 gene expression and prostaglandin E2 synthesis in human periodontal fibroblasts
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Field of Study	Orthodontics
Thesis Advisor	Assistant Professor SUPAPORN SUTTAMANATWONG, D.D.S., Ph.D.
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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของเอนโดทีลิน-1 ต่อระดับการแสดงออกของยีนไซโคลออกซีจีเนส-2 และการสังเคราะห์โพรสตาแกลนดินอี-2 ของเซลล์สร้างเส้นใยจากเนื้อเยื่อปริทันต์จากมนุษย์ โดยทำการทดสอบความมีชีวิตของเซลล์สร้างเส้นใยจากเนื้อเยื่อปริทันต์จากมนุษย์ด้วยวิธีเอ็มทีทีเพื่อหาเปอร์เซ็นต์ของความมีชีวิตหลังการกระตุ้นด้วยเอนโดทีลิน-1 ที่มีความเข้มข้นแตกต่างกัน หลังจากนั้นเซลล์สร้างเส้นใยจากเนื้อเยื่อปริทันต์ได้รับการกระตุ้นด้วยเอนโดทีลิน-1 ที่มีความเข้มข้น 10 พิโคโมลาร์เป็นเวลา 30 นาที, 1, 2, และ 4 ชั่วโมง เพื่อทดสอบผลของเวลา จากนั้นจึงกระตุ้นด้วยเอนโดทีลิน-1 ความเข้มข้น 1, 10, 100 พิโคโมลาร์ เป็นเวลา 1 ชั่วโมง เพื่อทดสอบผลของความเข้มข้น เทียบกับกลุ่มควบคุมที่ไม่ได้รับเอนโดทีลิน-1 โดยทำการสกัดอาร์เอ็นเอ การสังเคราะห์ซีดีเอ็นเอ และการทำ real time PCR เพื่อวัดปริมาณการแสดงออกของยีนไซโคลออกซีจีเนส-2 และทำเอนไซม์ลิงค์อิมมูโนซอร์เบนต์แอสเซย์ร่วมกับการวัดปริมาณโปรตีนไมโครบีซีเอ เพื่อวัดปริมาณโพรสตาแกลนดินอี-2 และใช้สถิติ one-way ANOVA ร่วมกับการทดสอบภายหลัง Games-Howell เพื่อวิเคราะห์ความแตกต่างระหว่างกลุ่ม ผลการทดสอบพบว่า เอนโดทีลิน-1 มีผลเพิ่มการแสดงออกอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ของยีนไซโคลออกซีจีเนส-2 เมื่อเวลาเพิ่มขึ้น แต่ไม่มีผลเพิ่มการแสดงออกของยีนไซโคลออกซีจีเนส-2 เมื่อความเข้มข้นเพิ่มขึ้น ส่วนปริมาณของโพรสตาแกลนดินอี-2 เพิ่มขึ้นตามการแสดงออกของยีนไซโคลออกซีจีเนส-2 แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ดังนั้น จึงมีความจำเป็นต้องมีการศึกษาเพิ่มเติมถึงผลของความเข้มข้นของเอนโดทีลิน-1 ต่อเซลล์สร้างเส้นใยจากเนื้อเยื่อปริทันต์จากมนุษย์

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KEYWORD:

Supachai Unmanatakoon : Effect of endothelin-1 on cyclooxygenase-2 gene expression and prostaglandin E2 synthesis in human periodontal fibroblasts. Advisor: Asst. Prof. SUPAPORN SUTTAMANATWONG, D.D.S., Ph.D. Co-advisor: Assoc. Prof. SIRIMA PETDACHAI, D.D.S., Ph.D.

The objective of this study is to clarify the effect of endothelin-1 on COX-2 mRNA expression and PGE2 production from human periodontal ligament fibroblasts. Effect of various concentration of ET-1 on cell viability of primary human periodontal ligament fibroblasts was determined by an MTT assay. Then, primary human periodontal ligament fibroblasts were treated with 10 pM ET-1 for 0.5, 1, 2, 4 hours to investigate the time-course effect. Furthermore, to investigate the dose-response effect, periodontal ligament fibroblasts were treated with 1, 10, 100 pM ET-1 for 1 hour. The untreated cells were set as control. RNA extraction followed by cDNA synthesis and real-time PCR were performed to assess COX-2 expression level. Enzyme-Linked Immunosorbent Assay along with micro BCA protein assay was performed to assess PGE2 production from the time-course experiment. One-way ANOVA followed by Games-Howell post-hoc analysis was used to analyze the difference between groups. The result showed that ET-1 significantly increased COX-2 expression in a time-dependent ($P < 0.05$) but not dose-dependent manner. PGE2 level was also increased at 1 hour of ET-1 treatment, though not statistically significant. Further investigation is required to determine the effect of ET-1 in human periodontal ligament fibroblasts.

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CHULALONGKORN UNIVERSITY

Field of Study: Orthodontics

Academic Year: 2020

Student's Signature

Advisor's Signature

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Supachai Unmanatakoon

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Chapter 1 Introduction

1. Background and rationale

In orthodontic tooth movement, the force applied to the tooth induces the release of many chemical messengers leading to changes in the periodontal ligament (PDL) and bone. Endothelin (ET) especially ET-1 has been proven to be involved in these processes (1-4). Prostaglandin E2 (PGE2) also plays an important role in stimulating PDL cells and bone remodeling (5, 6). The conversion of arachidonic acid to PGE2 requires cyclooxygenase-2 (COX-2). The effects of ET-1 on PGE2 production have been studied in various cell types (7-11). However, there is a lack of studies about the effects of ET-1 on COX-2 expression and PGE2 synthesis in hPDL cells. This study will clarify the roles of ET-1 in hPDL cells and provides information for further application of ET-1 to modulate orthodontic tooth movement.

2. Research questions

Does endothelin-1 alter COX-2 expression and PGE2 levels in human periodontal cells?

3. Research hypothesis

Research hypothesis 1

- Null hypothesis: ET-1 does not alter COX-2 expression in human periodontal cells
- Alternative hypothesis: ET-1 can alter COX-2 expression in human periodontal cells

Research hypothesis 2

- Null hypothesis: ET-1 does not alter PGE2 level in human periodontal cells
- Alternative hypothesis: ET-1 can alter PGE2 level in human periodontal cells

4. Research objectives

To investigate the effects of ET-1 to COX-2 expression and PGE2 level in human periodontal cells

5. Benefits of the study

This study will clarify the roles of ET-1 in PDL remodeling during orthodontic tooth movement and the relationships between ET-1 and PGE2 in PDL cells *in vitro*. This study can expand the knowledge of the mechanism of mediators during orthodontic tooth movement and can be further applied to accelerate the tooth movement.

6. Conceptual framework

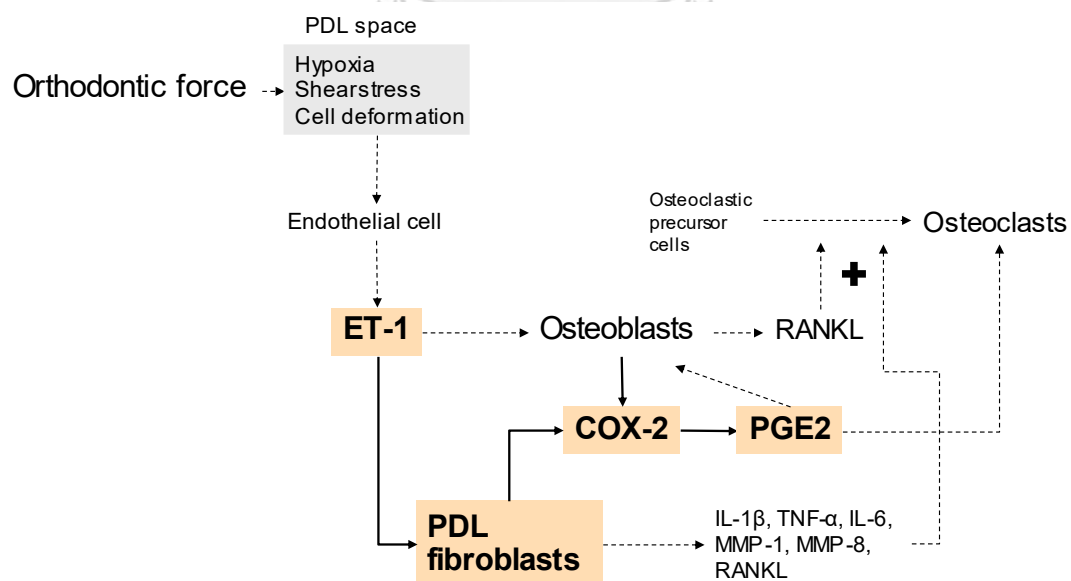


Figure 1 Conceptual framework

Chapter 2 Review literature

1. Endothelin & its receptors

Endothelin was first discovered from cultured porcine aortic endothelial cells and it functioned as vasoconstrictor peptides (12). The human endothelin gene encodes three types of endothelin: ET-1, ET-2, ET-3. Among these, ET-1 is the predominant member of the family. All of them exhibit strong and long-lasting vasoconstrictor activity (13). Even though their actions are similar, they are expressed in different organs (14, 15). The site of ET production is shown in Table 1. The endothelin precursors are processed by two proteases to create mature active forms. Preproendothelins are cleaved by furin-like protease into big endothelin, which then is processed by endothelin-converting enzymes (ECEs) to become the final product (Figure 1) (16). The effects of endothelin are expressed through the endothelin receptor, which can be divided into 2 types: endothelin A (ET-A) receptor and endothelin B (ET-B) receptor. Both receptors are G-protein-coupled transmembrane proteins, which function differently upon their location. Figures 2 and 3 show the different signal transduction between ET-A and ET-B receptors (17, 18)

Table 1 Sites of ET-1, ET-2, ET-3 production

ET-1	ET-2	ET-3
<ul style="list-style-type: none"> • Vascular endothelial cells • Smooth muscle cells • Airway epithelial cells • Macrophages • Fibroblasts • Cardiac myocytes • Brain neurons • Pancreatic islets • Bone • Periodontal ligament cell 	<ul style="list-style-type: none"> • Ovary • Intestinal epithelial cells • Bone 	<ul style="list-style-type: none"> • Endothelial cells • Brain neurons • Renal tubular epithelial cells • Intestinal epithelial cells • Bone

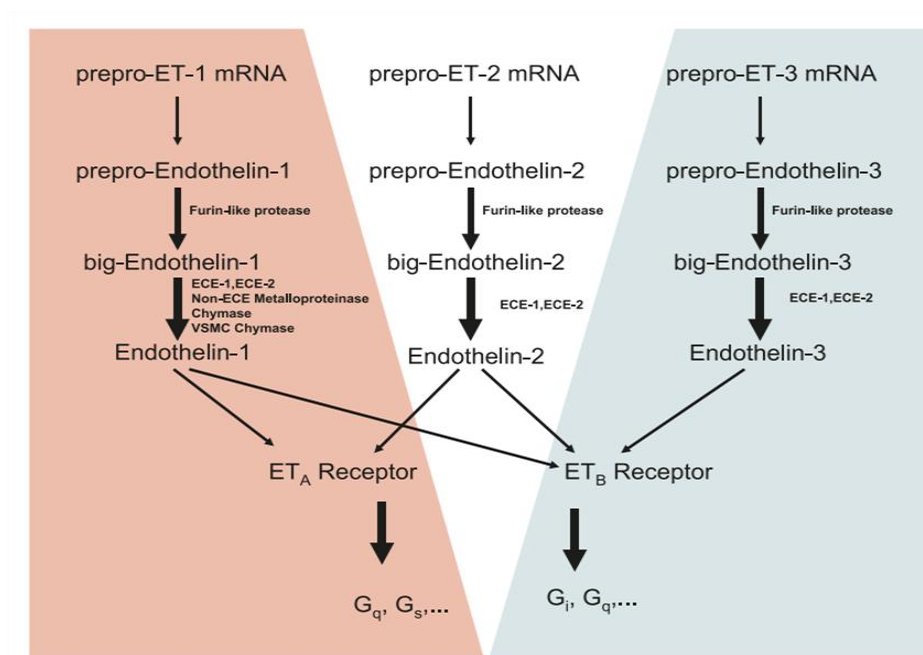


Figure 2 Sequences of endothelin processing production (14)

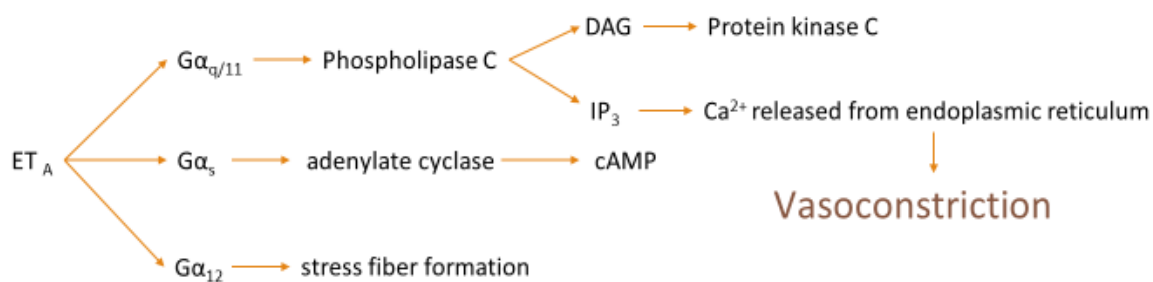


Figure 3 Endothelin A receptor signal transduction

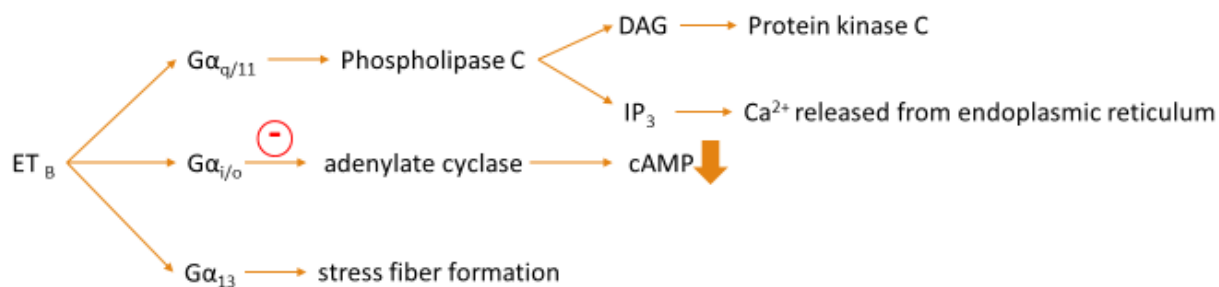


Figure 4 Endothelin B receptor signal transduction

Endothelin is expressed in both physiological and pathological conditions. Under normal conditions, it is regulated by various factors which usually take effect at the transcription process (16). Among the three types of ET, ET-1 is the predominant and most important isoform of endothelin in humans (19). ET-1 increases vascular resistance, facilitates cell proliferation and resistance to apoptosis, helps control glycemic and glucose uptake, and regulates renal function (14, 18). It has also been reported that ET-1 mRNA is upregulated in various cell types by transforming necrosis factor- β , tumor necrosis factor- α , interleukin, insulin, norepinephrine, angiotensin II, and thrombin (12, 20, 21). Shear force, stress, and stretch can also initially upregulate ET-1 mRNA in endothelial cells (22). Moreover, ET-1 mRNA level is upregulated by hypocapnia and downregulated in PDL cells by hypoxia, nitric oxide, prostacyclin, and atrial natriuretic factor (23-26). Furthermore, ET-A can be regulated by insulin and nitric oxide in smooth muscle cells (27, 28), while tumor necrosis factor- α and basic fibroblast growth factor can upregulate ET-B receptors in endothelial cells (29).

2. Roles of Endothelin on human PDL cells

Several studies have reported the production of ET-1 and its effects on different cell types (14, 16, 18). Human periodontal cells (hPDL cells), which are one of the key players in orthodontic tooth movement, were also reported. Fujioka et al. demonstrated that ET-1 can be detected in human

gingival crevicular fluid (30). They also reported the expression of ET-1 from hPDL cells, human gingival fibroblasts, and human keratinocytes, which showed the strongest expression (30). Besides, ET-1 can stimulate the production of proinflammatory cytokines from hPDL cells through several pathways (31-34). Tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 messenger RNA (mRNA) and protein levels in hPDL cells and hPDL stem cells increased dose- and time-dependently after treatment with ET-1 (32, 33). ET-1 also dose-dependently stimulated (IL)-1 β mRNA and protein level (34), matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-8 (MMP-8), and receptor activator of nuclear factor-kB ligand (RANKL) protein level in hPDL fibroblasts (31). These findings indicated the involvement of ET-1 in bone remodeling. Another effect of ET-1 on hPDL cells is promoting differentiation of PDL stem cells into osteoblasts by increasing osteocalcin (OCN), RUNX2, COL1 mRNA expression which are markers of osteoblast differentiation (32).

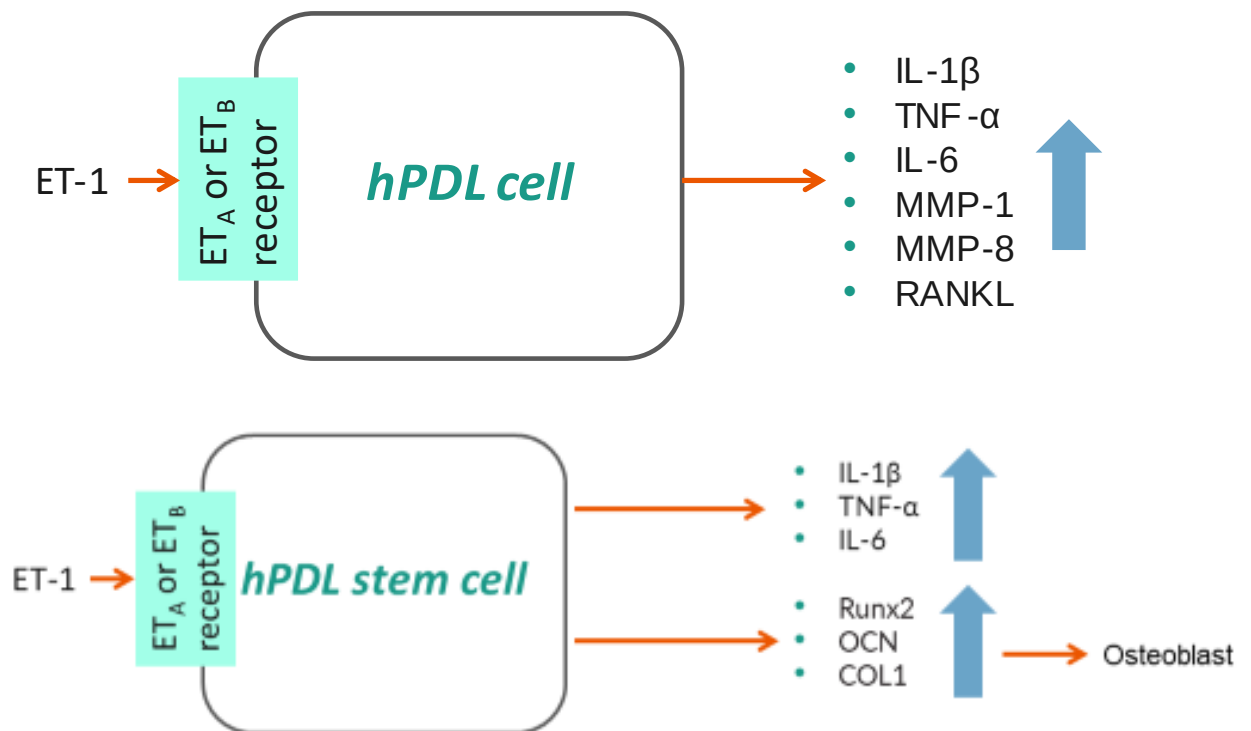


Figure 5 The effects of Endothelin-1 on human periodontal cell and human periodontal stem cells (32-34).

3. Endothelin & orthodontic tooth movement (OTM)

As the force is applied to the tooth, PDL fibroblasts, which exist in an active mechanical environment between the cementum of the root and the alveolar bone, become deformed mechanically (35). The compressive, tensile, and shearing forces in OTM cause the extracellular matrix remodeling, stimulate the PDL fibroblast cell membrane mechanosensitive receptors and ion channels and cytoskeletal reorganization, leading to changes in gene transcription, cell division, and differentiation differently between

compression and tension in bone remodeling (35, 36). Li et al. reported upregulated RANKL mRNA expression in 3D culturing hPDL cell model under high static compression (37). Kang et al. reported differential gene expression in the 2D and 3D cultured PDL cells after the application of identical compressive stresses (38). Le Li et al. also proposed that static compression or hypoxia situations increase reactive oxygen species expression (ROS) which stimulates NF- κ B migration to nuclei and further enhances COX-2, IL-1 β , and RANKL expression in PDL cells, osteoblasts, and stromal cells, leading to osteoclastogenesis (39). These results point out that on the pressure side in OTM, both compression and hypoxia regulate bone remodeling through PDL cells (39, 40) Vansant et al. stated that not only osteocytes and osteoblasts but also PDL cells are important mechanosensors and transducers of the applied strain which contribute to the activation of the cells and production of several mediators (40). They concluded that PDL cells are essential in cell activation and differentiation (40).

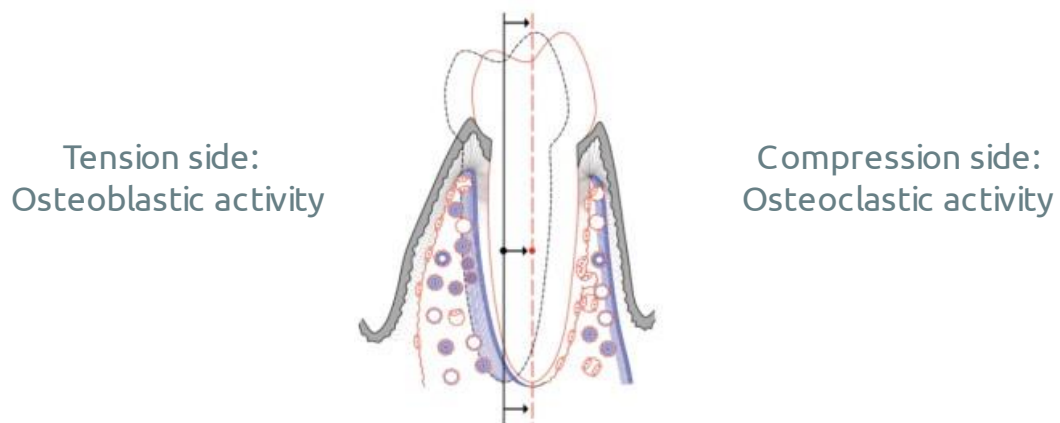


Figure 6 Different activity in bone after the force was applied on the tooth. When force is applied to the tooth, the side the tooth moves into is compression side, which its main activity is osteoclastic activity. The other side is tension side which has osteoblastic activity (35)

The effect of orthodontic force and changes in ET-1 distribution in PDL was first investigated by Sim et al. It was found that ET-1 changes its distribution in vascular endothelium, bone surface cells, and cementum surface cells in PDL of the rat and marmoset molars, both under normal condition and after loading intrusive force for three hours (41, 42).

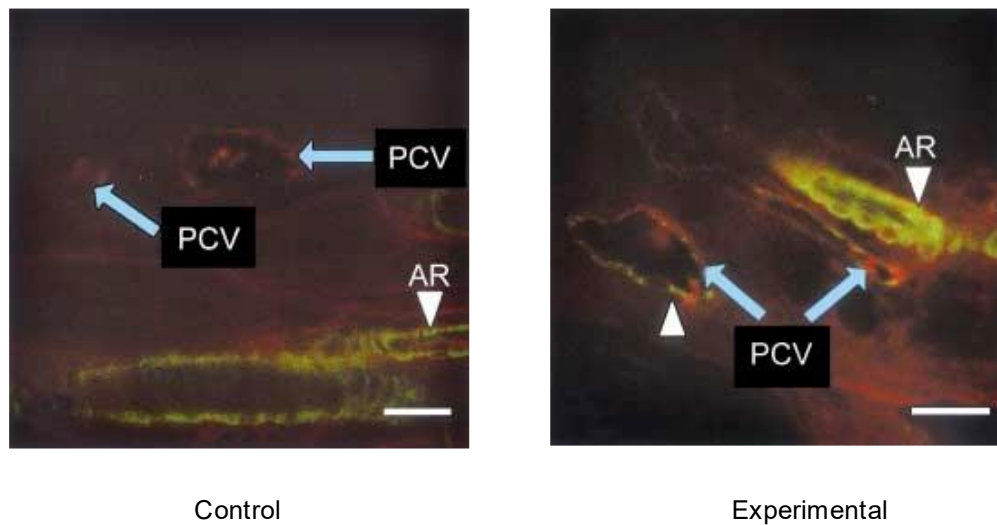


Figure 7 The immunolabelling of ET-1 in endothelium.

The immunolabelling of ET-1 from a small post-capillary-size venule and arteriole, presented as red in color, from periodontal tissue of the marmoset's molar. It was applied occlusal force for 3 hours (42).

Drevenšek et al. first suggested that ET-1, through ET-A and ET-B receptors, is involved in OTM (4). Inhibition of ET-1 receptors by tezosentan, an ET-A and ET-B receptor antagonist, enhanced the rate of tooth movement after 25 days of OTM in rats (4). However, the rate of tooth movement in rats was decreased after applying TBC3214, a selective ET-A receptor antagonist, in the late period of OTM (40 days), indicating the role of ET-1 in stimulating bone resorption via ET-A receptors (2). Sprogar et al. further reported that the level of ET-1, ET-A, ET-B gene expression was enhanced in the late period of tooth movement (2). TBC3214, a selective ETA antagonist, further reduced the rate of tooth movement at day 42 of OTM in the rat (3). ET-1 and ET-3 gene

expression is also observed to be involved in three phases of OTM in rats, and ET-1 gene expression is strongly increased in the late phase of OTM (1). Consequently, ET-1 and its receptors are involved in orthodontic tooth movement. The possible effects of ET-1, ET-A, ET-B on osteoblasts and osteoclasts during orthodontic tooth movement are shown in Figure 8.

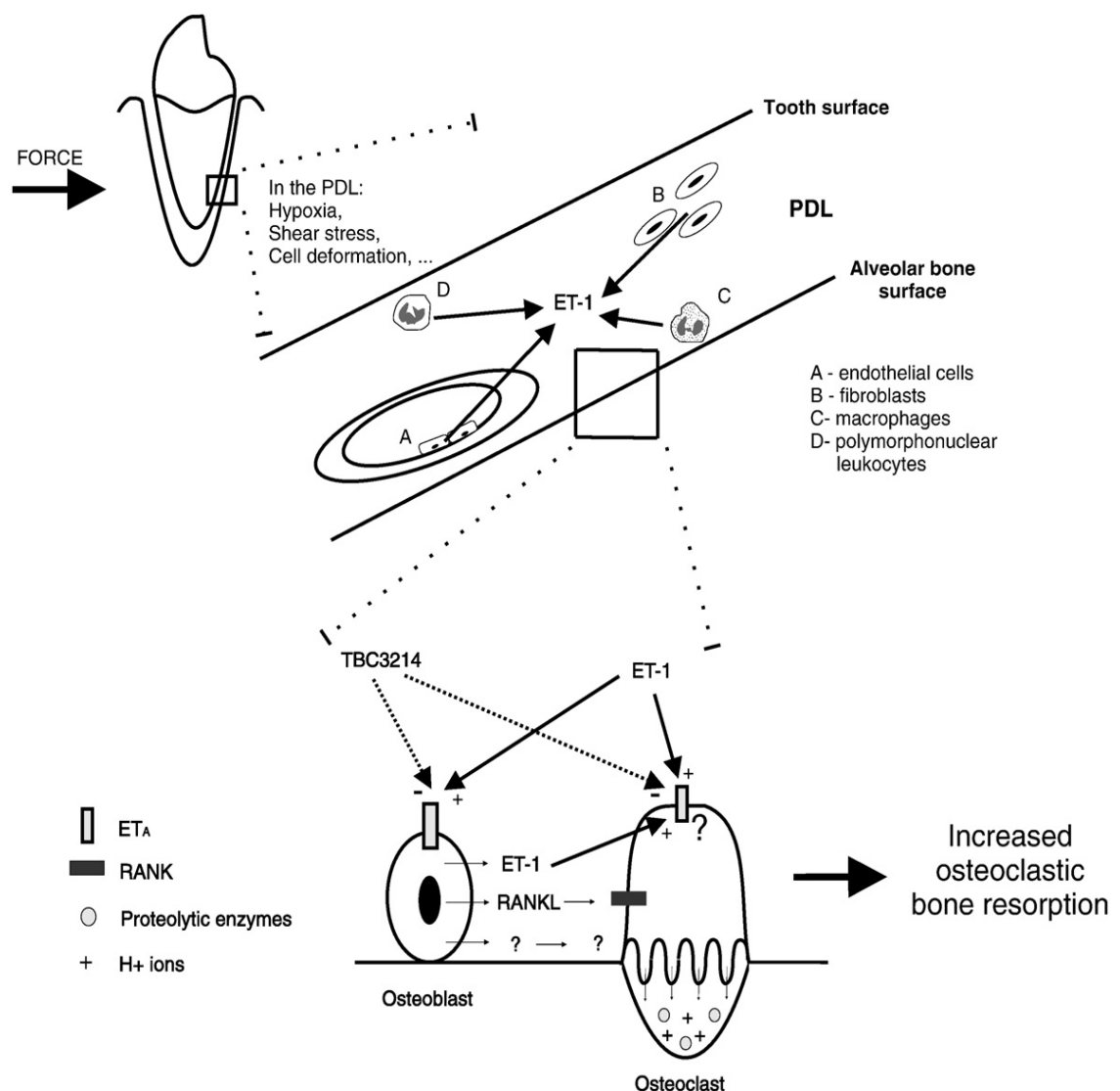


Figure 8 The proposed effect of ET-1 and ET-A receptors during orthodontic tooth movement (3)

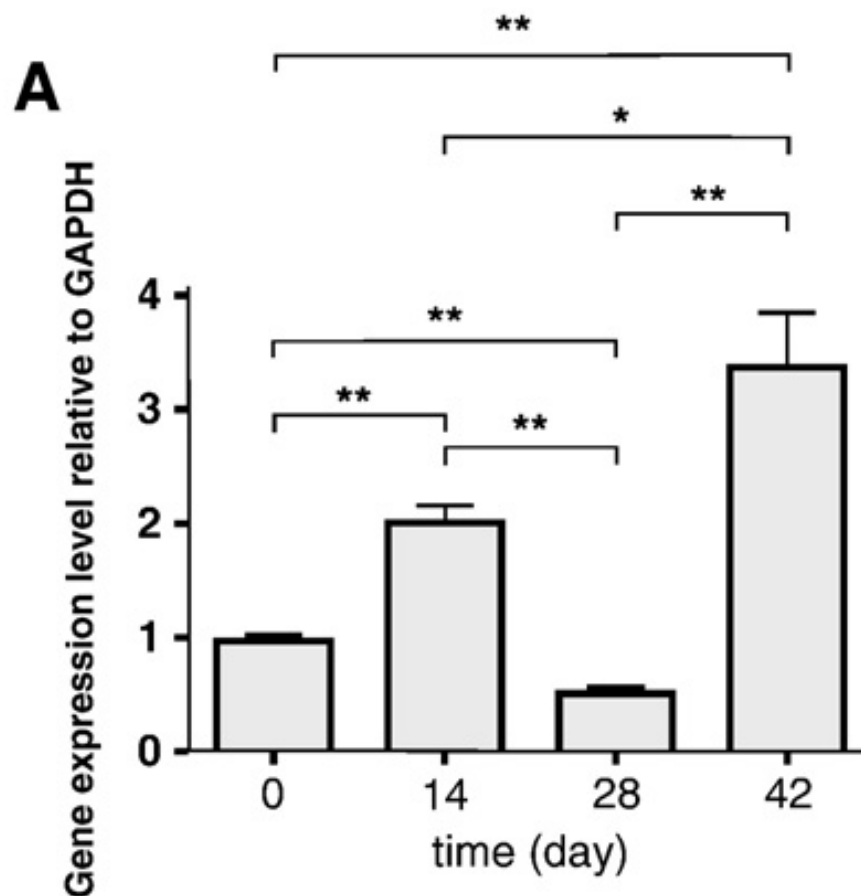


Figure 9 Gene expression of ET-1 in alveolar bone and periodontal ligament during orthodontic tooth movement in rat (3).

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4. Endothelin and PGE2 as mediators in osteoclastogenesis regulation

Since bone resorption is one of the processes in bone remodeling during orthodontic tooth movement, understanding osteoclast formation which is one of the cells involved in bone resorption is important. During tooth movement, the aseptic inflammation process occurs within periodontal supporting tissue, causing the remodeling (43). Several inflammatory

mediators including cytokines and neuropeptides involved in this process have been studied.

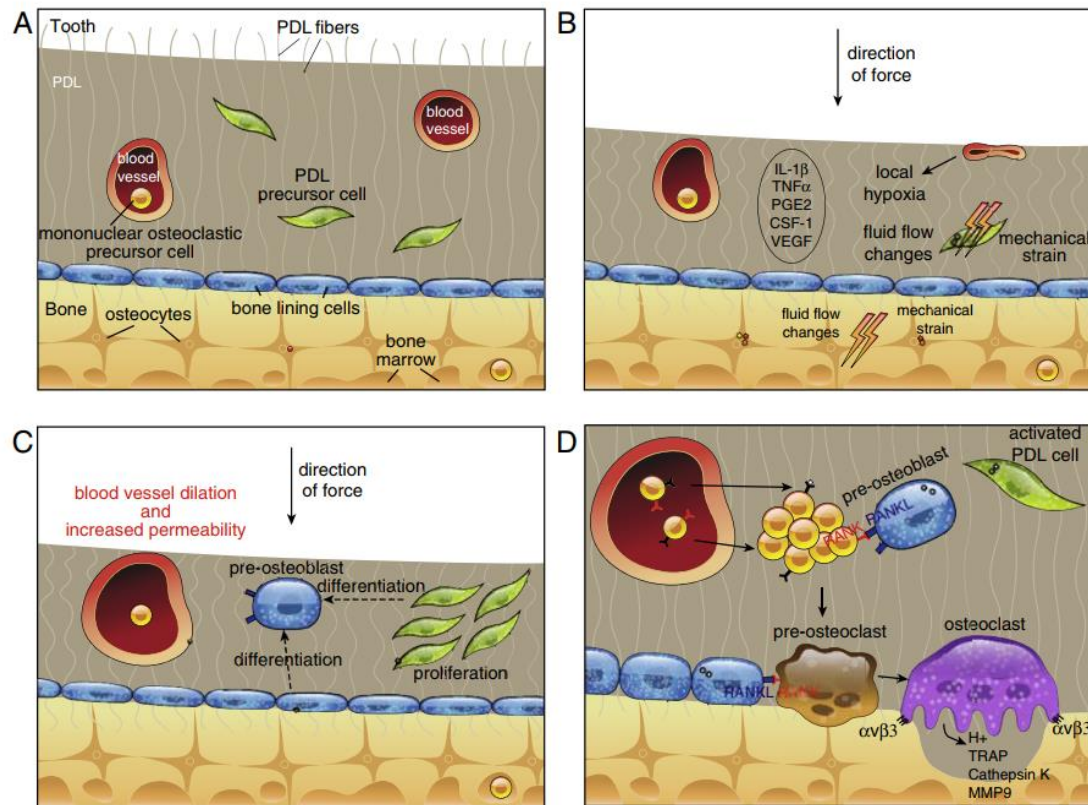


Figure 10 The cellular and molecular process in orthodontic tooth movement (44)

Prostaglandins, products of arachidonic acid metabolism, are produced by several mammalian cells, including osteoblasts and osteoclasts (43). The conversion of arachidonic acid to prostaglandin is catalyzed by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) of which PGE2 is the end-products (45).

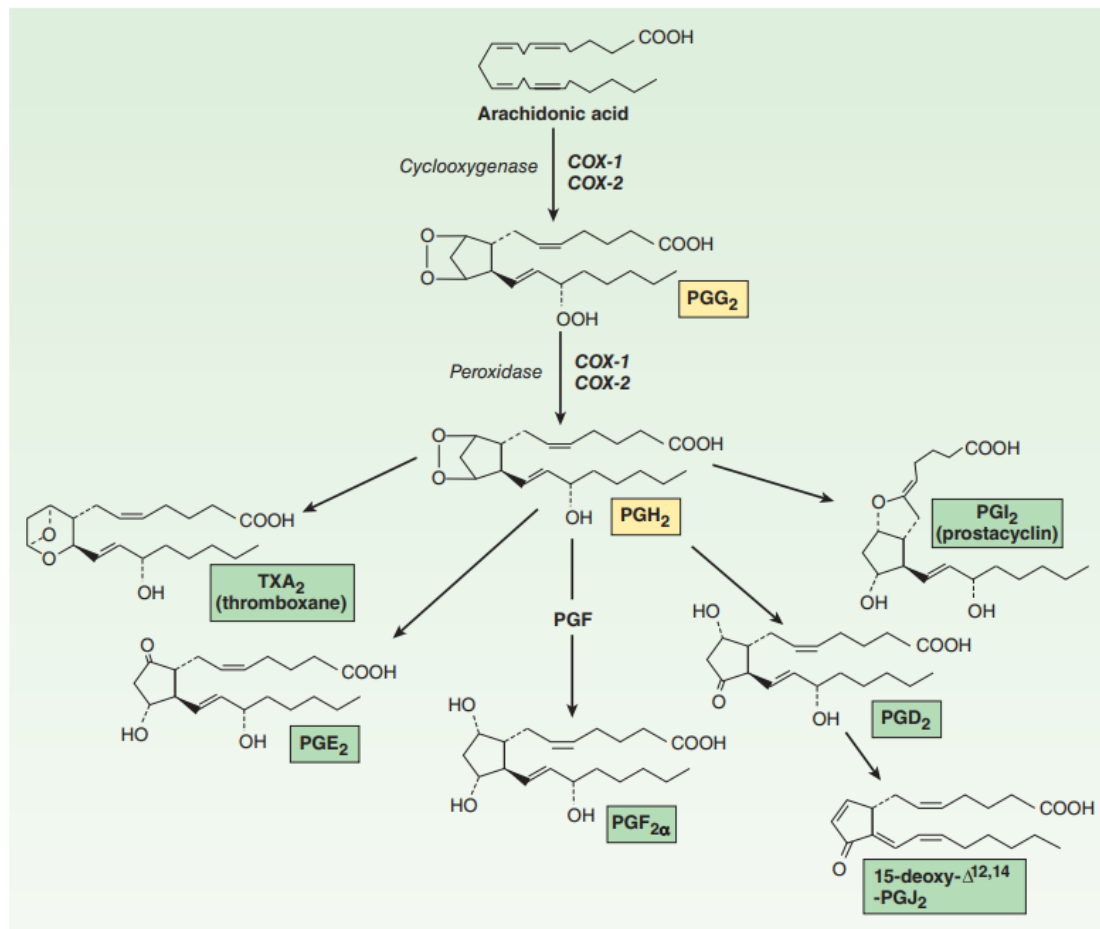


Figure 11 The biosynthesis of prostaglandin E₂. Adapted from Basic and clinical pharmacology by Katzung BG (46).

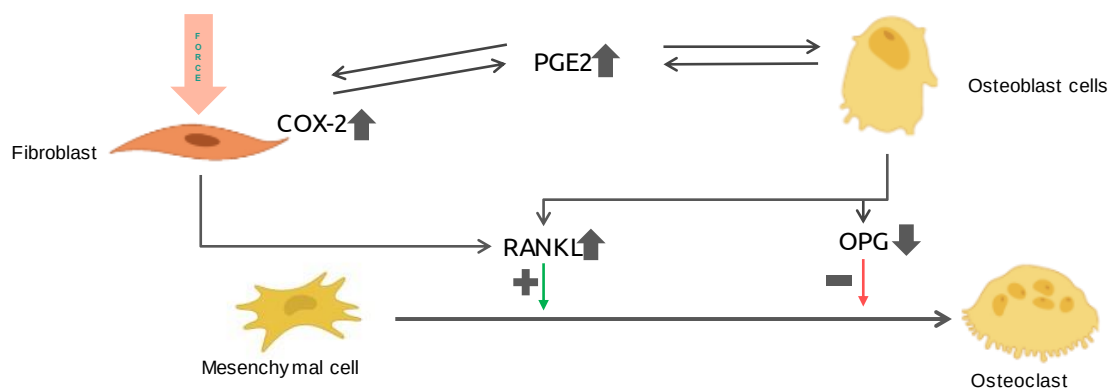


Figure 12 the role of Prostaglandin E₂ in inducing osteoclastogenesis (6, 47)

Prostaglandins cause a local, hormone-like effect (43). In bone and PDL, it has been reported that several cell types, such as osteocyte, bone lining cell osteoblast, and fibroblast, have the ability to release Prostaglandin (6, 44). Among prostaglandin derivatives, PGE2 has an effect on vasodilation, chemotactic properties, and osteoclastogenesis stimulation (43). Various molecules such as IL-1 α , IL-1 β , IL-6, TNF- α , Basic FGF, BMP-2, PTH, Vitamin D3 (1,25(OH)₂D₃), LPS, and PGE2, have been proven its effect to stimulate osteoblast in COX-2 or PGE2 production (6). Several studies reported the effects of PGE2 on osteoclast formation (48, 49). PGE2 can stimulate osteoclast formation via inhibiting OPG production and stimulate RANKL production through the cAMP-dependent pathway (48, 49). It also stimulates the formation of interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) from osteoblasts which in turn enhances osteoclast formation (6). PGE2 can also amplify its production from mouse osteoblastic MC3T3-E1 cells (50). Furthermore, PGE2, by itself, can enhance mouse bone marrow-derived macrophages, when treated with RANKL and human bone marrow cell cultures, when treated with M-CSF, TNF- α , and IL-1 into osteoclasts (51, 52). PDL cells are able to stimulate osteoclast formation via PG-E2 (43). Fukushima et al. reported the effects of IL-1 α in the co-culture of mouse spleen cells and hPDL cells in the formation of tartrate-resistant acid phosphatase positive (TRAP+) cells, which represent the osteoclastic bone resorption, via PGE2 and

COX-2 (53). PGE2 and IL-1 β also activated hPDL cells through RANKL to stimulate osteoclastogenesis (54). It has been demonstrated that mechanical stress can also enhance the production of PGE from hPDL cells in vivo (55) and PGE2 from hPDL cells in vitro (47, 56, 57).

Besides, it has been observed that ET-1, localized in mouse's metaphyseal bone marrow, aggregates in osteoblasts, osteoclasts, young osteocytes, and vascular endothelial cells, with the strongest expression in osteoclasts (15). In osteoclasts, ET-1 was intensely labeled especially in the cytoplasm, including ruffled borders and clear zones, plasma membranes, pale vacuoles, and lysosomal dense bodies (58). ET-1 was also found in the hypertrophic zone in cartilage which is close to vascular supply (15, 58). Roles of endothelin in regulating osteoclast function have been discussed by Alam et al. that ET-1 inhibited isolated osteoclast from osteoclastic bone resorption without inhibiting acid phosphatase secretion (59). ET-1 also inhibits osteoclast motility without significant effects on cell spread area (59). Since the concentration of ET-1 to inhibit the osteoclast activity was similar to that reported in the smooth muscle, it was believed that ET-1 might be a key regulator of osteoclast activity (59). Zaidi et al. also supported the inhibitory effect of ET-1 on osteoclastic bone resorption (60). They proposed that osteoclast margin ruffling and bone resorption by osteoclasts were also inhibited by ET-1 at concentrations similar to those function in

vasoconstriction action. They also reported that ET-1, Nitric Oxide (NO), prostacyclin, and reactive oxygen species, which are endothelial cell-derived products, have synergistic effects on osteoclasts. Endothelin and prostacyclin are known to inhibit cell margin ruffling, the Q effect (60). In contrast, nitric oxide increased the level of osteoclast cell retraction, the R effect, which indicates cell detachment or de-adhesion, while reactive oxygen species is likely to increase osteoclastic motility (60). Although ETA and ETB receptors, belong to the Ca^{2+} -mobilizing receptor family, endothelin has not been found to increase the cytosolic Ca^{2+} in isolated osteoclasts (60).

Several studies have demonstrated the relationships between endothelin and PGE2 in osteoclast formation. Spinella et al. reported that ET-1, through the binding with ETA receptor (ETAR), induces PGE2 production from ovarian carcinoma (7, 9). ET-1 also activates PGE2 production in renal mesangial cells, which in turn become contracted (8, 10). Furthermore, prostaglandin-dependent ET-1 stimulates bone resorption in neonatal mouse calvaria (11). However, the effect of endothelin on PGE2 production in periodontal ligament cells remains unknown.

Chapter 3 Materials and Methods

1. Study design

Experimental laboratory research

2. Study subjects & sample size

Primary human periodontal fibroblasts cells (hPDL) were obtained from the extracted premolar teeth which were extracted for orthodontic reasons. The study protocol was approved by the Ethics Committee of Faculty of Dentistry, Chulalongkorn University. The teeth obtained from 4 male and 7 female patients were non-carious and had healthy periodontium, from subjects aged between 12-25 years old (mean age: 16.2 years old). Subjects who had systemic diseases, the sign of inflammatory periodontal tissue such as bleeding on probing, pocket depth more than 4 mm., and those with previous orthodontic treatment were excluded. In each experiment, cells from 4 different subjects were used, except for dose-response experiment in which the cells were obtained from 3 different subjects.



Inclusion Criteria

- Noncarious and healthy periodontium



Exclusion Criteria

- Previous orthodontic treatment
- Systemic disease
- Sign of inflammatory periodontal tissue



Age: 12-25
Mean age: 16.1
Male: 4
Female: 7

Premolar tooth
Extracted for orthodontic reasons

Figure 13 Characteristics of sample used in the experiment.

3. Cell culture

Human PDL cells were obtained from the middle of the tooth root with a scalpel and placed in tissue culture dishes (60-mm dishes) with the Dulbecco's Modified Eagles Medium (DMEM, (Sigma-Aldrich St. Louis, MO, USA) comprising 10% Fetal bovine serum (FBS, Gibco, USA), 1% L-glutamine (Gibco, USA), and 1% Antibiotic-Antimycotic (Gibco, USA) under the humidified atmosphere with 37° C and 5% carbon dioxide. After reaching the confluence, the cells were subcultured with 0.25% trypsin (Gibco, USA). During the subculturing, the medium was renewed every 2 days. The cells from the third to the fifth passage was used in the experiments.

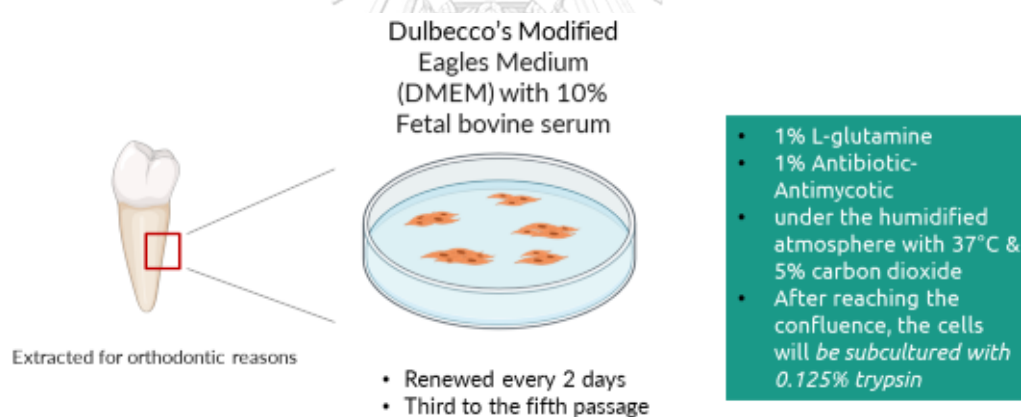


Figure 14 Cell culture protocol.

4. Cytotoxicity test

To investigate the viability of cells after treated with different concentrations of ET-1, an MTT cytotoxicity test was performed in triplicate. The hPDL cells were seeded in a 96-well plate at a density of 5,000 cells per well and allowed to attach overnight. The cells were then washed and treated

with ET-1 (1, 10, 100 pM) in DMEM with 1% Fetal bovine serum (FBS, Gibco, USA) for 24 hours. The cells treated with a concentration of 0 pM ET-1 for 24 hours were set as control. After that, the solution was removed and the MTT solution was then added to each well and incubated for 3 hours. Optical density was measured at 570 nm and the percent of cell viability comparing to control was calculated.

5. ET-1 treatment

After the non-toxic concentrations were confirmed, 10 pM ET-1 was selected to determine the time-course effect of ET-1 on COX-2 mRNA expression. The PDL cells were plated at 6×10^5 cells per plate in 60-mm tissue culture dishes in DMEM with 10% FBS. On the following day, the cells were washed and switched to a starvation medium (serum-free-DMEM) for 24 hours. After that, the cells were treated with 10 pM ET-1 (Sigma-Aldrich St. Louis, MO, USA) for 30 minutes, 1, 2, and 4 hours. The untreated cells were set as control. After that, the dose-response effect of ET-1 on COX-2 mRNA expression and PGE2 level was determined by treating the cells with 1, 10, and 100 pM for 1 hour.

6. RNA extraction, cDNA synthesis, and Real-time PCR assay

Total RNA was extracted using the Total RNA Mini Kit (Geneaid, Taiwan). The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm with a Thermo Scientific NanoDrop™ 2000

Spectrophotometer. The contaminated DNA was depleted by using DNase I treatment. One microgram of total RNA of each sample was reverse transcribed to single-strand cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT, Promega, USA) following the manufacturer's instruction.

The time-course and dose-response effect of ET-1 on COX-2 mRNA expression levels were quantitatively assessed by real-time PCR. The amplification of the cDNA template was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA). The mixture contained 5 μL of iTaq™ Universal SYBR® Green Supermix (2x), 0.25 μL of each primer, 2.5 μL of DNA template. Nuclease-free water was added to a final volume of 10 μL . The PCR program setting was 95° C for 5 minutes followed by 45 cycles for the amplification phase; each consisted of denaturation for 30 seconds at 95° C, annealing for 30 seconds at 56° C for GAPDH and 58° C for COX-2, and extension for 30 seconds at 72° C using Roche LightCycler 480. The primer sequences used for PCR amplification are shown in Table 2.

Table 2 The primer sequences used for PCR amplification

<i>Gene</i>	<i>Primer sequence</i>
GAPDH	Forward: 5'- TGAACGGGAAGCTCACTGG-3' Reverse: 5'- TCCACCACCCTGTTGCTGTA -3'
COX-2 (61)	Forward: 5' – GCTCAAACATGATGTTTGCATTC – 3' Reverse: 5'- GCTGGCCCTCGCTTATGA – 3'

7. Enzyme-Linked Immunosorbent Assay

Levels of PGE₂ released into the medium of treated cells were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, USA). First, reagents comprised of wash buffer, substrate solution, and PGE₂ standard are prepared at room temperature. Wash Buffer was prepared by adding 20 mL of Wash Buffer Concentrate to 480 mL of distilled water to prepare 500 mL of Wash Buffer. Substrate solution was prepared by mixing Color Reagent A (stabilized hydrogen peroxide) and B (stabilized tetramethylbenzidine) in equal volumes within 15 minutes of use. Two hundred microliter of the resultant mixture was required per well. To prepare PGE₂ Standard, the PGE₂ Standard was reconstituted with 1.05 mL of distilled water. This reconstitution produced a stock solution of 25,000 pg/mL. The standard was mixed to ensure complete

reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation before making dilutions. One hundred microliter of the stock solution was pipetted into the 2500 pg/mL tube followed by the addition of 900 μL of Calibrator Diluent RD5-56 and mixed well, then 500 μL of the mixture was pipetted into the remaining tubes. The 2500 pg/mL standard served as the high standard and the Calibrator Diluent RD5-56 served as the zero standard.

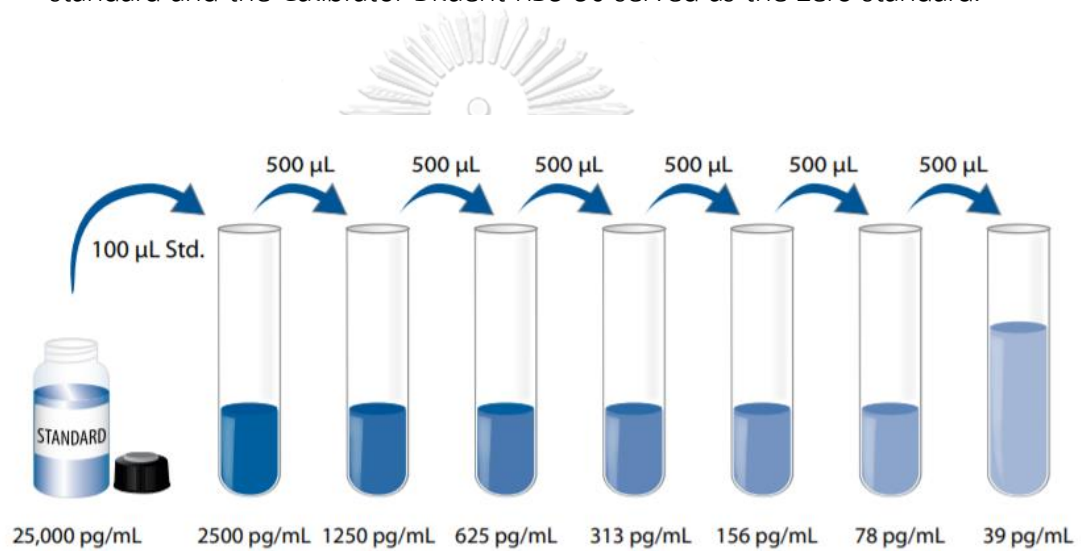


Figure 15 Sequences of PGE2 standard preparation for ELISA assay

(From Prostaglandin E2 Parameter Assay Kit product datasheet)

Then, cell supernatants from each sample preparation were centrifuged and 3-fold diluted by mixing 150 μL of the sample with 300 μL of Calibrator Diluent RD5-56. All reagents, working standards, and samples were used in the assay procedure in the 96-well plate. Two hundreds microliter of Calibrator Diluent RD5-56 was added to the non-specific binding (NSB) wells while 150 μL

of Calibrator Diluent RD5-56 was added to the zero standards (B0) wells. Then, 150 μL of each standard, control, or sample was added to the remaining wells. After that, 50 μL of the Primary Antibody Solution was added to each well (excluding the NSB wells). The plate was securely covered with a plate sealer and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. Then, 50 μL of PGE2 Conjugate was added to each well. A new plate sealer was used to cover the plate and then the plate was incubated for the next 2 hours at room temperature on the shaker. After that, each well was aspirated and washed. The process was repeated three times with a total of four washes by filling each well with 400 μL Wash Buffer. After the last wash, any remaining Wash Buffer was removed by aspirating and decanting. The plate was inverted and blotted against clean paper towels. Then, 200 μL of Substrate Solution was added to each well. The plate was incubated for 30 minutes at room temperature on the benchtop and protected from light. To stop the procedure, 100 μL of Stop Solution was added to each well. The plate was gently tapped to ensure thorough mixing. The optical density of each well was determined using a microplate reader set to 450 nm. The wavelength correction was set to 540 nm. Wavelength correction was done by subtraction of readings at 540 nm from the readings at 450 nm. The intensity of the color is inversely proportional to the concentration of PGE2 in the sample.

Total protein assay was performed by using BCA protein assay (Thermo scientific™ Pierce™ Micro BCA Protein Assay Kit, Waltham, MA, USA) following the manufacturer's instruction. Briefly, the 150 μ L of each 20-fold diluted standard or unknown sample replicates was pipetted into a microplate well. Then, 150 μ L of the working reagent was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. After that, the plate was cooled to room temperature. The absorbance was then measured at 562 nm on a plate reader. The average 562 nm absorbance measurement of the Blank standard replicates was subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates. The standard curve was prepared to determine the protein concentration of each unknown sample by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in μ g/mL.

8. Statistical analysis

Each experiment was repeated at least 3 times. Using SPSS version 22, One-way ANOVA test followed by Games-Howell post-hoc analysis was performed to compare mean differences in all experiments. The level of statistical significance was set at $P < 0.05$

9. Ethical consideration

The study was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2020-108). Informed consent was obtained from each subject.



Chapter 4 Results

1. Cytotoxicity test

The viability of cells after treatment with different concentrations of ET-1 was investigated by the MTT cytotoxicity test in triplicate. It was found that the percentage of PDL cell viability was not significantly affected by ET-1 treatment at 1 to 100 pM, comparing with control. From this finding, 10 pM of ET-1 was selected to investigate the effect on COX-2 mRNA expression and PGE2 production.

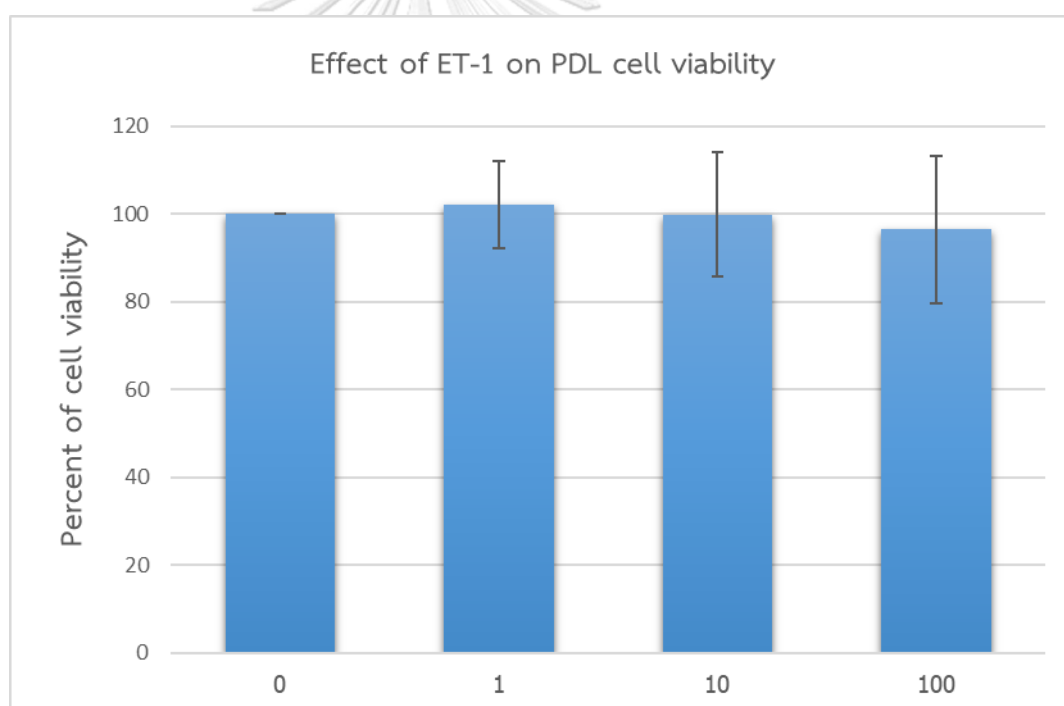


Figure 16 Effect of ET-1 on PDL cell viability.

Human periodontal ligament fibroblasts were treated with 1, 10, 100 pM ET-1 for 24 hours. MTT cytotoxicity test was used to determine cell viability. The percentage of cell viability of the untreated control group was calculated and presented as mean \pm SD. One-way ANOVA followed by Games-Howell post-hoc analysis was performed to determine the statistical difference between groups.

Table 3 Effect of ET-1 on PDL cell viability.

Concentration of ET-1 (pM)	0	1	10	100
Average	100.0	102.0	99.9	96.6
Standard deviation	0.0	9.9	14.1	16.8

2. Effects of ET-1 on COX-2 mRNA expression in PDL fibroblast cells

Real-time PCR revealed that COX-2 mRNA expression from human PDL fibroblast cells varied during the time course of treatment with 10 pM ET-1. After normalization to the expression of the housekeeping GAPDH, the COX-2 expression was increased significantly at 30 minutes and peaked at 1 hour. The ET-1-induced COX-2 expression was, however, gradually decreased at 2 and 4 hours. When comparing with basal expression at 0 hours, the expression was upregulated 3.3-, 3.9-, 2.5- and 2.2-fold, at 30 minutes, 1, 2 and 4 hours, respectively.

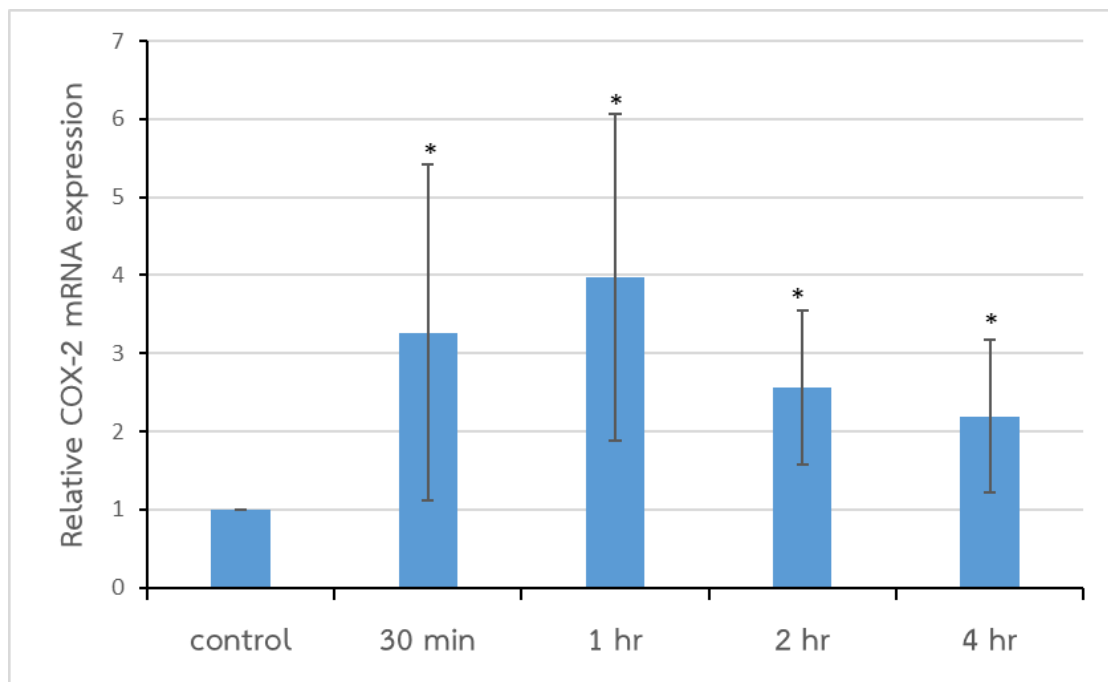


Figure 17 Time-course effect of ET-1 on COX-2 gene expression.

Human periodontal ligament fibroblasts were treated with 10 pM ET-1 for 30 minutes, 1, 2, and 4 hours. The untreated group was set as control. Real-time PCR with normalization with GAPDH gene expression was used to determine the level of gene expression. Fold change of gene expression was calculated and presented as mean \pm SD. One-way ANOVA followed by Games-Howell post-hoc analysis was performed to determine the statistical difference between groups. * $P < 0.05$ vs control

Surprisingly, no significant induction of COX-2 expression was found when dose-response investigation was performed using 1, 10 and 100 pM ET-1.

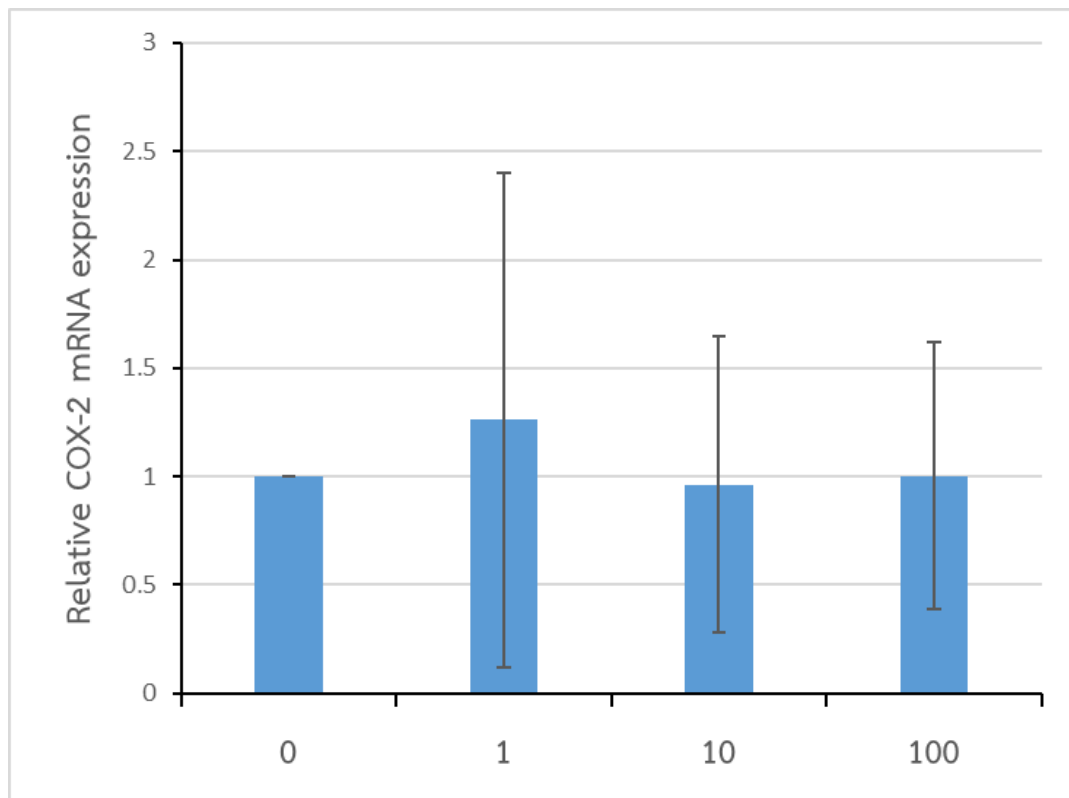


Figure 18 Dose-response effect of ET-1 on COX-2 gene expression.

Human periodontal ligament fibroblasts were treated with 1, 10, 100 pM ET-1 for 1 hour. The untreated group was set as control. Real-time PCR with normalization with GAPDH gene expression was used to determine the level of gene expression. Fold change of gene expression was calculated and presented as mean \pm SD. One-way ANOVA was performed to determine the statistical difference between groups.

3. Effect of ET-1 on PGE2 production in PDL cells

To observe the PGE2 level secreted from PDL cells from the time-course experiment, Enzyme-Linked Immunosorbent Assay as well as Micro BCA Protein Assay were performed. The amount of PGE2 was normalized by total protein amount and presented in fold changes comparing to control. It was found that PGE2 level was elevated in time-dependent manner from 30 minutes to 1 hour

and gradually decreased at 2 and 4 hours of ET-1 treatment. The PGE2 changes was 2.1-, 5.7-, 2.4-, and 2.8- fold higher than untreated cells. However, no statistical significant difference was found between different time points (P -value <0.05).

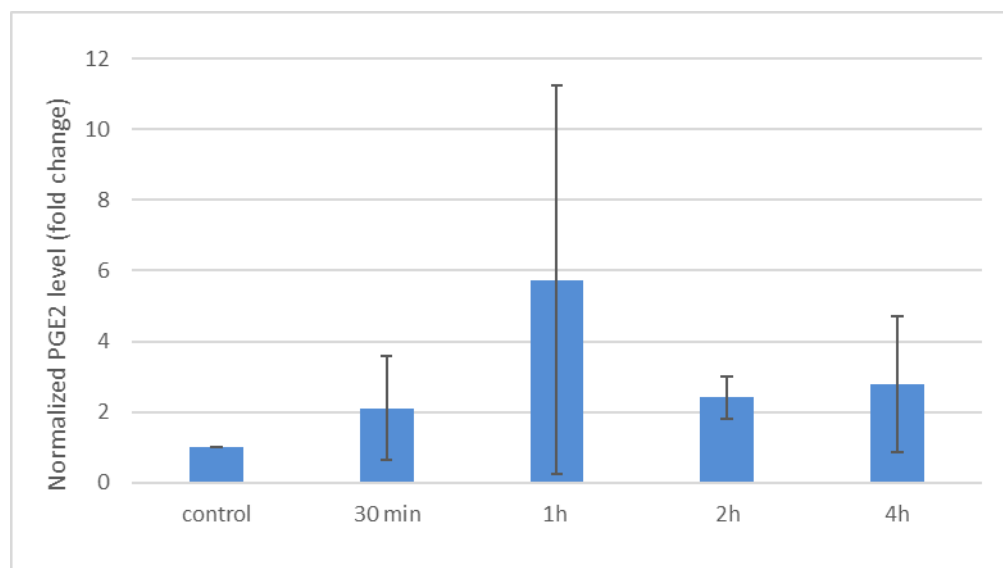


Figure 19 Time-course of ET-1 effect on PGE2 production in PDL cells.

Human periodontal ligament fibroblasts were treated with 10 pM ET-1 for 30 minutes, 1, 2, and 4 hours. PGE2 level in the media was determined by ELISA and normalized with total protein. The untreated group was set as control. Fold change of PGE2 production was calculated and presented as mean \pm SD. One-way ANOVA was performed to determine the statistical difference between groups.

Chapter 5 Discussion

1. Cytotoxicity test

The result from this study revealed that the concentration of ET-1 that was not toxic to the PDL cells was 1, 10, and 100 pM. However, our preliminary study revealed that ET-1 concentration of 1, 10, and 100 nM significantly decreased cell viability in a dose-dependent manner. Our preliminary results contrast with the previous study from Ruest et al. (31) who revealed that ET-1 at 20 and 100 nM dose-dependently increased cell proliferation of PDL fibroblasts which might be due to the different age groups of the subjects. Moreover, the passage used in our study was earlier than that of the previous study, which might be more sensitive to the exogenous ET-1. The result from several studies showed the proliferative effects of ET-1 in gingival fibroblasts from several animal and human sources (62-64). Ohsawa et al. showed the proliferative effects of ET-1 in cultured rabbit gingival fibroblasts at a concentration ranging from 10^{-8} to 10^{-6} M (64), while Ohuchi et al. showed significant cell proliferation after treating guinea pig gingival fibroblasts with 100 nM ET-1 (62). In human gingival fibroblast culture, Sano et al. showed the proliferative effects in a dose-dependent manner within the range of 0.1 nM – 1 μ M (63). These results suggested that ET-1 has proliferative effects on gingival fibroblasts but not on PDL fibroblasts. Furthermore, another study from Von Shroeder et al. (65) revealed the

proliferation in a dose-dependent manner of fetal rat calvarial cells after treatment with 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M ET-1. The result suggested that the ET-1 effects might be different in different cell types. Since our results indicated that the concentration of ET-1 at 1, 10, and 100 pM did not alter the viability of PDL cells, these concentrations were chosen in the following experiments.

The concentration of ET-1 used in this study was determined by MTT cytotoxicity assay. However, the actual concentration of ET-1 secreted from PDL cells during orthodontic tooth movement is unknown. There are a few studies related to the concentration of ET-1 in the gingiva or oral cavity. Fujioka et al. showed that the concentration of ET-1 in human GCF in healthy patients was 46.8 pg/ml or 18.8 pM, while in periodontitis patients the ET-1 concentration was 388.9 pg/ml or 155.9 pM (30). This means that the concentration of ET-1 in inflamed gingival tissues was almost 100 pM higher than in the healthy gingiva. Therefore, the concentration range used in this study corresponded to the level of ET-1 in the GCF of healthy patients.

2. Effects of ET-1 on COX-2 mRNA expression in PDL fibroblast cells

The ET-1 induction of COX-2 gene expression was varied during the time-course experiment. The time points used in this experiment were conducted previously by Chen et al. in 2003 and Song et al. in 2006 (66, 67).

Chen et al. studied the effects of 10 nM ET-1 in the induction of COX-2 and PGE2 in cultured feline esophageal smooth muscle cells (ESMC) (66) while Song et al. studied the induction of COX-2 by 10 nM ET-1 in cultured feline esophageal smooth muscle cells. Chen et al. studied the time points at 0, 0.5, 1, 2, 4, 8, and 16 hours while Song et al. used 0, 0.25, 0.5, 1, 2, 4, 8 hours (66, 67). Although our concentration of ET-1 was 1000-times lower than the previous studies, our results still corresponded to theirs that the peak COX-2 induction was between 1 to 4 hours after ET-1 treatment (66, 67).

In the dose-response experiment, COX-2 mRNA expression was peaked at 1 pM when comparing with 10 and 100 pM ET-1 when treated for 1 hour. The result was in contrast to the time-course experiment that at 10 pM ET-1 of 1-hour treatment, the COX-2 expression in the time-course experiment resulted in the highest fold-changes while in the dose-response experiment the expression was decreased. These might result from different responses to ET-1 by periodontal fibroblasts cells derived from different subjects. The average age of the subject used in the time-course experiment was 13.8, while that used in the dose-response experiment was 20.3.

According to Sumetcherngpraty et al. in 2021, the gene expression of endothelin receptors, both ETA and ETB receptors, was decreased in the adult group, comparing to the adolescent group (68). This suggests that patient ages might affect our result. Further study should be performed to

confirm these findings, by employing the subjects from the same age group as in the time-course experiment, or performing subgroup analysis between different age groups.

In other cell types, ET-1 can induce COX-2 mRNA expression and protein level in both dose- and time-dependent manner. For example, Peng et al. in 2008 reported that 10^{-9} to 10^{-6} M ET-1 dose-dependently increased COX-2 protein level at 24 h in human pulmonary epithelial cells (69). Furthermore, Pourjafar et al. also demonstrated that ET-1 dose-dependently increased COX-2 expression in mesenchymal stem cells (70). Moreover, several investigators reported the effects of ET-1 on the upregulation of COX-2 expression in glomerular mesangial cells. Hughes et al. reported the time-dependent effect of ET-1 on upregulation of COX-2 expression in rat mesangial cells (68). It was also found by Kester et al. that ET-1 can stimulate expression of prostaglandin endoperoxide synthase (PGHS)-2, which involves in the conversion of arachidonic acid to prostaglandin E2 (10). Pratt et al. also revealed that ET-1 stimulated the phosphorylation of ERK and p38, which involve in MAPK pathway and eventually induce the COX-2 expression in glomerular mesangial cells (71). In cardiac cell cultures, there are also reports of the effects of ET-1 on COX-2 expression. As reported by Rebsamen et al. in 2003, ET-1 induce COX-2 protein production time-dependently in neonatal rat ventricular cardiomyocytes (72). Hong et al. in 2014 also

reported the result from the rat neonatal cardiomyocyte cell culture. It was found that ET-1 upregulates COX-2 gene expression and protein level in both dose- and time dependent manner (73). Spinella et al. in 2004 investigated the effects of ET-1 on COX-1 and COX-2 expression in human ovarian cancer cells. They found that ET-1 induced COX-2 expression in Human ovarian carcinoma cell lines HEY and OVCA 433 in both dose- and time-dependent manner (74). These results from several authors indicated the relationships between ET-1 and COX-2 gene expression and protein level.

3. Effects of ET-1 on PGE2 level in PDL fibroblast cells

The level of PGE2 was increased at 30 minutes and 1 hour after ET-1 treatment and gradually decrease at 2 and 4 hours. These findings corresponded to the COX-2 level derived from the time-course treatment of 10 pM ET-1. These results indicated that ET-1 caused the PGE2 upregulation within one hour comparing to the untreated group. However, there was no statistically significant difference due to the small number of samples which resulted in a large standard deviation. Therefore, further study with more samples is required.

ET-1 effects on PGE2 production have also been reported in other cell types. For example, Hughes et al. in 1995 reported that ET-1 markedly increase PGE2 level from rat mesangial cells after treatment at 1-6 hours,

which was peaked at 2 hours of treatment (75). In 2008, Peng et al. reported the effects of ET-1 on PGE2 level in A549 cells. As measured by ELISA, the data showed that the effects of ET-1 on PGE2 were concentration-dependent (69). Since both COX-2 and PGE2 level was upregulated by ET-1, it was suggested that ET-1 might be involved in the inflammatory process of chronic obstructive pulmonary disease (69). Pourjafar et al. in 2016 reported that ET-1 significantly increased the PGE2 level in mesenchymal stem cells in a dose-dependent manner (70). Spinella et al. in 2004 revealed that ET-1 induced PGE2 released from human ovarian cancer cell lines time-dependently, which can be inhibited by BQ123 or BQ788, the ETA receptor antagonist (9, 74). These indicate the roles of ET-1 induced COX-2 and PGE2 expression via ETA receptor (9, 74).

4. The role of COX-2 and PGE2 in periodontal tissues and the application of ET-1 in orthodontic tooth movement

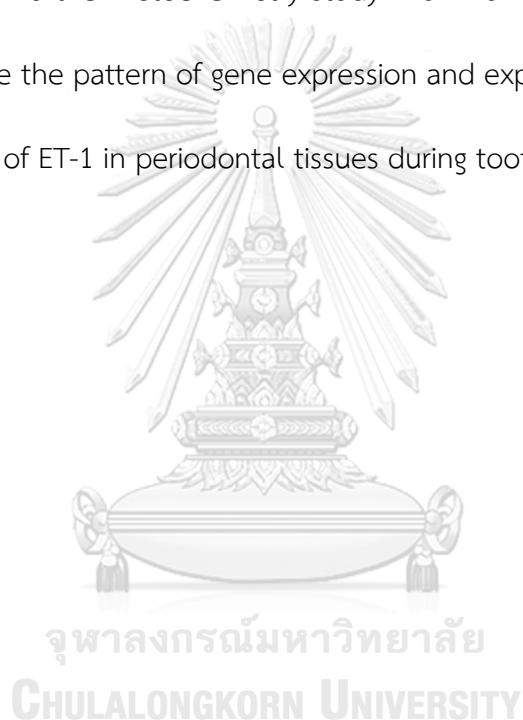
COX-2 and PGE2 have an important role in the bone resorption process. COX-2 expression was involved indirectly in the differentiation of pre-osteoclasts to osteoclasts (39). PGE2 induces RANKL release from osteoblasts, which in turn stimulates osteoclastogenesis (6). PGE2 also inhibited the production of OPG from osteoblasts, which result in a decreased number of antagonists of RANKL and increased osteoclastogenesis (48).

Furthermore, applying exogenous force to PDL cells can induce COX-2 and PGE2 levels (47, 56, 57). The upregulated level of PGE2 also induced the RANKL level which resulted in osteoclast differentiation (47, 56, 57). These previous studies suggested the important role of PGE2 in the process of orthodontic tooth movement.

There are several studies confirmed that exogenous prostaglandin can increase the rate of tooth movement. Leiker et al. demonstrated that exogenous prostaglandin by using 40 μ g per site can increase the rate of tooth movement in the rat (76). Yamasaki et al. in 1982 also demonstrated the effects of exogenous prostaglandin E2 to accelerate the tooth movement in monkeys (77). There is a study about the root resorption effect from Prostaglandin E2 injection in the rat by Pongsri Brudvik and Per Rygh in 1991. They found that the resorption was higher in the injection group than in the control group but no statistical significance was found (78). There is also a concern about pain reaction in a patient from using prostaglandin clinically since it was well-known as pain inducers (79). These suggest that although prostaglandin has the effects in acceleration of the tooth movement, this might be involved with pain sensation and increased risk of root resorption.

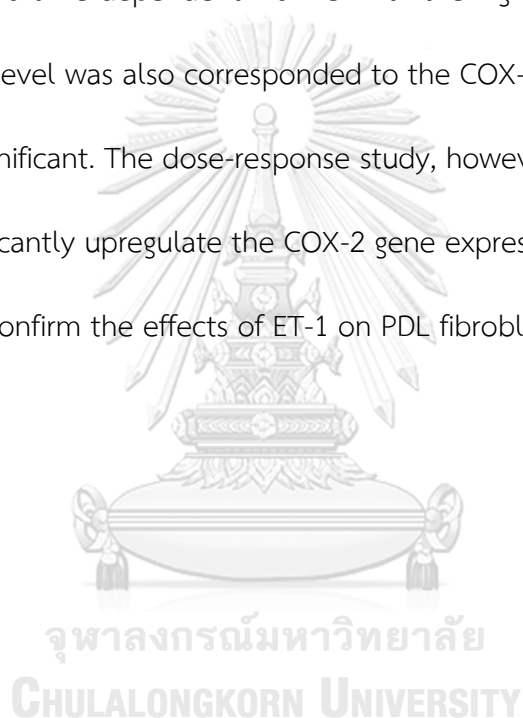
Our results suggest the possible use of ET-1 to induce the production of prostaglandin to increase the rate of tooth movement. Moreover, since prostaglandin E2 can be increased indirectly by ET-1, the unwanted effects

from prostaglandin injection can be avoided. However, since the phenomenon of orthodontic tooth movement involves the interaction among different cell types in periodontal space, our study which focused only on the effects from PDL fibroblasts may not reveal complete relationships between ET-1 and PGE2 in periodontal tissue during orthodontic tooth movement. Further histochemistry study in animal model may be performed to elucidate the pattern of gene expression and expand our understanding of the effects of ET-1 in periodontal tissues during tooth movement.



Chapter 6 Conclusion

In this study, the effects of ET-1 on COX-2 and PGE2 production in PDL fibroblast cells were investigated. It was confirmed by MTT assay that 1, 10, and 100 pM ET-1 are not cytotoxic. The time-course study revealed that ET-1 induced COX-2 gene expression in a time-dependent manner with the highest (3.9-fold) induction at 1 hour. The PGE2 level was also corresponded to the COX-2 gene expression, though not statistically significant. The dose-response study, however, showed that 1-100 pM ET-1 did not significantly upregulate the COX-2 gene expression. Further study should be performed to confirm the effects of ET-1 on PDL fibroblasts.



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