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THE APPLICATION OF PROSTACYCLIN IN PROMOTING ANGIOGENESIS OF DENTAL PULP TISSUE IN A THREE DIMENSIONAL ORGAN CULTURE SYSTEM

Mr. Sonntana Seang

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By	Mr. Sonntana Seang
Field of Study	Oral Biology
Thesis Advisor	Assistant Professor Doctor Chalida Limjeerajarus, Ph.D.
Thesis Co-Advisor	Professor Doctor Prasit Pavasant, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Dentistry
(Assistant Professor Doctor Suchit Poolthong, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Doctor Jeerus Sucharitakul, Ph.D.)

.....Thesis Advisor
(Assistant Professor Doctor Chalida Limjeerajarus, Ph.D.)

.....Thesis Co-Advisor
(Professor Doctor Prasit Pavasant, Ph.D.)

.....Examiner
(Associate Professor Doctor Thanaphum Osathanoon, Ph.D.)

.....Examiner
(Associate Professor Doctor Sireerat Sooampon, Ph.D.)

.....External Examiner
(Associate Professor Doctor Jarin Paphangkorakit, Ph.D.)

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บทนำ

ปัจจุบันการรักษยเชิงอนุรักษ์ทางเอนโดดอนต์ ได้รับความสนใจเนื่องจากสามารถเก็บรักษาความมีชีวิตของฟันเอาไว้ได้ เมื่อเทียบกับการรักษาคลองรากฟันด้วยวิธีปกติซึ่งส่งผลให้สูญเสียเนื้อฟันมาก กระบวนการสร้างหลอดเลือดใหม่มีความสำคัญกับการเจริญงอกใหม่ของเนื้อเยื่อในฟัน พรอสตาซัยคลินเป็นสารขยายหลอดเลือดที่ช่วยในกระบวนการสร้างหลอดเลือดใหม่ จากการศึกษาก่อนหน้าพบว่าพรอสตาซัยคลินสามารถเพิ่มการสร้างหลอดเลือดใหม่ในเซลล์เนื้อเยื่อในฟันและสามารถเพิ่มการสร้างเนื้อฟันชนิดตติยภูมิได้ ในการศึกษานี้ศึกษาถึงผลของไอโลพรอสซึ่งเป็นสารสังเคราะห์เลียนแบบพรอสตาซัยคลินที่สร้างขึ้นจากร่างกาย บนการเพาะเลี้ยงเนื้อเยื่อในฟันบนแผ่นตัดฟันชนิดสามมิติ

ระเบียบวิธีวิจัย

แผ่นชิ้นตัดฟันจากฟันกรามของผู้ป่วย จำนวน 12 ซี่ ที่ยังมีเนื้อเยื่อในฟันติดอยู่จะถูกตัดให้มีความหนา 2 มม. โดยนำไปเพาะเลี้ยงในถาดเลี้ยงเซลล์ที่บรรจุอาหารเลี้ยงที่ใส่ยาไอโลพรอสโดยไม่ใส่โกรทแฟกเตอร์ เป็นเวลา 1 หรือ 3 วัน การทดสอบทางกล้องคอนโฟคอลชนิดสามมิติเพื่อทดสอบความมีชีวิตของเซลล์เนื้อเยื่อในฟัน หลังจากนั้นแผ่นชิ้นฟันจะนำไปฝังด้วยพาราฟินและตัดชิ้นเนื้อฮิสโตและการย้อมสีทางอิมมูโนต่อไป

ผลการวิจัย

พบว่าด้วยวิธีการเลี้ยงแบบสามมิติโดยไม่มีโกรทแฟกเตอร์ในน้ำเลี้ยง เซลล์เนื้อเยื่อในฟันบนแผ่นตัดฟันมีชีวิตอยู่ได้ถึง 3 วัน เมื่อใส่ยาไอโลพรอสพบว่าชิ้นเนื้อเยื่อในฟันเกิดการสร้างหลอดเลือดใหม่ขนาดเล็กเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ มีการเพิ่มการแสดงออกของวาสคุลาร์เอนโดทีเลียลโกรทแฟกเตอร์และวอนวิลลีแบรนด์แฟกเตอร์ นอกจากนี้ที่วันที่สามยังพบการเพิ่มการสร้างคอลลาเจน เมื่อทำการยับยั้งการทำงานของรีเซบเตอร์พรอสตาซัยคลินโดยการใส่สารยับยั้งเอนไซม์โปรตีนไคเนส เอ ซึ่งเป็นเอนไซม์สำคัญในกระบวนการเหนี่ยวนำสัญญาณจากรีเซบเตอร์ ทำให้กระบวนการสร้างหลอดเลือดใหม่ถูกระงับ

บทสรุป

การทดสอบประสิทธิภาพยาในมนุษย์ก่อนนำไปใช้รักษาจริงทำได้จำกัด ชิ้นตัวอย่างฟันสามารถหาเพิ่มได้ง่ายเนื่องจากรับมาจากฟันกรามที่ถอนอยู่แล้วของผู้ป่วย แผ่นชิ้นเนื้อเยื่อฟันสามมิติจึงเป็นโมเดลการทดลองในระดับห้องปฏิบัติการที่สามารถทำซ้ำได้ ไม่ซับซ้อน งานวิจัยนี้แสดงให้เห็นว่าการเลี้ยงแผ่นฟันสามมิติ สามารถเลี้ยงได้โดยไม่ต้องใส่โกรธแฟกเตอร์ลงไปในอาหารเลี้ยง จึงสามารถลดความแปรปรวนของการแปลผลงานวิจัย และพบว่าพรอสตาซัยคลินมีบทบาทเพิ่มการสร้างหลอดเลือดใหม่ในเนื้อเยื่อในฟันมนุษย์ กล่าวได้ว่าพรอสตาซัยคลินเป็นสารที่มีความเป็นไปได้ในการนำมาใช้เพื่อเพิ่มการสร้างหลอดเลือดใหม่ เพื่อใช้ในการรักษาเชิงอนุรักษ์ทางเอนโดดอนต์วิทยาต่อไป

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5676053832 : MAJOR ORAL BIOLOGY
KEYWORDS: ANGIOGENESIS / HUMAN DENTAL PULP / PROSTACYCLIN / REVASCULARIZATION / TOOTH SLICE

SONNTANA SEANG: THE APPLICATION OF PROSTACYCLIN IN PROMOTING ANGIOGENESIS OF DENTAL PULP TISSUE IN A THREE DIMENSIONAL ORGAN CULTURE SYSTEM. ADVISOR: ASST. PROF. DR. CHALIDA LIMJEERAJARUS, Ph.D., CO-ADVISOR: PROF. DR. PRASIT PAVASANT, Ph.D., 82 pp.

Background: Dental pulp vitality can be threatened by carious or iatrogenic exposure that can lead to pulp tissue loss. The formation of a vascular network is crucial to provide a suitable environment for tissue regeneration. Thus, promoting angiogenesis is essential for successful pulpal repair in regenerative endodontics therapy. Iloprost, a prostacyclin analog, promotes vascularization in several organs such as, heart, lung, and corneal. Previously, it has been demonstrated that, iloprost induced pulpal blood flow and enhanced dentin formation in a rat mechanical pulp exposure model. However, the angiogenic effect of iloprost on the human dental pulp vasculature remains unknown.

Objective: The present study investigated the effect of iloprost on promoting dental pulp angiogenesis using the tooth-slice organ culture system.

Materials and methods: Tooth-slices with intact dental pulp tissues were prepared from extracted human third molars. Dental pulp tissue viability was analyzed by live/dead cell confocal microscopy. The tooth-slices were cultured without iloprost (control group) or with iloprost at a dose of 10^{-6} M. The microvessel density and vascular endothelial growth factor (VEGF) expression in the cultured tooth-slices were determined by immunohistochemical staining. The collagen deposition was determined by Masson's Trichrome and immunofluorescent staining. A PKA-inhibitor was used to identify the molecular mechanisms regulated by the cAMP-dependent protein kinase A (PKA) that govern iloprost in promoting angiogenesis.

Results: The dental pulp tissue architecture in the human tooth-slices model were maintained and remained viable when cultured in serum-free media. Iloprost increased the microvessel density as shown by a higher number of von Willibrand Factor-positive blood vessels when compare with the control tooth slice. A significant increased in the VEGF expression and the collange 1 (COL1) production were also observed in the tooth-slices cultured with iloprost. The effect of iloprost on angiogenesis and collagen synthesis were abolished following inhibition of protein kinase A activity.

Conclusion: Human tooth-slices provide a valuable and easy-to-obtain model to investigate the effect of bioactive molecules used in dental pulp regeneration. This study, for the first time, showed that tooth slices could be kept viable under serum-free conditions for up to 3 days. Iloprost promoted angiogenesis, increased new vessel formation, and induced collagen deposition. This study proposes the clinical value of iloprost as a drug for inducing angiogenesis that can increase the success of pulp regeneration.

Field of Study: Oral Biology
Academic Year: 2017

Student's Signature .....
Advisor's Sign ture .....
Co-Advisor's Signature .....

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CHAPTER I

Introduction

The dental pulp is a specialized living connective tissue which located in a tough encasement of hard tissue that provided a strong mechanical support and protection. Dental pulp bears the source of nutritious support to the tooth, and it is also a sensing organ that can response to different stimulus, including pathogenic stimuli [4, 5]. It has been known that the adult dental pulp contained a population of putative post-natal stem cells, which capable of self-renewal capacity with a multi-lineage differentiation [6-8]. Upon stimulation, dental pulp cells (DPCs) can differentiate into various types of cell including the endothelial and the odontoblast-like cells that capable of capillary forming and mineralized matrix secretion, respectively [9-12]. Stem/progenitor cells of the dental pulp were localized in the perivascular area [13]. In response to the pulpal injury, these cells can be migrated to the site of the injury [14]. Additionally, when the appropriate growth factors were applied to the exposed pulp, these growth factors shown to speed up the healing process, and also induce the dental pulp cell to differentiate and form the mineralized dentin [15, 16].

Dental pulp regeneration is preferred to conventional root canal treatment as it preserves the vitality of the tooth [17]. When an injury occurs to the dental pulp, the odontoblast layers are destroyed. As a result, cells multi- or mono-potent progenitors in the dental pulp tissue played the role in tissue healing and promoted reactionary dentin formation [18]. In the adult dental pulp, human dental pulp cells (hDPCs) possessed the important role in dental pulp regeneration. Among the mixed

populations in the dental pulp stem cells, one subpopulations, with negative PCNA labelled of the cells played important role in the early repair process [19]. At the late phase of healing, the increase of positive PCNA-labelled cells was observed, implying that the cells were migrating from apical regions to the exposure area. To further enhance the tissue healing property in dental pulp tissue, certain biomolecules that could increase cells migration and lead to tissue healing are needed.

The formation of a vascular network is crucial to provide a suitable environment for tissue regeneration [20]. Upregulating proangiogenic factor expression promotes angiogenesis [21]. Prostaglandins and other eicosanoids are bioactive lipids that affect normal development, tissue homeostasis, inflammation and revascularization [22, 23]. Prostaglandins play an important role in wound healing in dental pulp tissue. Prostacyclin (PGI_2), a prostaglandin member of the eicosanoid family, stimulates angiogenesis [24]. PGI_2 is prominently produced by endothelial cells, and endogenous PGI_2 plays a physiological role in endothelial cell survival by promoting and maintaining the capillary microcirculation [3, 25].

Iloprost is a long-acting prostacyclin analog and is used to treat pulmonary arterial hypertension, Raynaud's disease, and other diseases where the lack of blood flow damages the tissues. Iloprost works by stimulating the blood vessel dilation and allow the blood to flow through again to the damaged tissues [26]. Recent findings have demonstrated that iloprost upregulated vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) expression in hDPCs *in vitro via* the protein kinase A (PKA) pathway [2]. Iloprost also increased pulpal blood flow and enhanced dentin formation in a rat

mechanical pulp exposure model [2, 27]. Collectively, these data suggest the possible use of iloprost in clinical treatment. However, there are a limited number of model systems to study angiogenesis in a 3-dimensional (3D) environment. The tooth slice model is a useful laboratory system in translational studies related to dental pulp tissue engineering because it mimics the *in vivo* situation [11, 28, 29]. Apart from our available knowledge on functional and therapeutic significance of iloprost in generating calcified dentin matrix *in vivo* [2, 27], little is known about the precise mechanisms by which iloprost contributes to the angiogenesis of blood vessel and tissue healing in human dental pulp. The objectives of the present study were to evaluate the effect of iloprost on inducing angiogenesis and promoting dental pulp cells wound healing by using a human tooth slice 3-D organ culture model and a scratch wound healing essay, respectively.

Keywords: Angiogenesis, ECM, human dental pulp, human tooth slice culture, migration, MMP-9, prostacyclin, wound healing.

Research question

Can iloprost promote human dental pulp cells wound healing, and induce angiogenesis in human dental pulp tissue in a tooth slice 3-D organ culture system?

Objectives and hypothesis

Objective 1

To develop a method for maintaining the viability of the human tooth slice in serum free media (SFM).

Hypothesis

The viability of the human tooth can be maintained in a serum free condition.

Experimental design

- A 2-mm thick tooth slices were prepared from the extracted human third molar. The tooth slices with intact pulp tissue were cultured in SFM for 1 or 3 days.
- Histology and immunohistological analysis were performe to access the morphological change of pulp/dentin complex in the culture. Dental pulp tissue viability was evaluated by the live/dead cell immunofluorescent staining.

Objective 2

To investigate the angiogenic effect of iloprost on promoting angiogenesis in human tooth slice 3-D organ culture system.

Hypothesis

The application of iloprost can promote the angiogenesis of the human dental pulp

tissue in tooth slice 3-D organ culture model.

Experimental design

- The human tooth slices were cultured in the presence or absence of iloprost at a concentration of 10^{-6} M for 1 and 3 days. Collagen 1 (COL1) expression was evaluated by Masson's trichrome staining and COL1 immunofluorescent staining. The microvessels density were evaluated by immunofluorescent staining of von Willebrand factor (vWF).
- Mechanistic study was performed by pretreating the human tooth slices with IP-receptor antagonist or PKA-inhibitor prior to iloprost stimulation. The effect of inhibitors on COL1 content and microvessel formation were evaluated by immunofluorescent staining.

Objective 3

To investigate the role of prostacyclin (iloprost) on promoting human dental pulp cells (hDPCs) migration.

Hypothesis

The application of prostacyclin (iloprost) can accelerate hDPCs wound closure in the wound scratch-test assay.

Experimental design

- The hDPCs were seeded in a 12-well at a density of 37,500 cells/cm^2. The wound field was created on a confluent cells monolayer with a sterilized pipette tip. Wound closure was analyzed using the image analysis software, Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

- Mechanistic study was performed by pretreating the hDPCs with the IP-receptor antagonist or PKA-inhibitor prior to iloprost stimulation.
- The Cell viability was determined using an MTT assay. The mRNA expressions were determined by real-time quantitative polymerase chain reaction (qPCR). The protein expression level was analyzed by ELISA.

Expect benefit

This study is the first study to highlighting the potential use of iloprost on enhancing wound healing in human dental pulp cells and promoting microvessel formation (angiogenesis) in the human dental pulp tissue in the tooth slice organ culture model.

Limitation

This study was performed *in vitro,* thus the result might not be an exact representation for what occur *in vivo* or physiological condition. Moreover, it is also difficult to establish *in vivo* relevant *in vitro* systems, because cultivation of an isolated tissue/organ usually differs strongly from the corresponding organ type in an organism.

Keywords

Angiogenesis, ECM, human dental pulp, human tooth slice culture, migration, MMP-9, prostacyclin, wound healing.

CHAPTER II

REVIEW LITERATURES

2.1 Dental pulp biology

Anatomically, the dental pulp is the soft tissue located in the center of the tooth and it is encapsulated by hard dentin. The mature dental pulp bears a strong resemblance to the embryonic connective tissue with a rich microcirculatory components and a layer of specialized cells, the odontoblasts along its periphery [30]. The connection between the dental pulp and the surrounding tissue is through the root apexes. The establishment of the vascular networks in the dental pulp is influenced by the geometry of pulp environment, where the main feeding arterioles entering the root canal through the apical foramen, running longitudinally toward the coronal pulp, and forming a robust vascular networks [4]. The high microcirculatory of these vascular networks is functioned to provide oxygen and nutrients supply to the pulp tissue [4, 30]. Therefore, the physiological state of these vascular networks is important for the survival of the dental pulp tissue and its function. The primary function of the dental pulp is formative. It gives rise to odontoblast that not only form dentin but also interact with dental epithelium early in tooth development to initiate the formation of enamel [31]. Secondly, dental pulp serves as a sensory organ related to tooth sensitivity, hydration, and defense [32, 33].

2.2 Mechanisms of dental pulp repair

2.2.1 Dental pulp, diseases and responses

The dental pulp is encased in a rigid structure comprising dentin and enamel structure, bearing the source of nutritious support to the tooth, and it is also severed as

a sensing organ that can response to different stimulus, including pathogenic stimuli. Dental pulp is located in a very unique environment in which the responses to the pathology are different than in other organs and tissues. To date, it is a well-known fact that the dental pulp is incompressible, the total volume of blood within the pulp space cannot be greatly increased. Thus, alteration of microcirculatory functions, such as pulpal blood flow, intra/extravascular pressures, blood volume, and capillary permeability, play an important role in initiating pulpal disorders and in contributing to the pathophysiological processes of dental pulp [4, 21, 30].

During the normal functions of mastication and speech production, the dental pulp is relatively well protected from injuries, since pulp tissue is surrounded by a tough encasement of hard tissues. As long as this hard tissue barrier is pristine, the pulp tissue will not be exposed to adverse influences by deleterious components present in the oral environment. However, in clinical situations, teeth suffer the loss of its structural integrity, outer enamel or cementum layers are broken through, creating a potential exist for noxious element in the oral environment to gain access to the pulp and adversely influence the tissue along exposed dentinal tubules [32].

The pathology of dental pulp represents a cascade of inflammatory reactions of pulp cells, microcirculation and nerves whenever dentin and pulp is affected by caries or mechanical, chemical, thermal, microbial irritants, and physical trauma (Table 1). The dental pulp may be exposed to a number of irritants that are noxious to the health of the pulp and it may be either constant irritants or specific events that interfere with the pulp blood supply [32-35]. Each type of irritant or injury will have a different effect on the pulp; in general, the effects will be acute/chronic inflammation or necrosis. Of note, it has been shown that teeth with pulps are much more resistant to

bacterial invasion into the dentinal tubules than are teeth with root canal fillings, thereby suggesting that the vital pulp plays an important role in this defense process [36].

Table 1 Major etiological factors of pulp disease

Microbial	Traumatic	Iatrogenic
- Caries	- Concussion/luxation injuries	- Trauma from operative procedure
- Periodontal disease	- Crown/root fractures	- Marginal leakage
		- Dental material toxicity

It has also been documented that the sensory nerve activity has an importance role in alteration of the pulp microcirculatory. The activation of the sensory nerve activity in response to pulp injury cause the intradental sensory nerves to release neuropeptides, such as substance P and calcitonin gene-related peptide, resulted in alteration of microcirculatory haemodynamics. The vasoactive property of these neuropeptides, released from sensory nerves, lead to an increase in the pressure of the pulp, resulting in a decrease in blood flow and an increase in flow resistance in the venules. The flow stasis causes an aggregation of red blood cells and elevation of blood viscosity. It also produces tissue hypoxia or ischaemia, which suppress cellular metabolism in the inflamed area of the pulp, resulting in tissue necrosis [5, 37, 38]. Therefore, modulating the pulpal microcirculatory function by an effective drug during the inflammatory phases may play a key in enhancing the survival rate of the pulp tissue and the long term-prognosis of the tooth.

2.2.2 Initiation of dentinogenesis as a part of defense mechanism and repairing process

Dental pulp restricted in a rigid dentin wall, which plays a role in protecting dental pulp from harmful pathogens and physical stimuli. Dentinogenesis involves in controlling reactions that result in conversion of unmineralized predentin to dentin when apatite crystals are formed [15, 31]. In pathological condition when the dentin wall is disrupted by traumatisms or carries, dentinogenesis requires recruitment and induction of pulp's specialized cells, the odontoblasts, and the undifferentiated mesenchymal cells (may differentiate into dentin-forming cells upon stimulation), which are derived from cell within the pulp cell population(s) [15, 27, 31], follow by differentiation of these cells into functioning odontoblasts. This phenomenon enables the healthy pulp to repair, regenerate, and partially compensate for the loss of enamel or dentin.

2.2.2 Conversion/maturation of predentin to dentin

Mineralization is initiated by phosphorylated extracellular matrix proteins localized within collagen gap zones that bind calcium and phosphate ions in an appropriate conformation to nucleate the formation of apatite crystals [39]. Likewise, the formation of dentin by mature odontoblasts involves secretion of several extracellular matrix proteins that ultimately results in collagen-based deposition of carbonate apatite mineral. During dentinogenesis, the unmineralized predentin, consisting largely of type I collagen fibrils is progressively formed outside the odontoblasts. These collagen fibrils progressively thicken, as the odontoblasts build this fibrillar matrix, they recede and leave behind odontoblastic processes, and the forming fibrillar tissue in the predentin zone is then converted to dentin. However, it

has been known that collagen alone does not spontaneously mineralize. In this, many non-collagenous proteins, which are expressed in odontoblasts, have been proposed to play important roles in this process. These proteins include, osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP), dentin morphoprotein 1 (DMP-1), and dentin sialophosphoprotein (DSPP) [39, 40].

Dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) are two non-collagenous extracellular matrix proteins produced by odontoblasts. DMP1 and DSPP are belonging to the SIBLING (Small Integrin Binding Ligand N-linked Glycoprotein) family, a group of acidic and highly phosphorylated proteins involved in mineralization process of dentin [41-43]. DMP1 is expressed in both pulp and odontoblast cells, its expression is required in both early and late odontoblasts for normal odontogenesis to proceed. In particular, *Dmp1*-null mice showed defects in odontogenesis and in dentin mineralization [42]. The *In vivo* experimental study, the over expressing or re-expressing DPM1 into *Dmp1*-null mice, it has been shown that transgenic expression of DMP1 was able to rescue both abnormalities in dentin/predentin thickness as well as the defect in mineralization in *Dmp1*-null mice [43]. This study implicated the importance role of DMP1 as a key regulator of odontoblast differentiation, maturation of the dentin.

Similarly, to DMP1, DSPP is a proteolytic cleavage protein, which undergo proteolytically cleaved into two products, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). DSPP is mainly expressed in teeth by the odontoblasts and preameloblasts and believed to play a crucial role in converting predentin to form mineralized dentin [44, 45]. *Dspp*-deficient mice develop tooth defects similar to human dentinogenesis imperfecta with enlarged pulp chambers, increased width of

predentin zone, hypomineralization, and pulp exposure. Taken together, all these reports indicated a potential function for DMP1 and DSPP in tooth mineralization.

2.3 Current progress in dental pulp therapy

2.3.1 Endodontics treatment

Deep cavities and traumatic injury are the main reasons for endodontics treatment. Presently, endodontic treatment is one of the most commonly used techniques in clinical management of irreversible pulpitis and dental trauma. The goal of endodontic treatment is to remove the contaminated and injured dental tissue, and restore the created void with synthetic dental material to eliminate future contamination. With advancements in antibiotic therapies, dental materials, and endodontic technology, the success rate of endodontic therapy has increased dramatically [46, 47]. Despite the satisfactory result following endodontic treatment, it can often result in discoloration of the tooth grown, mainly due to (1) pulpless teeth lose ability to maintain their mineral integrity, (2) staining from endodontic drugs and filling materials. Additionally, to this date, there is no restorative material that could mimic all physical and mechanical properties of tooth and pulp tissue [48, 49]. Based on these issues and concerns, the ability to maintain or renew dental pulp vitality would be preferable to current endodontic treatments. Ultimately, it is considered important to find and develop novel method in pulp-dentin regeneration, as well as a thorough understanding of the various biological processes involved in the mechanisms of reactionary or reparative dentinogenesis, and pulp wound healing.

2.4 Dental pulp regeneration therapy

2.4.1 Vital pulp therapy

Dental pulp plays an important role in the formation and nutrition of dentin as well as in the innervation and defense of the teeth. Therefore, the vitality of dental pulp is essential for long-term tooth survival and function in both primary and permanent teeth. Vital pulp therapy aims to preserve pulp tissue that has been compromised and to stimulate the remaining pulp to regenerate the dental-pulp complex. In addition to these, in primary teeth it is important to preserve the tooth until its natural exfoliation time, thus preserving arch integrity. Clinically, there are two therapeutic approaches in vital pulp therapy: **(1)** indirect pulp capping for deep cavities and **(2)** direct pulp capping or pulpotomy in case of pulp exposure. Indirect pulp capping is recommended as the most appropriate procedure for treating primary teeth with deep caries and reversible pulp inflammation. This approach is achieved by applying a protective agent on the thin layer of dentin remaining over a nearly exposed pulp, in order to allow the underlying dental pulp to recover [50, 51]. In contrast, direct pulp capping is the strategy where a protective biocompatibility agent is placed directly on the exposed pulp to facilitate the formation of reparative dentin, and to protect the underlying pulp from further injury [51]. Regard to these strategies, pulp capping with medication or specific growth factors have also been reported. The *in vivo*, direct pulp capping of bone morphogenetic proteins (BMP)-2, -4, and -7 has been shown to promote reparative dentin formation and induce differentiation of adult pulp cells into odontoblasts [15, 52, 53]. On the other hand, the direct pulp capping with fibroblast growth factor (FGF)-2 and transforming growth factor (TGF)-β has

been shown to accelerate the pulpal healing and promote the reparative dentin bridges [16, 54].

As previously mentioned, dental pulp possesses the ability to form a dentin-like matrix (tertiary dentin) as part of the defense mechanism and repair in the dentin-pulp organ [32, 55]. Whenever the dentin-pulp complex is affected by dental trauma, severe carries lesions or deep cavity preparation, three different physiopathologic conditions might be observed at the dentin-pulp border:

(1): In the case of mild injuries (e.g., slowly progressing dentinal caries, cavity preparation), the odontoblasts responded by up-regulating its secretory activity and leaded to deposition of reactionary dentin matrix beneath the injury. The process of reactionary dentinogenesis involved in the up-regulation of odontoblast activity, often in quiescent cells at the stage of physiological secondary dentinogenesis, in response to the injury [32, 55]. The nature of the signaling process from this stimulus may be rather variable and has been hypothesized to result from the release of growth factors and other bio-active molecules, such as epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2), insulin-like growth factor (IGF)-1/2, platelet-derived growth factor (PDGF), placenta growth factor (PlGF), and TGF-β from the dentin matrix after injury [56, 57]. DMP1 and DSPP have also been shown to actively take part to the dentin biomineralization by upregulation its level of expression in carious dentin compared to the control group of normal dentin. Immuno-labeling of DMP1 and DSPP was also shown to present in peri/intratubular dentin and along the walls of odontoblast processes at a certain distance from the carious lesion, suggesting its protective role in defense of non-affected dentin against a potential damage, after a noxious stimulus has eroded the mineralize structure of the teeth [58].

(2): In severe dentinal injuries without pulp exposure (e.g., rapidly progressing carious lesions or severe tissue damage in cavity preparation), odontoblasts are destroyed subjacent to the affected dentin. In an appropriate metabolic state of the dentin-pulp complex, while the carious lesions did not appear to invoke an inflammatory response, even in the absence of any pulp-capping agent, the induction of reparative dentinogenesis may take place at the site of injury [59]. This event is initiated by a new generation of odontoblast-like cells that might have differentiated from progenitor cells within the pulp and secreted a reparative dentin matrix [55].

(3): In the case of injury leading to pulp exposure (e.g. patient with severe form of dental carries, exposed cavity preparation, or dental trauma), odontoblasts are severely destroyed at the exposed area. While the tissue debris at the exposed dentin did not appear to invoke an inflammatory response (in case of irreversible pulpitis, endodontic treatment is indicated), auto-induction of reparative dentinogenesis may be initiated [59]. In addition, when an appropriate pulp capping material is placed in contact with the damaged pulp, aiming to restrict bacterial contamination of exposed dental pulp, the formation of dentinal bridge around the wound site is significantly stimulated [52, 59, 60]. Initially as part of the wound healing process in the repairing pulp, the cells under the wound surface proliferate, migrate and elaborate new collagen in contact with the firm necrotic zone of damaged pulp, or the material interface. The necrotic zone, as well as the new collagen layer attracts mineral salts to form calcified matrices of fibrodentin. Finally, a layer of odontoblast-like cells is formed in association with the primitive matrix and tubular matrix is secreted in a polar predentin-like pattern [16, 54, 55].

2.4.2 Revitalization of the dental pulp

In 1971, Nygaard-Östby & Hjortdala performed studies that can be considered as a ground breaking in pulpal regeneration [61]. They demonstrated that after the entire pulp tissue was removed and the contamination was controlled, the presence of the blood coagulum inside the pulp chamber enhanced the reparative processes and the tissue formation was promoted. Since then, evidence of successful revascularization in both controlled animal and human case series has been reported [62-65]. The important feature of dental pulp revascularization is that after the root

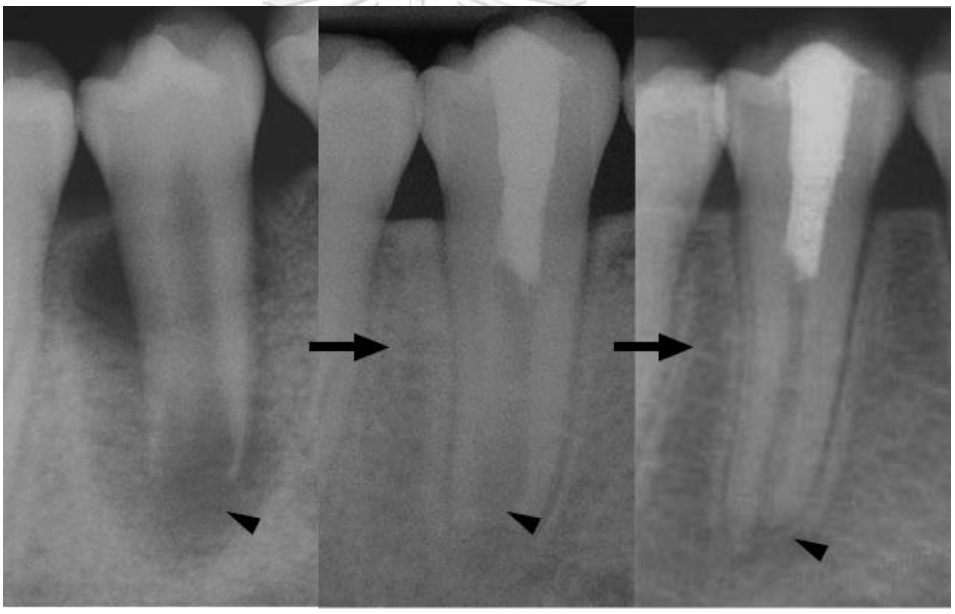


Figure 2.1 Revascularization of an immature tooth with a necrotic infected canal with apical periodontitis. At 7 months the apex shows healing the apical periodontitis and some closure of the apex. At 24 months apical healing is obvious, and root wall thickening and root lengthening have occurred, indicating that the root canal has been revascularized with vital tissue. Adapted from [57].

canal infection is controlled, the necrotic uninfected pulp acts as a scaffold for the in-growth of new tissue from the periapical area. The blood clot is then introduced so as to mimic the scaffold that is in place with the ischemic necrotic pulp. The invading cells within the blot clot matrix will further initiate new tissue formation inside the root canal. The approach of pulp revitalization shown to enhance the survival rate of immature permanent teeth with pulp necrosis and this approach also allowed the dentinal walls of the immature root to continue to develop and produce a stronger mature root that is better able to withstand against fracture [62-65] (figure 2.1).

Presently, it is certain that the pulp space has returned to a vital state after revascularization, but based on recent research in avulsed teeth and on study on infected teeth, it is likely that only in a few cases the pulp is actually the tissue. In many cases, the ingrowth tissue within the root canal space is more similar to periodontal ligament than to pulp tissue, and there is also a little chance of tissue formation, fully entering the pulp space after revascularization [63, 66]. Together with all these previous findings and results, it is logical to assume that, because there are no capillaries in the blood clot, hence the important cellular elements are dependent on the diffusion of nutrients supplied at the early phase before coagulum and later phase by tissue fluid. Therefore, these observations suggested that successful dental pulp revitalization is not depend on the formation of the blood clot alone. It has been demonstrated that long-term function of three-dimensional tissues construct is depended on the degree of vascularization, which is required for cell survival and tissue growth in organ regeneration [67, 68]. Thus, encouraging angiogenesis is a key essential in dental pulp revitalization, and the different methods of enhancing the vascularization of dental pulp engineered constructs will be reviewed next.

2.5 Strategies for improving the physiological relevance of dental pulp engineering

2.5.1 The role of vascular networks in dental pulp engineering

The goal of regenerative endodontics is to generate vital dental pulp tissue that is morphologically and functionally similar to the physiological pulp. The approach of revitalization in endodontics therapy is beneficial and extremely desirable, not only to maintain a pulp space free of infection, but to allow the root and dentinal structure to continue to develop and strengthen with a better long-term prognosis. To achieve this goal, a multidisciplinary approach both *in vitro* and *in vivo* that integrates engineering and biological methodologies is necessary.

Successful three-dimensional tissue construct relies on the early establishment of an effective vascular network that was able to supply the tissue generation with oxygen and nutrients. In addition, the supply of nutrients and oxygen to the targeted engineering tissue construct are critical for a sustainable the high metabolic activity of cells presenting in the matrices at the early post-transplantation stage [68, 69]. It has been noted that during the dentin formation, peripheral capillaries invaded into the odontoblast layer and located close to the predentin where they formed a dense vascular network consisting of fenestrated capillaries. This vascular network facilitated a rapid and sufficient supply of raw materials and nutritional requirements for the odontoblasts to synthesize dentin [70].

2.5.2 The angiogenic growth factors

It has been known that the presence of bioactive molecules, especially growth factors including those of the TGF-β family, sequestered within the dentin matrix provides an endogenous source of cell-signaling molecules for trans-dentinal

stimulation of tertiary dentinogenesis after injury to the tooth. Angiogenesis is defined as the formation of new capillaries from pre-existing blood vessels. The processes of angiogenesis and neovascularization are essential for wound healing and repairing process of the pulp-dentin complex. There is a number of bioactive molecules, especially growth factors including those of the TGF-β super family and vascular endothelial growth factor (VEGF), sequestered within the dentin matrix. The release of these molecules and growth factors, provided an endogenous source of cell-signaling molecules for transdentinal stimulation of reparative dentin formation as a consequence of injury in the dentin–pulp complex [53, 56, 71]. Additionally, those bioactive molecules and growth factors of the dentin could be isolated from the dentin matrix as a solubility form, also known as the soluble dentin extracellular matrix components [71, 72]. In the *in vivo* mechanical pulp exposure model, the treatment of the soluble dentin matrix shown to induce the differentiation of odontoblast-like cell, promote mineralization of the dentin, and enhance local vascularization at the sites of injury [57, 72].

VEGF has been known to have a major role in angiogenesis, and capable of promoting the formation of new capillaries. Its function is essential to endothelial cell proliferation, migration, survival, and tissue generation [73]. The potential use of VEGF in therapeutic angiogenesis has been extensively explored in the treatment of several vascular diseases, such as myocardial ischemia and infarction [74]. The *in vivo*, intracoronary administration of VEGF in chronic myocardial ischemia showed a significant improvement in myocardial blood flow and regional myocardial function [75].

FGF-2 is another potent angiogenic factor and also a stimulator of endothelial proliferation. The function of FGF-2 is important in wound healing, acting as an angiogenic cytokine promoting endothelial cell proliferation and induces capillaries sprouting [76, 77]. *In vitro*, FGF-2 is a potent mitogen for different cell types, including vascular endothelial cells and fibroblasts. The culture of endothelial cells in the presence of FGF-2 shown to induce an angiogenic phenotype consisting of increased proliferation, migration, proteinase production, and expression of specific integrins [78]. Increased in the expression of FGF-2 mRNA and protein level have been observed in several systems, including heart, brain and endothelial cells following injury-induced increases FGF-2 [79]. High expression of FGF-2 protein in wound is proposed to act as an autocrine growth-promoting stimulus for the endothelial cells [78, 79].

2.5.3 Iloprost-induced increase angiogenic factors

Controlling angiogenesis using angiogenicity-related factor activation is one of the novel approaches in regenerative medicine [1, 80]. Prostacyclin (PGI_2) is a prostaglandin member of the family of lipid molecules known as eicosanoids. PGI_2 is known to modulate a variety of physiological responses, including vascular permeability, vasodilation, and anti-platelet aggregation [3]. PGI_2 is prominently produced by endothelial cells and the endogenous production of PGI_2 within the specific tissue/organ play a physiologic role that protects and maintains the capillary microcirculation from deposition of platelet aggregates [25]. The classical signaling pathway of PGI_2 uses a G-protein-coupled receptor named IP (figure 2.2), whose activation leads to increased production of intracellular cyclic-AMP (cAMP) and consequent activation of the protein kinase A (PKA) cascade. This pathway is

postulated to be responsible for the multiple effects of PGI_2 [1, 3, 25]. In addition to these conventional functions of PGI_2, it has been demonstrated that the activation of

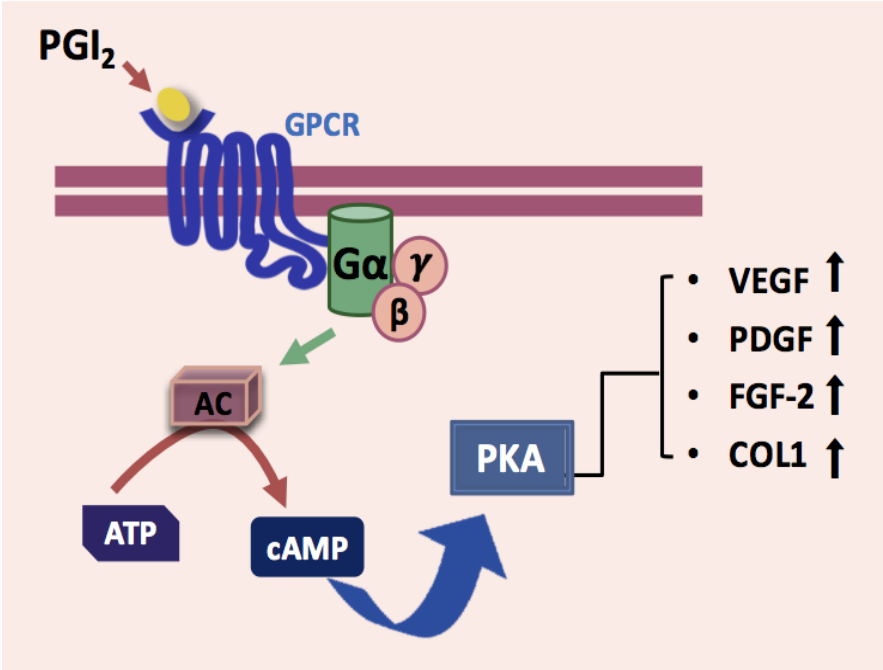


Figure 2.2 Generalized schematic of cAMP-dependent protein kinase A (PKA) pathway. GPCR associate with heterotrimeric G-proteins. G-proteins composed of three subunits: alpha, beta, and gamma. The subunits are tethered at the membrane surface by covalently attached lipid molecules. Upon ligand binding to the GPCR the receptor undergoes a conformational change whereby the α subunits of the G protein subsequently dissociate to activate PKA downstream signaling cascades [1-3].

PGI_2 pathway produced a significant enhancement of proangiogenic factors, such as VEGF, FGF-2, PDGF mRNA expression *in vitro* and facilitated the increase in vascular permeability in the inflame pulp *in vivo* [1, 2, 24, 81]. Furthermore, it has been shown that PGI_2 pathway is an important signaling mechanism, which is

responsible for the angio/mitogenic effect of PGI_2 in endothelial progenitor cells and the high production of PGI_2 level is required for endothelial regeneration and function after injury [1, 82].

Physiologically, PGI_2 is short-lived and undergoes spontaneous inactivation within minutes *in vivo* [25]. Therefore, several chemically stable PGI_2 analogs with the resembled pharmacological properties have been synthesized [83] (figure 2.3).

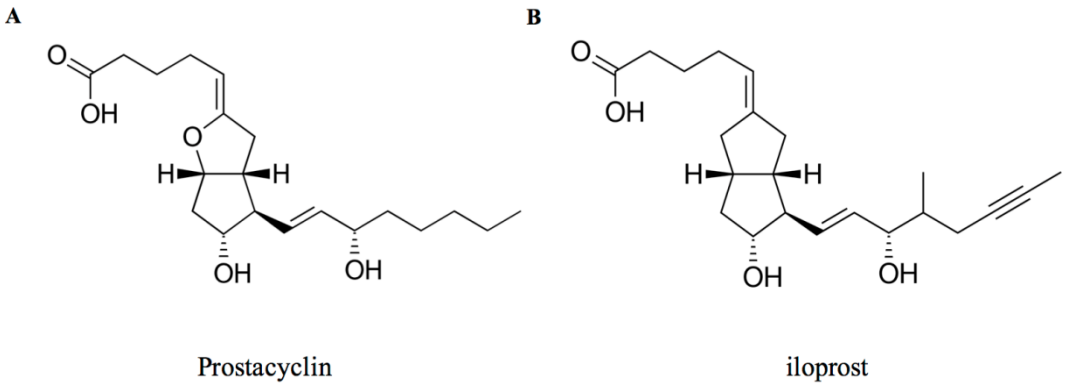


Figure 2.3 Molecular structures of PGI_2 (A) and iloprost (B). PGI_2 and its analog (iloprost) share a resemble primary structure that determines their binding to the IP receptor, including the essential carboxyl group, and hydrogen-bonding OH groups.

Iloprost, currently the most stable and long acting analogue of PGI_2, has been widely used in the treatment of pulmonary arterial hypertension and other vascular-related diseases [84]. PGI_2/iloprost pathway is known as the important signaling mechanism, which responsible for the angio/mitogenic effect of PGI_2/iloprost in endothelial progenitor cells. Additionally, the high production of PGI_2 level within tissue is required for endothelial regeneration and function after injury [75, 79]. It has been shown that, in pathological condition when the dental pulp is inflamed, the cells in the

inflamed dental pulp tissue respond to injury at the exposed dentin area by endogenously releasing PGI_2, which in turn promoting the proangiogenic factors and facilitating the healing process [9, 12]. Recent findings from *Limjeerajarus et al.* have demonstrated that the administration of iloprost leaded to the up-regulation of mRNA expression of several proangiogenic factors, such as VEGF, FGF-2 and PDGF in hDPCs (Figure 2.2) and increased pulpal blood flow in rat mechanical pulp exposure model [2]. Furthermore, iloprost could stimulate the odontogenic response in hDPCs by up-regulation of odontogenic-related markers, such as DMP-1, ALP, COL1, and BMP-4. In addition, iloprost enhanced the formation of tertiary dentin *in vivo* [27]. Thus, encouraging the early angiogenesis even by boosting up the level of PGI_2 with an appropriate iloprost administration after pulp exposure, a successful revitalization of dental pulp tissues could be achieved.

2.6 Matrix metalloproteinase (MMP) in matrix remodeling and healing

The synthesis and deposition of extracellular matrix (ECM) is a crucial feature in wound healing and repair process of an organ. The ECM is assembled from the components that synthesized and deposited by specialize cells, which provide structural and functional integrity to connective tissues and organs [85]. The ECM synthesis and deposition mostly occur in response to growth factors, cytokines, and mechanical signals that triggered *via* the cell surface receptors [86]. In addition to its role in structural and barrier functions, ECM also severs as reservoir for a variety of bioactive molecules [87].

The degradation and remodeling of ECM by proteases, particularly matrix metalloproteinases (MMPs), is regard as an essential key feature of leukocyte influx, angiogesesis, tissue reepithelialization, and remodeling. MMPs are a family of over

20 zinc-containing endopeptidases that are capable of degrading various ECM components [88, 89] (table 2). During angiogenesis, degradation of the vascular basement membrane and remodeling of the ECM by MMPs is required for the formation of the newly formed blood vessels and also to facilitate the endothelial cells to invade into the surrounding tissue during the event of angiogenesis. [85-87]. Beside its function in degrading ECM components, MMPs activity is additionally required for making available growth factors and cytokines. Several growth factors, for example insulin-like growth factor-binding proteins (IGF-BPs) can be proteolyzed by MMPs to generate a IGF ligand [89-91]. Likewise, cleavage of the proteoglycan perlecan by MMPs-1, 3 and cleavage of decorin by MMPs-2, 3, 7 can release fibroblast growth factor (FGF) and transforming growth factor-beta 1 (TGF-**β**), respectively [90-92]. MMP-3 and MMP-7 have also been shown to cleave the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF) and releasing active HB-EGF [91].

Table 2 List of the mammalian MMPs family members

Mammalian MMPs and substrates.

MMP	Non-traditional substrate	Resultant effector	Matrix substrates
Minimal domain			
MMP-7/Matrilysin	Pro-a-defensin [41•]	α-Defensin	Proteoglycans, laminin, fibronectin, gelatin, collagen III/IV/V/IX/X/XI, fibrin/fibrinogen, entactin, tenascin, vitronectin
	Decorin [13]	Bioavailable TGFβ	
	Cell surface bound Fas-L [27•,28•]	Active soluble Fas-L	
		Inactive soluble Fas-L	
	B4 integrin [31]	Release of B4 integrin	
	E-cadherin [32,33•]	Bioactive E-cadherin ectodomain	
	Plasminogen [60,61•]	Angiostatin	
	Pro-TNFα [39•]	Bioavailable TNFα	
	Pro-MMP-2,7	MMP-2,7	
MMP-26/ Matrilysin-2/Endometase	α1-proteinase inhibitor [62]	Inactive serpin	Gelatin, collagen IV, fibronectin, fibrinogen [62–64]
	MMP-9 [63]	Pro-MMP-9 [63]	
Collagenases			
MMP-1/Collagenase-1	Perlecan [12]	Bioavailable FGF	Collagen I/II/III/VII/X, gelatin, entactin, aggrecan, tenascin
	IGFBP-2,3	Bioavailable IGF	
	α1-antichymotrypsin	Inactive serpin	
	α1-proteinase inhibitor	Inactive serpin	
	Pro-MMP-1,2	MMP-1,2	
	Pro-TNFα	Bioavailable TNFα	
MMP-8/Collagenase-2	α1-proteinase inhibitor	Inactive serpin	
	Pro-MMP-8	MMP-8	
MMP-13/Collagenase-3	α1-antichymotrypsin	Inactive serpin	
	Pro-MMP-9,13	MMP-9,13	

Table 2 (continued)

Stromelysins			
MMP-3/Stromelysin-1	Perlecan [12]	Bioavailable FGF	Proteoglycans, laminin, fibronectin, gelatin, collagen III/IV/V/IX/X/XI, fibrin/fibrinogen, entactin, tenascin, vitronectin
	Decorin [13]	Bioavailable TGFβ	
	Pro-HB-EGF [18]	HB-EGF	
	Pro-IL1-B [21]	IL1-β	
	Plasminogen [59]	Angiostatin	
	E-cadherin [33•,65]	Bioactive E-cadherin ectodomain	
	IGFBP-3	Bioavailable IGF	
	α1-antichymotrypsin	Inactive serpin	
	α1-proteinase inhibitor	Inactive serpin	
	Pro-MMP-1,3,7,8,9,13	MMP-1,3,7,8,9,13	
	Pro-TNFα	Bioavailable TNFα	
MMP-10/Stromelysin-2	Pro-MMP-1,8,10	MMP-1,8,10	
MMP-11/Stromelysin-3	α-1-proteinase inhibitor	Inactive serpin	Laminin, fibronectin, aggrecan
	IGFBP-1	Bioavailable IGF	
Gelatinases			
MMP-2/Gelatinase A	Decorin [13]	Bioavailable TGFβ	Gelatin, elastin, fibronectin, collagen I/IV/V/VII/X/XI, laminin, aggrecan, vitronectin
	Pro-TGF-B2 [20•]	TGF-β2	
	Pro- IL1-B [21]	Active IL1-β	
	MCP-3 [36••]	Inactive chemoattractant	
	IGFBP-3/5	Bioavailable IGF	
	ProTNFα	TNFα	
	FGF-R1 [23]	Bioactive FGF-R1 ectodomain	
	Pro-MMP-1,2,13	MMP-1,2,13	
MMP-9/Gelatinase B	Unknown [37••]	Bioavailable VEGF	
	Pro-TGF-B2 [20•]	TGF-β2	
	Pro-IL1-B [21]	IL1-β	
	Cell-surface bound IL-2Ra [22•]	Release of IL-2Rα	
	Plasminogen [60,61•]	Angiostatin	
	α1-proteinase inhibitor [66•]	Inactive serpin	
	Pro-TNFα	TNFα	
Membrane associated			
MMP-14/MT1-MMP	Pro-MMP-2,13	MMP-2,13	Gelatin, fibronectin, vitronectin, collagen, aggregan
	Cell-surface bound CD44 [67]	Release of CD44	
	Cell-surface bound tissue transglutaminase (tTG) [68]	Release of tTG	
MMP-15/MT2-MMP	Cell surface bound tTG [68]	Release of tTG	
MMP-16/MT3-MMP	Pro-MMP-2	MMP-2	
	Cell surface bound tTG [68]	Release of tTG	
MMP-24/MT5-MMP	Pro-MMP-2 [69,70]	MMP-2	
MMP-17/MT4-MMP	Pro-MMP-2 [71]	MMP-2	Gelatin [71]
MMP-25/MT6-MMP	Pro-MMP-2 [72]	MMP-2	Gelatin, collagen IV, fibrin, fibronectin, Laminin-1 [73]
MMP-23			Gelatin [74]
Other MMPs			
MMP-12/ Metalloelastase	Plasminogen [57,58]	Angiostatin	Elastin, fibronectin, fibrin/fibrinogen, laminin, proteoglycan,
MMP-19/RASI			Gelatin, tenascin, fibronectin, Collagen IV, laminin, entactin, Fibrin/fibrinogen, aggrecan, COMP [75,76]
MMP-20/Enamelysin			Amelogenin, Aggrecan, COMP [74]
MMP-28/Epilysin			

Table 2.6.1 This table lists all of the mammalian MMPs, along with their matrix substrates and non-traditional substrates with the resultant effector following proteolysis noted [89].

MMP-9, a 92 kDa gelatinase B can degrade ECM components with high specific activity for denatured collagens. MMP-9 can cleave type IV, V, XI of native collagen and elastin [93]. MPP-9 has a restricted pattern of expression, varied from

development to maturity and it is frequently expressed at sites of active tissue remodeling and neovascularization [93, 94]. Mice carried the MMP-9 mutated gene displayed a delay in endochondral ossification with an abnormal phenotype of skeletal growth plate vascularization. This was consistent with the *in vitro* finding that growth plates (hypertrophic cartilage) from MMP-9 null mice in culture show a delayed release of an angiogenic activator [94]. Several angiogenic molecules secreted by hypertrophic chondrocytes in culture have also been reported, including transferrin and a 120 kDa factor that is up-regulated by vitamin D [95]. Taken together from these observations, it suggested that the phenotype causes by a dysregulation of the MMP-9 expression in animal (growth plate), may explain the important function of MMP-9 in proteolysis of the ECM, releasing stored growth/signaling molecules both at the early of the developmental stage and later at the maturity.

2.7 *In vitro* model for study dental pulp angiogenesis

Two dimensional (2-D) or monolayer cell culture has been established and practicing for more than 10 decades. 2-D culture of hDPCs by which pulp cells are grown under controlled conditions with various treatments of different growth/differentiation factors could induce hDPCs to express typical markers of differentiation [4]. For example, osteo/odontogenic and neurogenic induction media could induce dental pulp stem cells to express markers of their lineage specific differentiation, such as ALP/BSP/OCN/RUNX2/DSP/DMP1 and Nestin/β3 tubulin, respectively [5, 14]. Although, 2-D culture system shown to facilitate pulp cells to express the markers of interest, this culture system is only inadequate representations in 2-D environment, which was different from the biological of the dental pulp tissue. Therefore, the correlation of biomarkers from 2D cultures to *in vivo* clinical scenarios

has been questioned. It has been known that there are speculations that the intracellular signaling pathways that direct the odontoblast polarity and determine the ability of cells to initiate odontogenic expression were orchestrated differently in 2D and 3D culture system [96, 97]. Moreover, the contact interaction between the odontoblast and the dentin matrix is required for these cells to maintain their phenotypic morphology and secretory activity.

As mentioned in the above review in **2.5.3**, iloprost involved in the vascularization, and capable of promoting the angiogenic genes expression in several systems. However, there is a lack of evidence demonstrating the efficacy of iloprost in promoting angiogenesis of the dental pulp tissue in human; therefore more studies are needed to elucidate the prognostic value of iloprost in promoting angiogenesis of the human dental pulp tissue. Equally important, it is necessary to establish the culture model that can reflect the multicellular microenvironment similar to the human pulp-dentin complex *in vivo*.

2.8 Tooth slice 3-D organ culture

To date, many experimental observations have repeatedly demonstrated about the usefulness of human tooth slice model in dental pulp tissue engineering [11, 97-100]. The developments of this technique have highlighted some of the shortcomings of 2D cell culture, including the maintenance of cell-cell and cell-ECM signals. Moreover, utilization of tooth slice organ culture allowed investigators to study the close relation of pulp-dentin complex over a long period of time, as well as to evaluate the angiogenesis-based therapeutic strategies for promoting angiogenesis of human dental pulps [11, 97-100]. For this reason, tooth slice organ culture became a useful tool for *in vitro* study of pulp-dentin complex. By adapting of the tooth slice

culture method [16, 80] with some modifications, the tooth slice 3-D organ culture system was developed in this study to evaluate the angiogenic potency of iloprost in promoting angiogenesis of the human dental pulp tissue. Moreover, this system will allow us to study the physiological and histological change of pulp-dentin complex over a range of time dependent fashion.

CHAPTER III

RESEARCH METHODOLOGY

Patient selection and sample collection

Human impacted third molars

Sample

Human tooth slices from the non-carious teeth.

Ethical considerations

Human impacted third molars

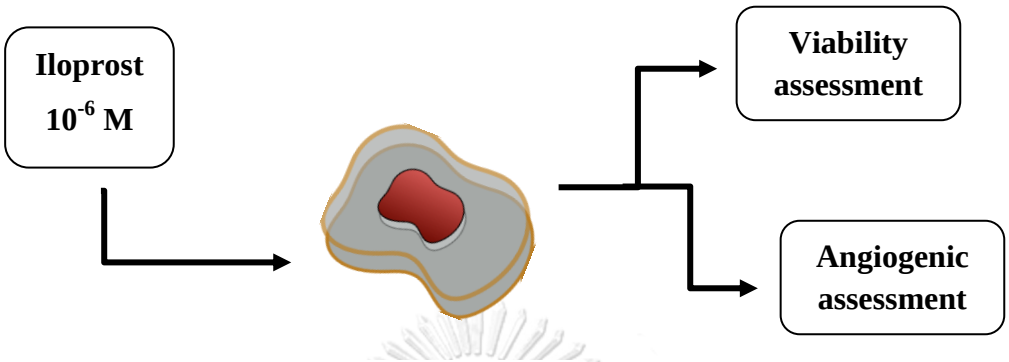
1. The study protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (Protocol HREC-DCU 2015-097).
2. The study process causes no harm to the patients as the teeth used in the study are the impacted third molar, which was the indication for tooth extraction in orthodontic procedure.
3. The legal guardians were informed about the details of the study process before signing the consent forms.

Inclusion criteria

Impacted human third molar from the non-carious permanent teeth of

- healthy adult patients (age 18-30 years)
- adult with no systemic disease
- adult with no local anesthetic or drug allergy

Experimental design



MATERIALS AND METHODS

3.1 Tooth slice preparation

Human intact impacted third molars were collected from healthy adult patients (age 18–30, under local anaesthetic) in the Department of Oral and Maxillofacial Surgery at the Faculty of Dentistry, Chulalongkorn University. Immediately after extraction, the teeth were stored on ice in DMEM containing 10% fetal bovine serum, 2 mM L-Glutamine (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 mg/ml streptomycin. The 2 mm thick tooth slices were prepared as describe previously with some modifications [83-84]. Freshly extracted molar will be transversally cut at the cemento-enamel junction with a diamond saws. To preserve the pulp tissue inside the pulp chamber, lowspeed cutting machine was operated at the lowest speed under cooling with sterilized PBS supplement with 1% antibiotic. One or, in some instances, 2 slices were obtained from each tooth. In addition, there were some instances in which more than 1 tooth will be collected from the same person. However, to minimize bias and to ensure the independence of specimens, tooth slices obtained from the same tooth, or from the same person, will not be included in the same experimental group.

3.2 Tooth slice culture

Immediately after cut, the tooth slices were washed 3 times with sterilized PBS, 2 times with SFM and cultured in SFM (Figure 4.1.1). The tooth slices were randomized and cultured for 1 and 3 days in a 12-well culture plate and maintained in a standard incubator of 37 °C in a humidified atmosphere containing 5% CO_2. The slices were cultured without (control) or with iloprost (10^{-6} M) for 1 or 3 days. The

10^{-6} M concentration was chosen as the most effective dose base on the previous finding [2]. The culture media were replaced every 2 days, together with iloprost added for treatment group. Freshly cut tooth slices were used as baseline control in morphological study (figure 4.1.1).

In some experiments, tooth-slices were pretreated with 10 μM of a Protein Kinase A (PKA) inhibitor (cAMPS-Rp, TOCRIS, Bristol, UK) for 30 mins prior to iloprost stimulation.

3.3 Histology and imagine

3.3.1 Immunohistochemistry and immunofluorescent staining

After 1 or 3 days of culture, the tooth-slices were fixed in 10% neutral buffered formalin (Surgipath® Leica, IL). Freshly cut tooth-slices were used as non-cultured controls. The samples were rinsed and demineralized in hydrochloric acid decalcification solution (Decalcifier II®, Leica, IL). The specimens were dehydrated in a graded ethanol series and embedded in paraffin. Serial paraffin-sections 2-μm thick were obtained from the tooth-slice samples using a tissue microtome (Leica RM2255, Germany).

Heat-induced epitope retrieval was performed using 10 mM sodium citrate buffer at pH 6.0 at 100 °C prior to the antibodies treatment. The immunohistochemistry staining was performed as described previously [27]. Briefly, after antigen retrieval, the sections were rinsed in distilled water and treated with methanol containing 0.4% hydrogen peroxide for 15 mins to inhibit endogenous peroxidase. The sections were rinsed and incubated in 5% bovine serum albumin (w/v) for 2 hrs at room temperature. The sections were then incubated with rabbit

polyclonal anti-vWF (1:500) (ab6994, Abcam, UK), rabbit polyclonal anti-VEGF (1:200) (Dako, Glostrup, Denmark), or mouse monoclonal anti-COL1 (1:500) (ab6308, Abcam, UK), at 4 ^{0}C overnight. After washing in PBS, the sections were incubated for 1 hr with peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark), and developed using 3–30 diaminobenzidine (Sigma-Aldrich) for 5 mins. The sections were counterstained with hematoxylin. For immunofluorescence staining, sections were incubated with primary antibodies and subsequently incubated with FITC- or Rhodamine-conjugated secondary antibody (1:250). The sections were counterstained with 4′,6-Diamidino-2-phenylindole (DAPI). Isotype antibodies were used as the control.

The specimens were examined using a light microscope equipped with a motorized stage focus control system, and a color digital video camera with a microcomputer (Carl Zeiss, Germany). The quantification of the immuno-positive cells and microvessel density was performed using ImageJ software [101]. The immunofluorescent stained sections were examined using a fluorescence microscope (Zeiss Axio Observer Z1, Germany). Quantification of the COL1 intensity was performed using the ImageJ (National Institutes of Health, Bethesda, MD) software program.

3.3.2 Live and dead cell immunofluorescent staining

After 1 and 3 days of culture, the tooth slices were immersed in 1 mL of freshly prepared live/dead staining solution containing 10 mM calcein-AM (CaAM) (Life Technologies,CA) and 1 mM EthHD-1 (Life Technologies, CA), and incubated for 1 hr at 37°C. After incubation, the tooth-slices were transferred to a fresh plate, washed

with PBS and were immediately observed using a LSM 800 confocal microscope system (Carl Zeiss, Germany).

To visualise CaAM (green fluorochromes), the 488-nm wave length of the Argon laser, a main beam splitter 488/543, a beam splitter 545 nm, and a band pass 505–530 nm were used to document the first channel.

To visualise EthHD-1 (red fluorochromes), the 543-nm wave length of HeNe1 laser, a main beam splitter 488/543, a beam splitter 545 nm, and a long pass 585 nm were used on the second channel.

The number of Live- (CaAM) and dead- (EthHD-1) labeled cell in each sample were documented from random fields within the pulp tissue. Digital Z-stack images from three layers per field with a total of three fields from each sample were quantified. The quantification of live and dead cell was analysed using ImageJ software. The cell viability was expressed as a ratio of live cell to a total cell number. Freshly cut tooth-slices were used as baseline control for the live/dead cell staining.

3.3.3 Histological analysis

At the end of each experimental time point, the tooth slice samples were collected and prepared for paraffin embedding. The serial paraffin-sectioned of 2-μm were obtained using tissue microtome. The sections were then received either hematoxylin-eosin (H&E) or Masson’s trichrome staining for light microscope analysis. Freshly cut tooth slice were used as baseline control for tissue morphology. The immunohistological analysis method is indicated as show in figure 3.1.

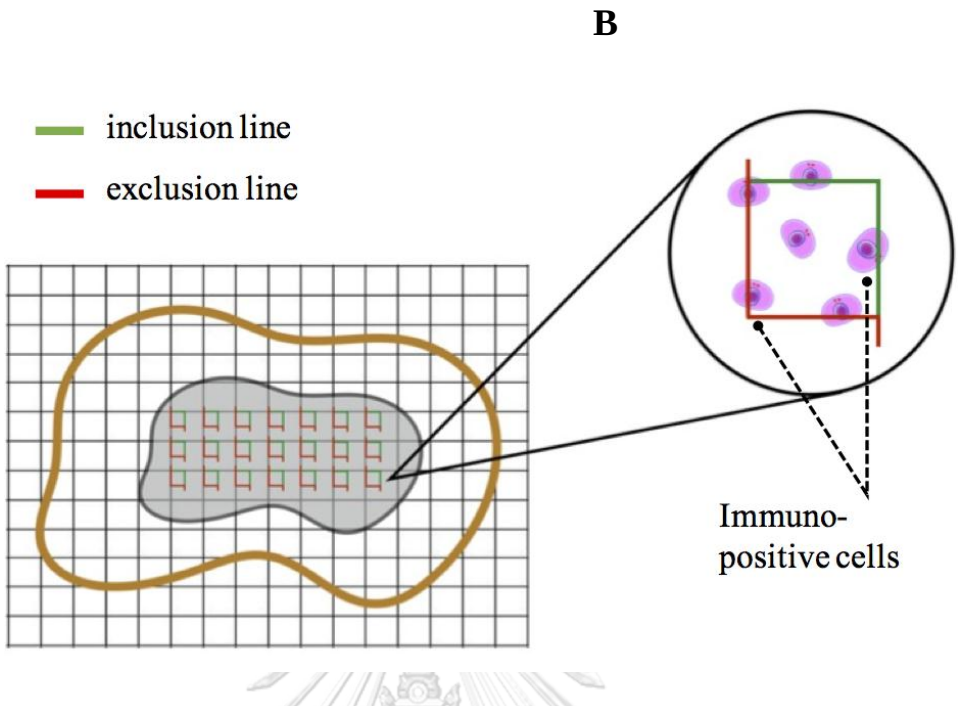


Figure 3.1 Stereological counting in the pulp tissue. (A) The area corresponding to the pulp tissue superimpose with rectangular grid that consists of grid areas. The grid defines the positions of counting frames (50 µm x 50 µm). (B) A high magnification image of the section superimpose with a counting frame (50 µm x 50 µm) consisting of two inclusion lines (green) and two exclusion lines (red).

3.4 Human dental pulp cells isolation and culture

The isolation of the hDPCs protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (Protocol number HREC-DCU 2015-097). Impacted human third molars without lesion were obtained from 3 healthy consenting patients (age 18-30) in the Department of Oral Surgery at the Faculty of Dentistry, Chulalongkorn University. Pulp tissue was isolated and cultured in a 3.5mm culture dish in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, MA, USA) containing 10% fetal bovine serum (FBS), 2mM L-Glutamine (Invitrogen,

Carlsbad, CA, USA) and antibiotics (100units/ml penicillin and 100mg/ml streptomycin). Cells were maintained at 370C in a humidified atmosphere containing 5% CO_2. The HDPCs from passages 3-6 were used in the study.

hDPCs (3 x 10^5 cells) were seeded in a 6-well plates and maintained in normal growth medium for 24 h. The cells were then cultured in serum-free medium and subsequently treated with 10^{-6} M iloprost (Ilomedin; Bayer AG, Germany). This selected concentration was based on previous findings (Limjeerajarus, Osathanon, et al., 2014). The culture media were replaced every 2 days, together with iloprost added for treatment group. Cell viability was determined using an MTT assay (Sigma Aldreich, USA). The mRNA expression was determined by real-time quantitative polymerase chain reaction (qPCR). The culture medium was collected and evaluated for MMP-9 protein secretion using ELISA after 24 h of iloprost treatment.

3.5 Inhibitors treatment

hDPCs were pre-treated for 30 minutes prior to iloprost treatment with the following chemical inhibitors; a prostacyclin receptor (IP) antagonist CAY10449 (Cayman, MI, USA), a protein kinase A (PKA) inhibitor (Calbiochem, Darmstadt, Germany), or forskolin (Santa Cruz, TX, USA). The concentration of the inhibitors and activator was 3nM, 10 μM, and 10 μM for IP-receptors antagonist, PKA-inhibitor and forskolin, respectively (Limjeerajarus, Osathanon, et al., 2014; Manokawinchoke, Pimkhaokhum, Everts, & Pavasant, 2014).

3.6 Scratch-test assay (wound healing assay)

The hDPCs were seeded in a 12-well at a density of 37,500 cells/cm^2 and cultured in DMEM containing 10% FBS for 24 h. Cells were starved in serum free

media (SFM) for 24 h prior to the treatment. The wound field was created on a confluent monolayer cells with a sterile 200µl pipette tip. The cell debris was washed away 2x with SFM and the cells were cultured at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. The cells were treated with iloprost(10^{-6} M) for 72 h. The wound field was measured at 0 h, 24 h and 72 h using inverted microscope equipped with digital camera. The wound closure was analyzed using the image analysis software, Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

3.7 Real-time qPCR analysis and enzyme-linked immunosorbent assay (ELISA)

RNA isolation was performed using a commercial RNeasy Plus Mini Kit (QIAGEN, Hileden, Germany) according to the manufacturer's instruction. One microgram of each RNA sample was converted to cDNA by avian myeloblastosis virus reverse transcriptase, ImProm- II (Promega, Madison, WI, USA). Absorbance ration measures using a NanoDrop spectrophotometer (NanoDrop ND-2000, Thermo Scientific) were used to access the purity of the nucleic acid samples with a 260/280 ratio of ~1.8 and ~2.0 were considered as a purified DNA and RNA respectively.

The oligonucleotide sequences of the realtime-PCR primers for matrix metalloproteinase (MMP) -1, -2, -9, tissue inhibitor of metalloproteinase (TIMP) -1, -2, -3, focal adhesion kinase (FAK), and collagen 1 (COL1) are listed in table 3. Real-time quantitative PCR (qPCR), the amplification of the cDNA template was performed using a SYBR Green I Master kit (Roche Diagnostic, USA) on CFX Connect™ Real–Time PCR Detection System (Bio-Rad, Hercules, CA). The amplification profile was 95 ^{o}C/10 s, 60 ^{o}C /10 s, and 72 ^{o}C /20 s for 45 cycles. Gene

expressions were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The culture medium was collected and assayed to quantify the MMP-9 protein levels using ELISA kits (R&D Systems, MN, USA) according to the manufacturer instruction.

3.8 Statistical analysis

The data are presented as mean ± standard deviation. The data are shown as mean ± SD. Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances will be used in all experiments. The Student's t-test for independent samples was employed for two group comparison. One-way ANOVA with post-hoc Tukey LSD test was used to compare three or more groups. The statistical analyses were performed using SPSS v.21 (IBM, New York, USA) at a significance level of 0.05. The microvessel density, VEGF and COL1 quantification results were evaluated non-parametrically using the Kruskal Wallis test followed by the Mann Whitney's test for comparison between the groups. The data that does not achieve normality will be analyzed using non-parametric methods. For non-parametric tests will employed using the Kruskal Wallis test followed by the Steel-Dwass test for comparison of the changes in the treatment groups in relation to the control. Differences at $p < 0.05$ were considered to be significant.

Table 3 The oligonucleotide sequences of realtime-PCR primers

Gene	Accession no.	Primer sequence	Size (bp)
GAPDH	NM_001289746.1	Forward: 5'-CACTGCCAACGTGTCAGTGGTG-3' Reverse: 5'-GTAGCCCAGGATGCCCTTGAG-3'	121
COL1	NM_000088.3	Forward: 5'-GTGCTAAAGGTGCCAATGGT-3' Reverse: 5'-ACCAGGTTCACCGCTGTTAC-3'	128
FAK	NH_004967.3	Forward: 5'-CAATCCCACACATCTTGCTGA-3' Reverse: 5'-AGCCGGCAGTACCCATCTATT-3'	186
MMP-1	NM_002421.3	Forward: 5'-AGAGAGCAGCTTCAGTGACA-3' Reverse: 5'-CTTGAGCTGCTTTTCCTCCG-3'	87
MMP-2	NM_004530.5	Forward: 5'-TTGACGGTAAGGACGGACTC-3' Reverse: 5'-ACTTGCAGTACTCCCCATCG-3'	153
MMP-9	NM_004994.2	Forward: 5'-TTTGACAGCGACAAGAAGTG-3' Reverse: 5'-CAGGGCGAGGACCATAGAGG-3'	208
TIMP-1	NM_003254.2	Forward: 5'-ACTTCCACAGGTCCCACAAC-3' Reverse: 5'-GCATTCCTCACAGCCAACAG-3'	167
TIMP-2	NM_003255.4	Forward: 5'-CGACATTTATGGCAACCCT-3' Reverse: 5'-ATTCCTTCTTTCCTCCAACG-3'	152
TIMP-3	NM_000362.4	Forward: 5'-CTTCCGAGAGTCTCTGTGGCCTT-3' Reverse: 5'-CTCGTTCTTGGAAGTCACAAAGC-3'	230

CHAPTER IV

RESULTS

4.1 The tooth-slice in 3D organ culture was kept viable in a serum free condition

The tooth slices with intact pulp tissue were kept viable in culture for 3 days (figure 4.1.2, 4.1.3). The odontoblast layer along the dentin wall remained intact and appeared as tall, columnar cells with continuous processes into the dentinal tubules. Blood vessels were observed with a continuous endothelial layer at the boundary between the lumen and the vessel wall (figure 4.1.2, 4.1.3). After 3 days in culture, no changes were observed in either cell or tissue morphology (figure 4.1.3, 4.1.4). The viability assay indicated that the number of live and dead cells from day 1 and day 3 was not different to that of the non-cultured freshly obtained control tooth-slices (figure 4.1.5).

A

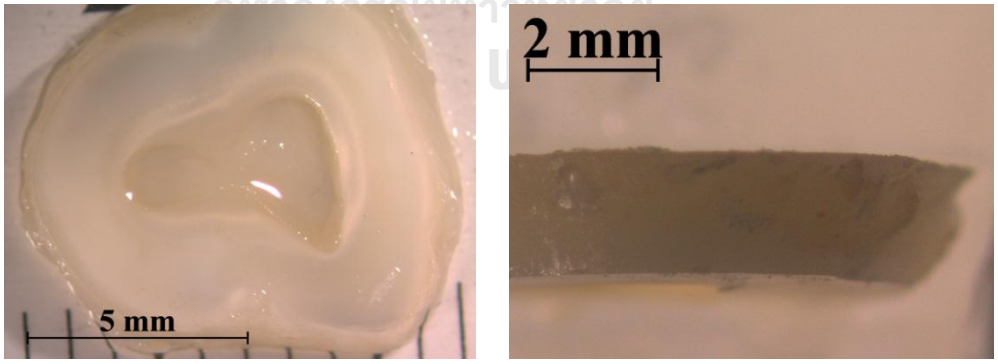


Figure 4.1.1 Tooth slice morphology and dimension after the initial preparation, but before culture. Freshly cut 2 mm thick tooth slice with intact pulp tissue were prepared from an extracted human third molar.

B

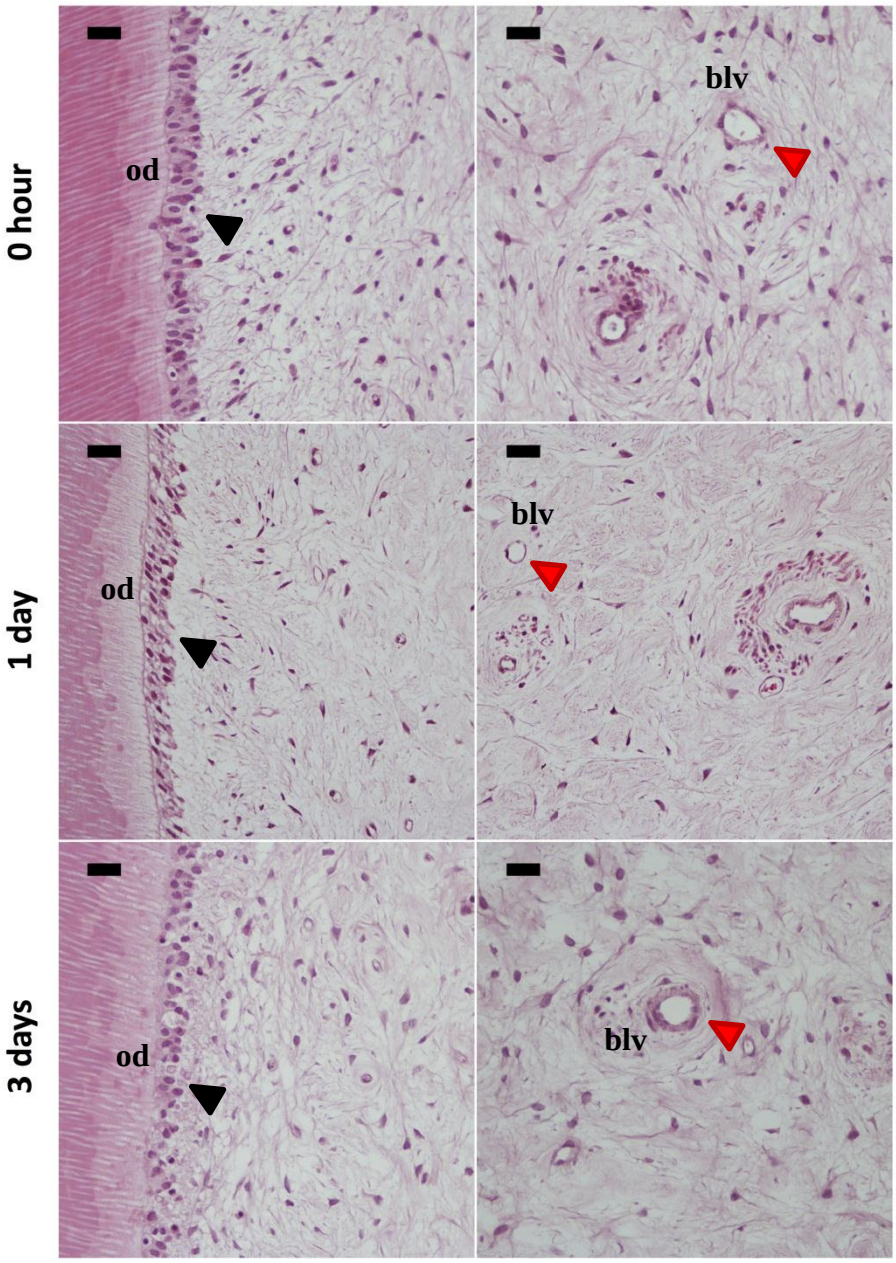


Figure 4.1.2 Histological sections of the human tooth slice before and after cultured at day 1 and day 3 (stained with hematoxylin and eosin). The dark and the red arrow heads are point to the odontoblast layer and the blood vessel, respectively. Scale bar = 20 µm. od, odontoblast; blv, blood vessel.

C

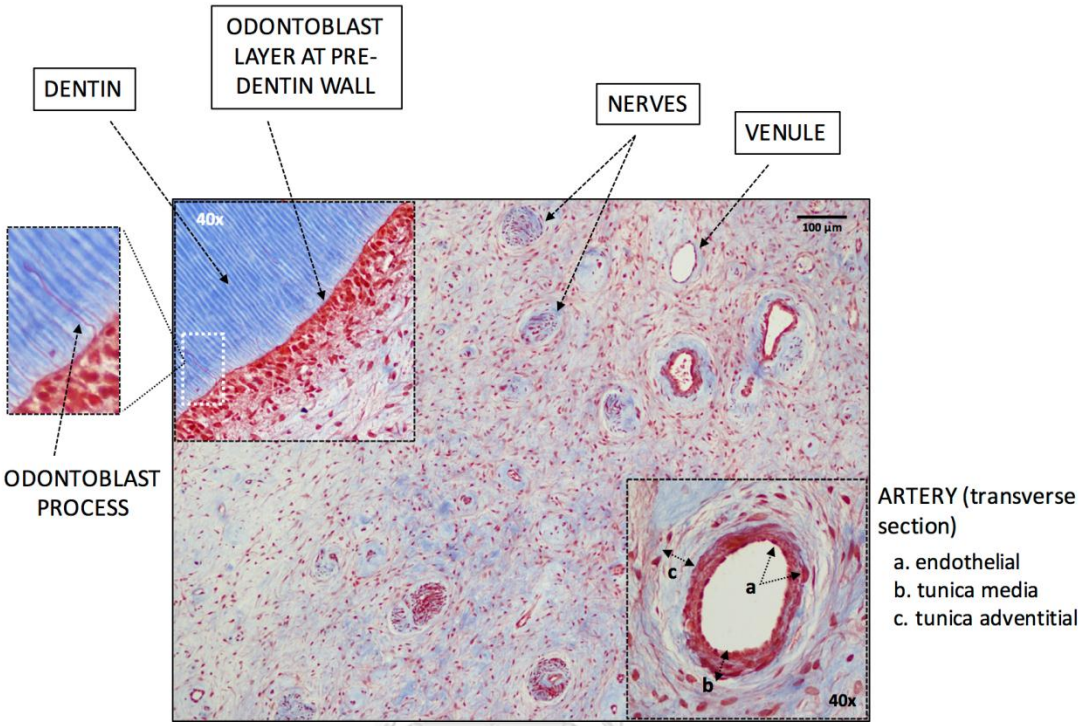


Figure 4.1.3 Histological sections of the human tooth slice at 3 days culture. Stained with Masson's trichrome staining.

D

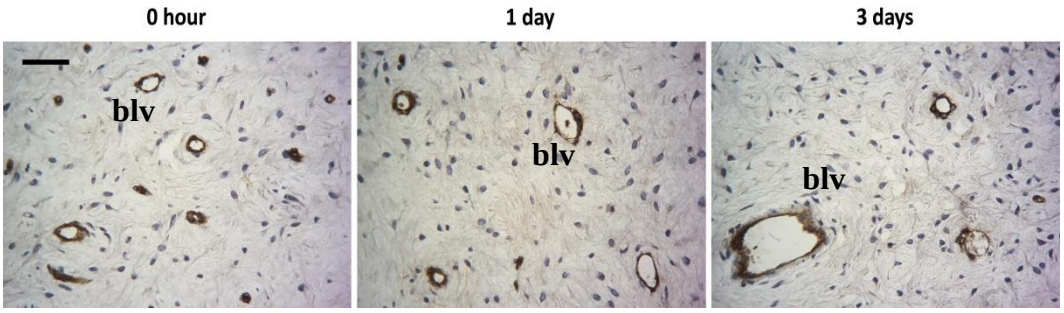


Figure 4.1.4 Immunohistochimical staining of vWF were performed to identify the present of the endothelial cells and the blood vessels (brown) in human tooth slice. Scale bar = 50 µm. blv, blood vessel.

E **F**

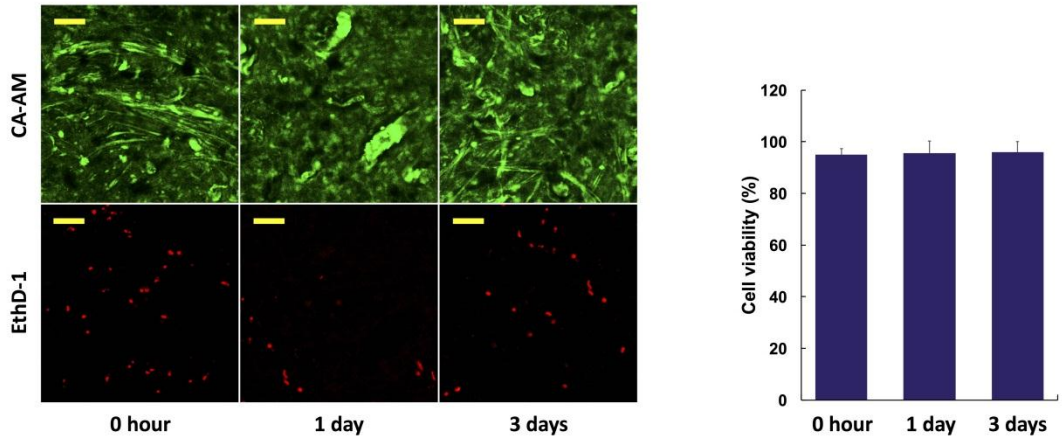


Figure 4.1.5 Live and dead cell confocal microscopy analysis of dental pulp tissue viability were performed at day 1 and day 3 (E). The green represents Calcein AM (live) and the red represents EthD-1(dead). Scale bar = 50 µm. The quantification graph of live/dead cells (n=3) (F).

4.2 Iloprost promoted dental pulp angiogenesis

To evaluate the angiogenic potential of iloprost on dental pulp tissues, the tooth slices were cultured with iloprost at a concentration of 10^{-6} mol/L. Immunofluorescent staining using anti-vWF demonstrated a significant increase in microvessel density in the iloprost-treated group compared with the untreated control group. This was apparent at both after day 1 and day 3 ($P < 0.05$ and $P < 0.001$, respectively) (figure 4.2.1). Iloprost significantly enhanced VEGF expression as shown by the increased number of VEGF-positive cells after 1 and 3 days in culture in a time-dependent manner ($P < 0.001$) (figure 4.2.2).

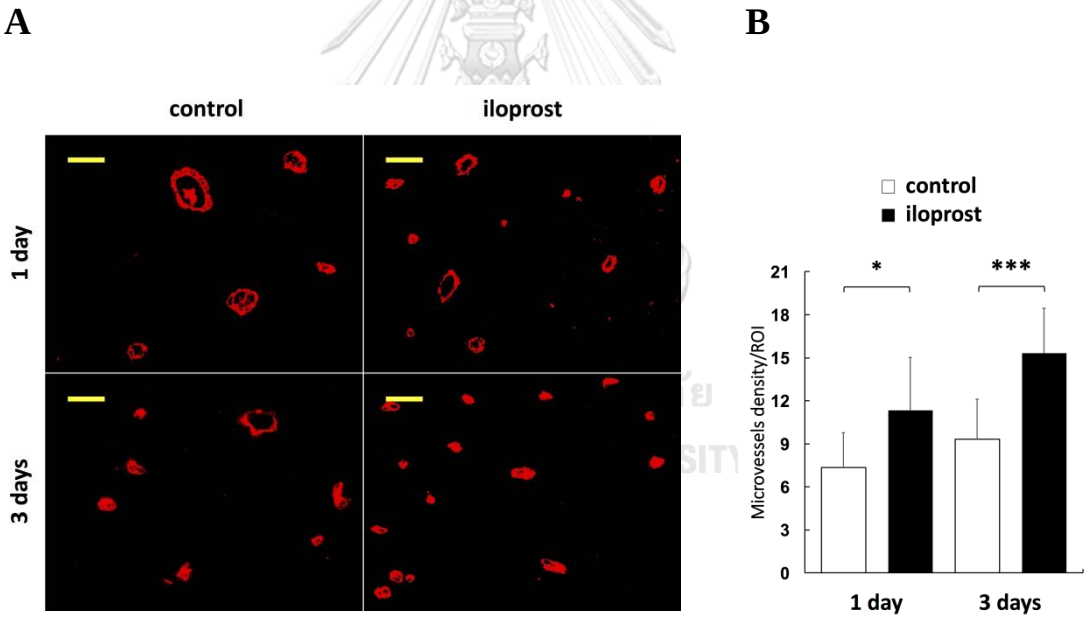


Figure 4.2.1 . Dental pulp tissue microvessel density in the tooth slice culture is shown by immunofluorescent staining of anti-vWF (red) (scale bar = 50 µm) (A). Quantitative measurement of vWF-positive vessels density is shown as mean ± standard deviation (n = 3) (*P < 0.05 and ***P < 0.001) (B). ROI, region of interest.

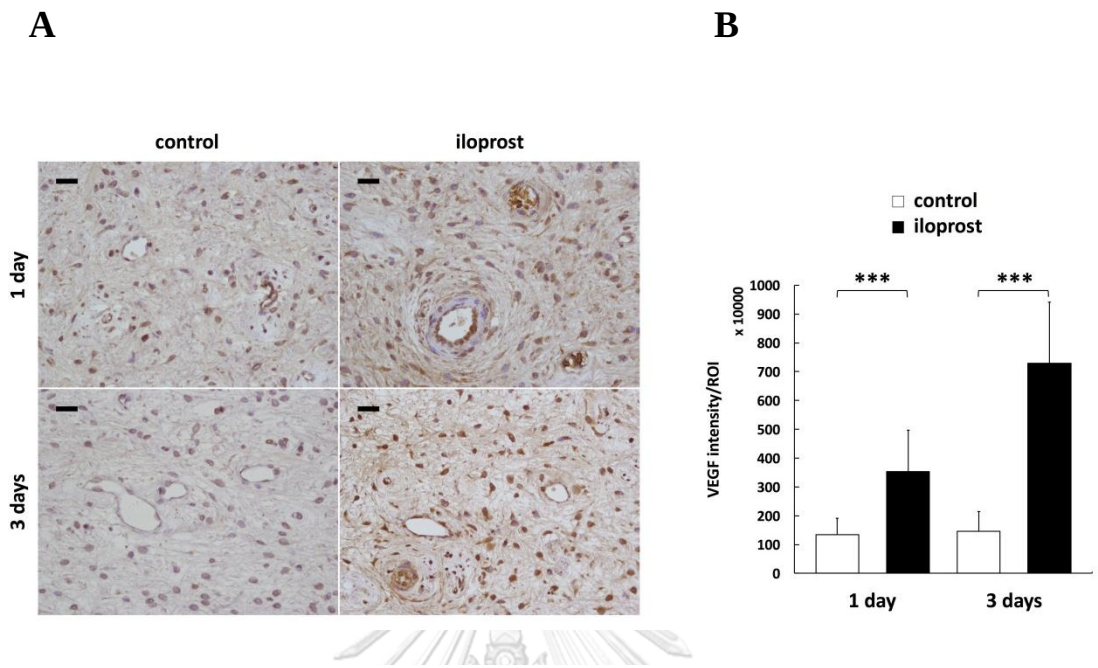


Figure 4.2.2 Effect of iloprost on VEGF expression as demonstrated by immuno-histochemical staining in the tooth slice culture system (scale bar = 20 μm) (A). Quantification of VEGF intensity is shown as mean standard ± deviation (B), n = 3, (***P < 0.001).

4.3 Iloprost stimulated collagen synthesis

The most abundant extracellular matrix component is collagen type I. Iloprost induced collagen deposition as shown by Masson's trichrome and COL1 immunofluorescent staining. The tooth slices cultured with iloprost demonstrated more intense collagen staining compared with the controls at both 1 and 3 days (figure 4.3.1 and 4.3.2). Tooth slices pretreated with the PKA inhibitor before iloprost incubation showed a reduced number of vWF-positive (figure 4.3.4) cells and reduced COL1 expression (figure 4.3.3 and 4.3.5) compared with the iloprost-treated group.

A

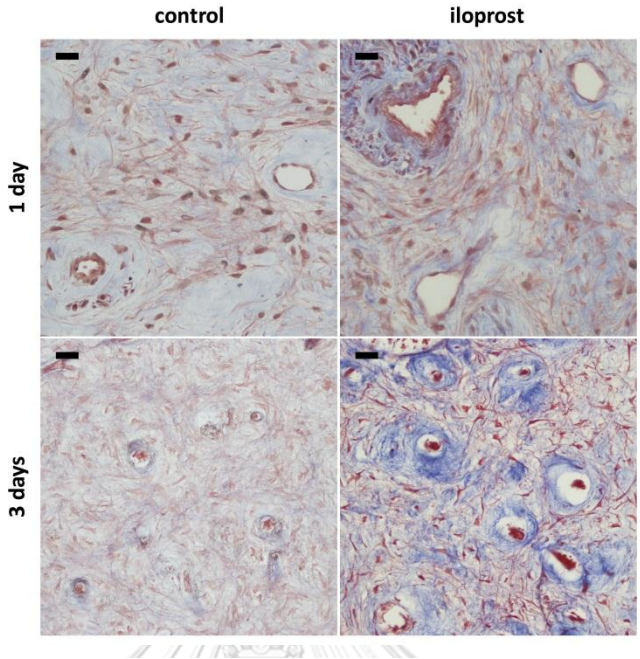


Figure 4.3.1 Effect of iloprost on COL1 expression in human dental pulp tissue in the tooth slice culture system as demonstrated by Masson's trichrome staining (Scale bar = 20 µm).

B

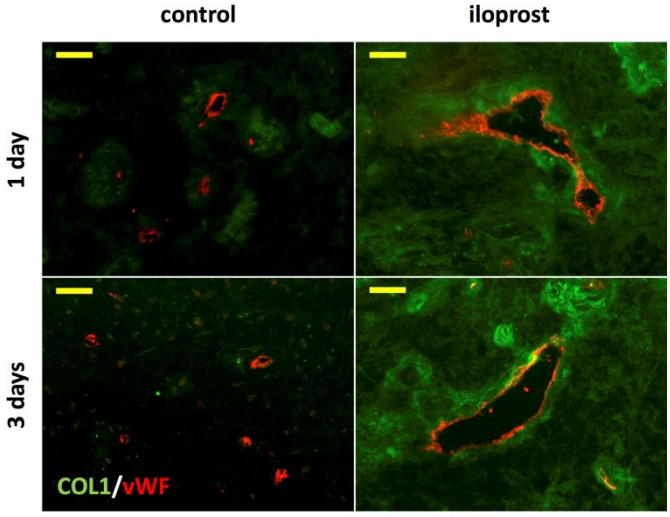


Figure 4.3.2 Effect of iloprost on COL1 expression in human dental pulp tissue is demonstrated by a double immunofluorescent staining using anti-COL1 and anti-vWF antibodies (Scale bar = 50 µm).

C

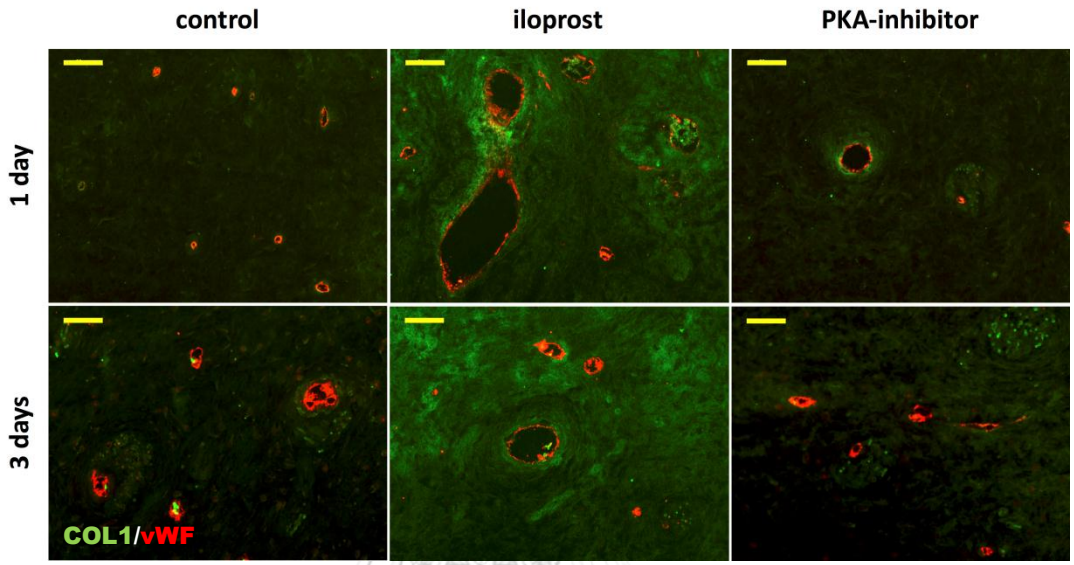


Figure 4.3.3 The inhibition effect of PKA-inhibitor on COL1 expression at day 1 and day 3 is demonstrated by a double immunofluorescent staining using anti-COL1 and anti-vWF antibodies (Scale bar = 50 µm).

D

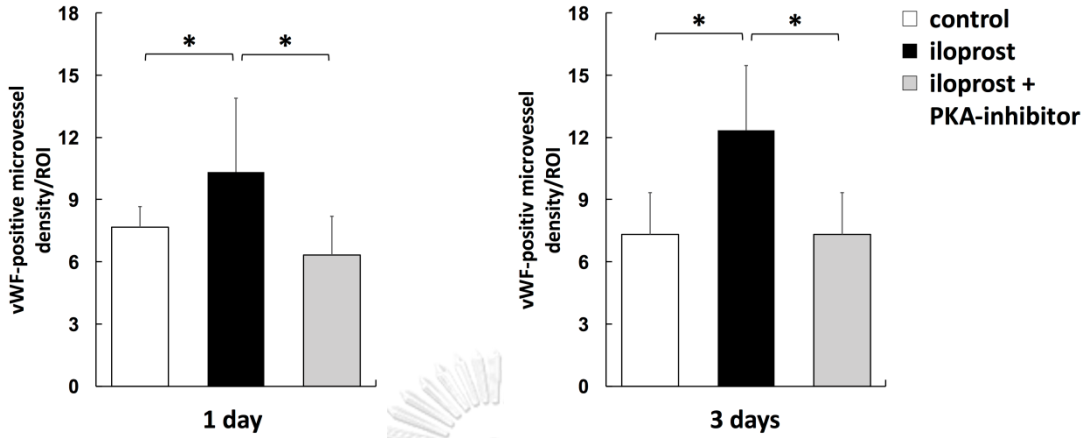


Figure 4.3.4 The inhibition effect of PKA-inhibitor on microvessel formation. Quantitative measurement of vWF-positive vessels density is shown as mean standard ± deviation, n = 3 (*P < 0.05).

E

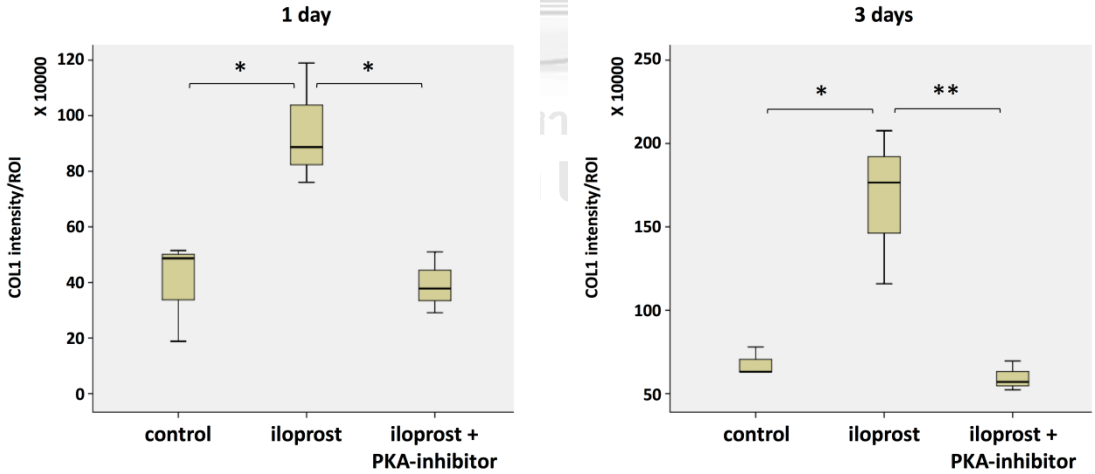


Figure 4.3.5 The inhibition effect of PKA-inhibitor on COL1 expression. Quantitative measurement of COL1 density is shown as mean standard ± deviation, n = 3 (**P < 0.001).

4.4 PGI_2 accelerated cells migration in the *in vitro* wound healing model

The effect of PGI_2 on promoting hDPCs migration was determined at 0 h, 24 h and 72 h. Iloprost significantly accelerated the migration in hDPCs compared with control as shown by the closure of the scratch area (Figure 4.4.1A and B).

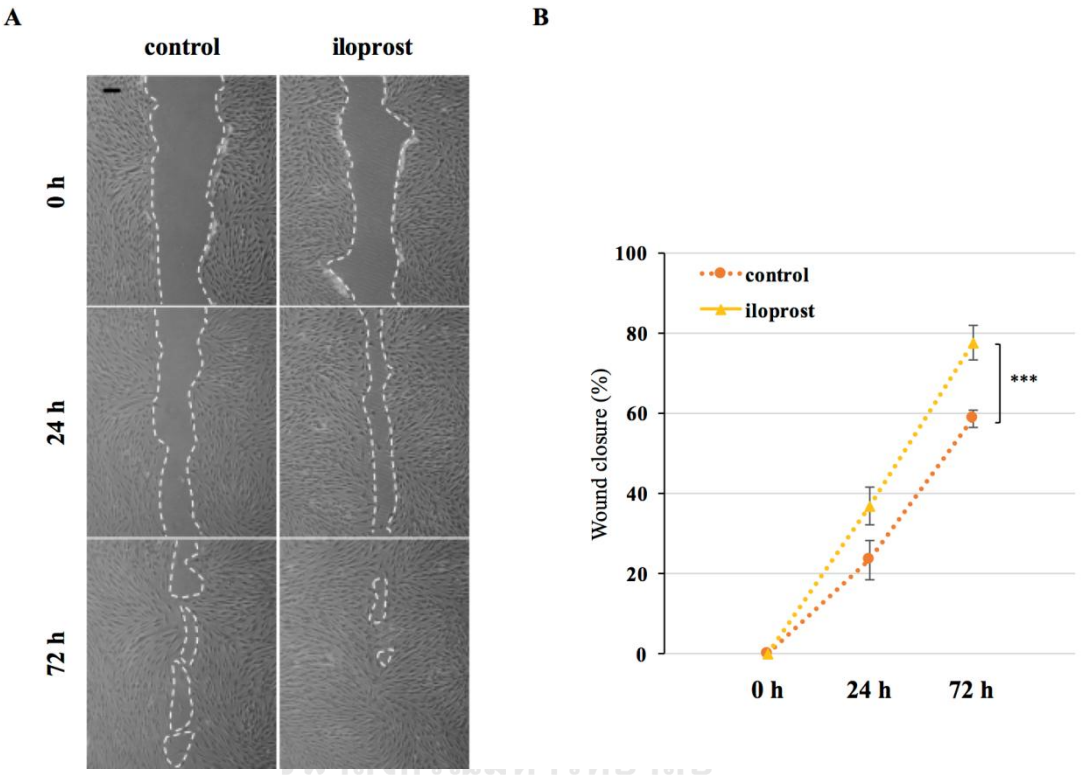


Figure 4.4.1 hDPCs migration in wound scratch-test assay was determined at 0 h, 24 h and 72 h, scale bar is 200 μm (A). The quantitive analysis of wound closure area (percentage) was shown as average ± SD (n=3). Asterisks indicate a statistically significant difference compared with the control (B).

4.5 PGI_2 up-regulated MMP-9 expression in hDPCs

The expression of some genes that can be associated with wound healing was explored. The results from qPCR showed that iloprost significantly up-regulated MMP-9 mRNA expression at 24 h while there was no difference compared with

control at 72 h (Figure 4.5.1). MMP-1 and MMP-2 expression were not affected by the treatment of iloprost. Other wound healing-related genes such as FAK, TIMP1 or COL1 were also not affected by the treatment of iloprost (Figure 4.5.1).

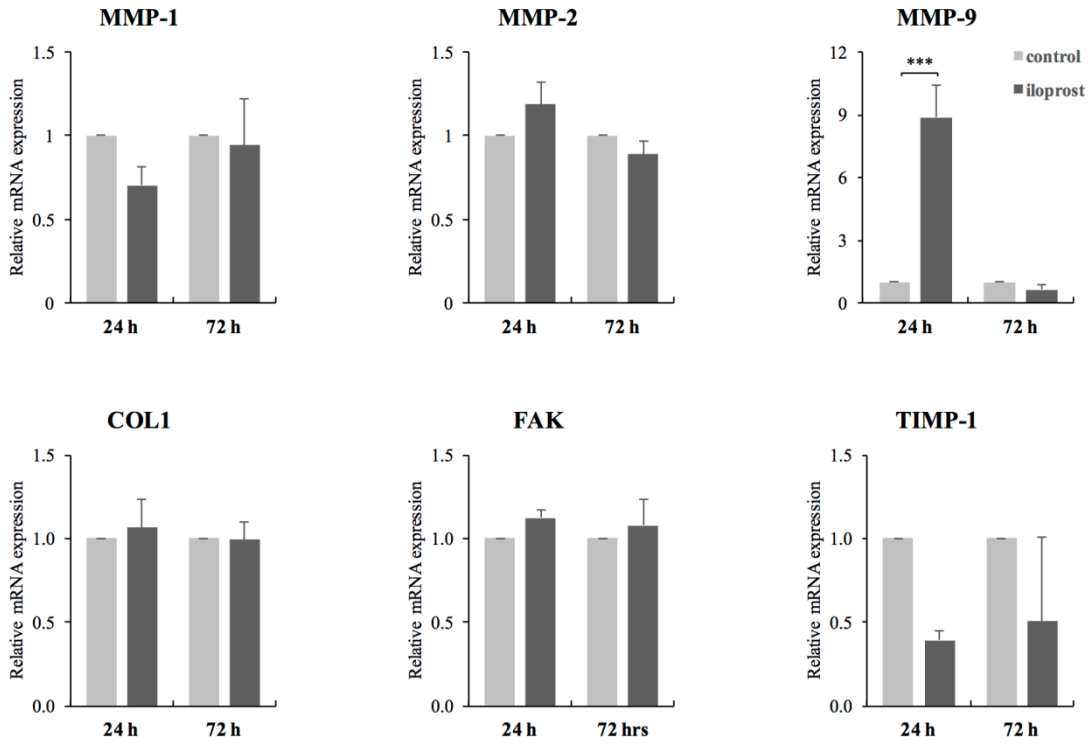


Figure 4.5.1 The mRNA expression of MMPs, COL1, FAK and TIMP-1 in hDPCs was analyzed at 24 h (n=3) (A). The data are presented as fold increase normalized to the control (no treatment) at 24 h. Bars showed the average ± SD. Asterisks indicate a statistically significant difference compared with the control.

4.6 The up-regulation of MMP-9 was directed by IP-PKA signaling pathway

To further obtain information on the mechanism underlying the iloprost induced up- regulation of MMP-9 expression, an IP antagonist and PKA inhibitor were added to the hDPCs (Figure 4.6.1A).The increased expression of MMP-9 induced by PGI_2

was abolished when the PKA-inhibitor or IP-receptor antagonist were used (Figure 4.6.1A).

Next, we analysed the actual protein expression of MMP-9 and the effect of PGI_2 in hDPCs. The increase of MMP9 protein expression was observed at 72 h, but not at 24 h (Figure 4.6.1B). Since the IP receptor was modulated by PKA signaling pathway, the effect of PKA inhibitor on hDPCs migration was also evaluated by scratch-test assay at 0 h, 24 h and 72 h. PKA inhibitor or IP-receptor antagonist attenuated the effect of PGI_2 on promoting hDPCs migration when compared with the iloprost-treated group (Figure 4.6.1C and D).

4.7 The activation of PKA reverse the effect of PGI_2-MMP9 signaling

To confirm the involvement of PKA on PGI_2-MMP9 signaling, forskolin, an agent that activates adenyl cyclase and the PKA pathway, was employed. The MTT assay in hDPCs in culture showed that forskolin in any of the concentration used did not affect the cells viability (Figure 4.7.1A). The result showed that the application of forskolin, a PKA activator, up-regulated MMP-9 in a dose-dependent manner and promoted hDPCs migration (Figure 4.7.1B, C and D), in contrast to the effect of the PKA inhibitor.

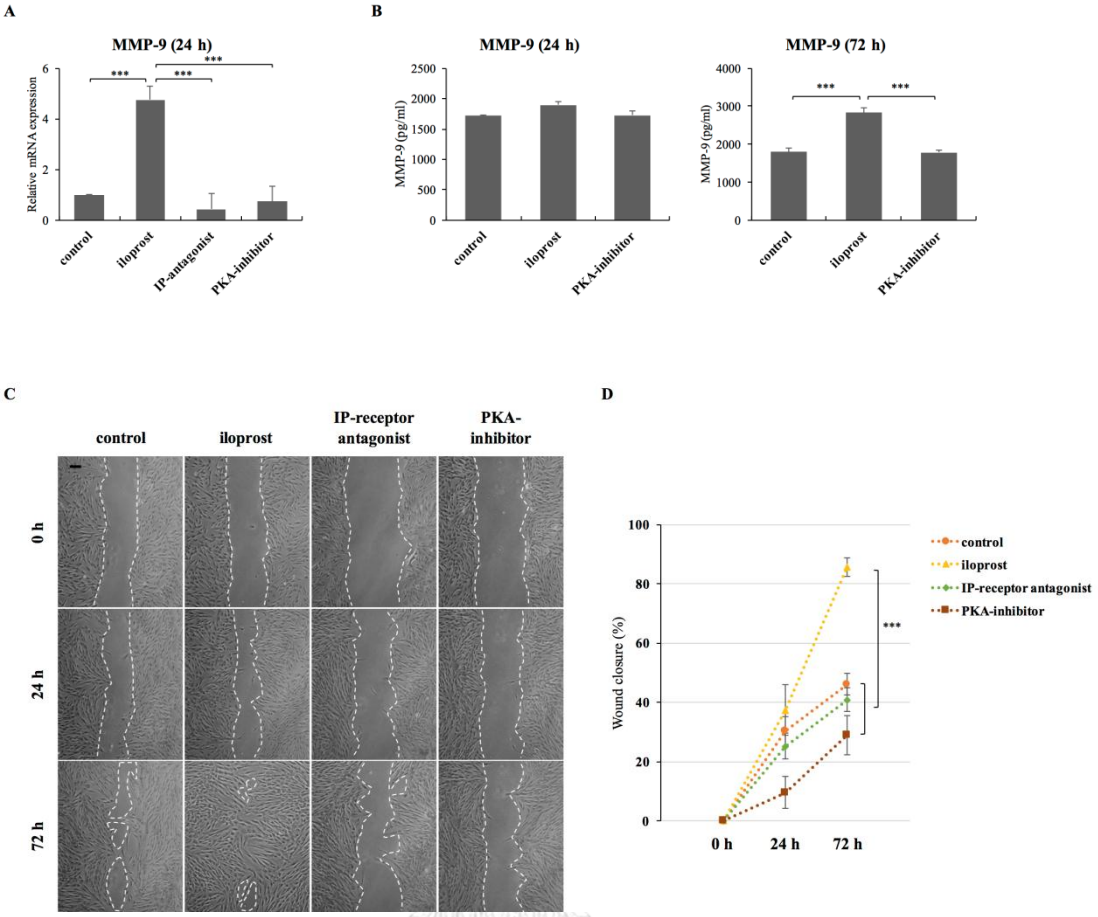


Figure 4.6.1 iloprost-induced increases MMP-9 mRNA expression was abolished by the pre-treatment of IP-receptor antagonist (CAY 10449) (A). The MMP-9 protein expression was determined by ELISA after the treatment with iloprost or pretreated with PKA-inhibitor prior to iloprost stimulation at 24 h and 72 h (B). The effect of pretreating PKA inhibitor prior to iloprost was confirmed by scratch assay at 0 h, 24 h and 72 h. The scale bar is 200 μm (C). The quantitive analysis of wound closure area (percentage) was shown as average ± SD (n=3) (D). Asterisks indicate a statistically significant difference compared with the control.

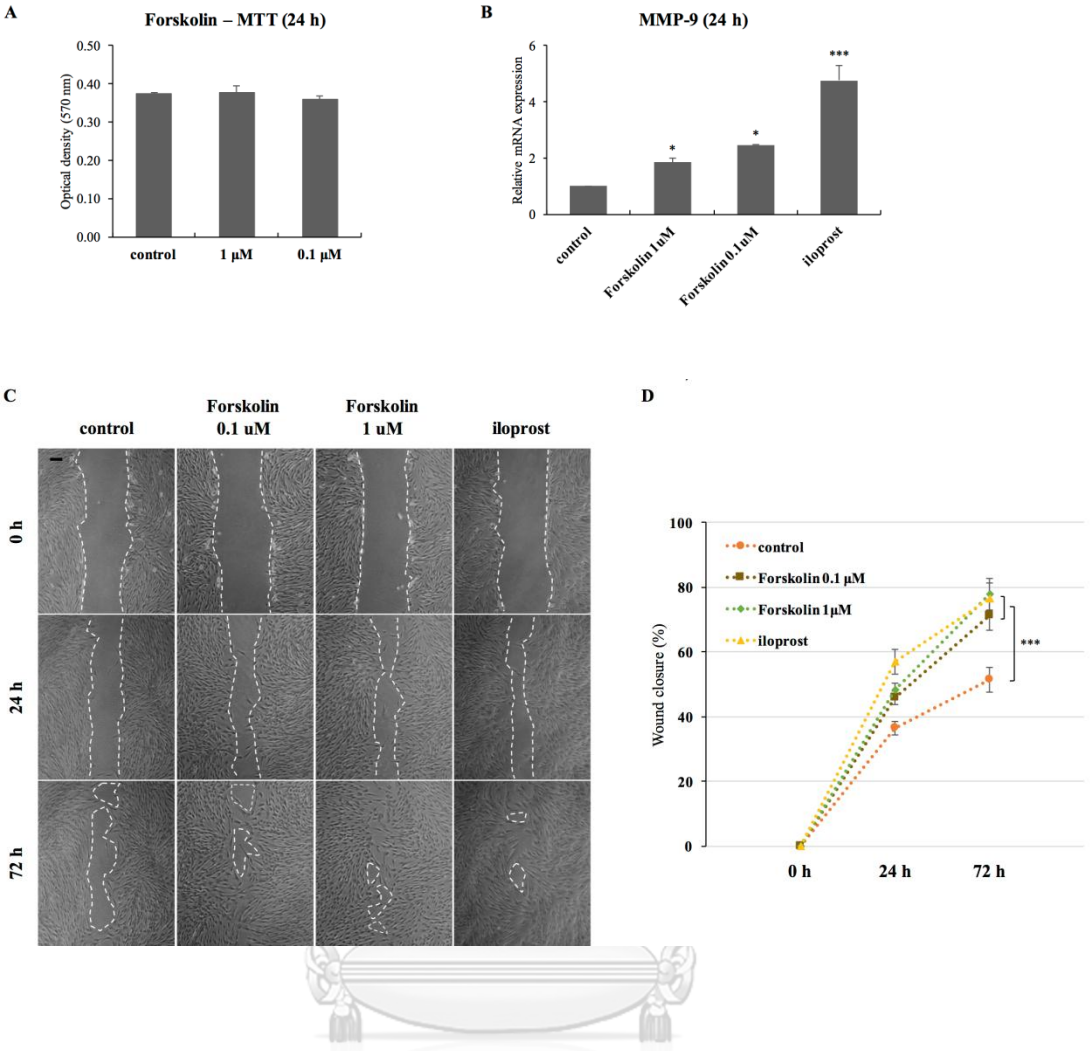


Figure 4.7.1 The cell cytotoxicity of forskolin was evaluated by MTT at 24 h (A). The effect on MMP-9 expression on the treatment of forskolin was determined by qPCR analysis (B). The data are presented as fold increase normalized to the control (no treatment) at 24 h. The scratch wound healing assay in hDPCs at 24h and 72 h compared with control (0 h), the scale bar is 200 µm (C). The quantitative analysis of wound closure area (percentage) was shown as average ± SD (n=3) (D). Asterisks indicate a statistically significant difference compared to the control.

CHAPTER V

DISCUSSION AND CONCLUSION

The present study investigated the effect of iloprost, a PGI_2 analog, on enhancing angiogenesis. To achieve dentin-pulp regeneration with dentin, functional blood vessels, and innervation, more sophisticated tissue engineering approaches are required. The dental pulp in the tooth slice model represents the mixed population of cells found in the dental pulp tissue, ie, odontoblasts, dental pulp stem cells, pulp fibroblasts, and blood vessels, which mimics the clinical situation [11]. Culturing the dental pulp in a 3D system mimics the complex *in vivo* tissue microenvironment with its cell-cell and cell-matrix interactions [102]. Several studies have also been demonstrated about the use of growth factors and serum in tooth slice culture for stimulating angiogenesis and maintaining the tissue viability [11, 100, 103]. However, when it comes to a clinical translation, using growth factors is a technic sensitive procedure and cost effective treatment. Furthermore, serum containing media may change the activation level of certain cell signaling molecules that may affect the treatment outcomes and data interpretation [11, 100, 103, 104]. In our study, the advantage of using a serum-free culture model is the lack of compounds present in serum that may influence cell behaviour [104]. Maintaining the viability of the isolated tooth slices with intact dental pulp tissue for the length of the experiments was a key factor in this study. We demonstrated that the tooth slice pulp tissue remained viable during the 3 days of culturing. Thus, viability was not affected in the absence of serum. A possible explanation is that supporting factors were released

from the intact pulpal tissue, and those factors may have helped in preventing the cells from dying.

Angiogenesis is an important process in the early phase of wound healing [105]. To regenerate the dentin/pulp complex, biomolecules that can induce angiogenesis are needed. VEGF plays an important role in neovascularization. VEGF enhances endothelial cell proliferation, migration, and new vessel formation [106]. It was recently reported that the addition of VEGF at the early phase of wound healing increased odontogenic expression in dental pulp stem cells [107]. In our study, iloprost induced the expression of VEGF in a time-dependent manner, suggesting the possible role of iloprost in enhancing angiogenesis in the dental pulp.

The vWF, a glycoprotein found on the surface of endothelial cells, also regulates angiogenesis (18). It was reported that VEGF upregulated vWF in endothelial cells [108]. The vWF is routinely used to identify microvessels in tissue sections [109]. In the present study, iloprost increased microvessel number in the tooth slices, ie, promoting angiogenesis in dental pulp tissues. Thus, upregulating VEGF expression induced angiogenesis and the increase in vWF increased microvessel density.

Angiogenesis also controls the formation and maturation of collagen fibers at the later phase of wound healing [110]. The vWF binds to exposed collagen at the site of vascular injury during thrombin formation [111]. In the present study, iloprost induced both COL1 and vWF expression, with higher expression of COL1 in association with vWF-positive blood vessels. Co-localization of vWF and collagen around the vessels suggested that angiogenesis is associated with the synthesis of new

collagen fibers as observed on both 1 and 3 days. Taken together from these results, it indicates the possible role of iloprost in enhancing angiogenesis, wound healing, and tissue remodeling after the upregulating of both vWF and COL1. Further investigation to clarify the underlying mechanisms of vWF and COL1 upregulation by iloprost is required.

Although iloprost is the FDA approval drug and considered safe to be used systemically in pediatric and adult patients, when administrated at high doses some adverse effects such as hypotension, dyspnea and nausea have also been observed [112]. In our study we also observed that the induction effect of angiogenesis and collagen synthesis was more pronounce at day 3 in the tooth slices treated with iloprost. This finding suggested that maintaining a proper level of drug concentration during the culture by the addition of iloprost is important for stimulating angiogenesis. Consistent with this observation, we previously demonstrated that VEGF expression in hDPCs in culture can be prolonged with a control release of iloprost form PLGA microsphere [113]. Iloprost/PLGA microsphere delivery system also increased pulpal blood flow in a prolonged manner and increased tertiary dentin formation in a rat tooth mechanical pulp exposure model [113].

PGI_2 acts by activating IP receptors and the PKA pathway [114]. PGI_2 regulated endothelial remodeling at the laminin basement membrane of endothelial cells via PKA signaling [115]. Furthermore, VEGF regulated vWF expression in endothelial cells through PLC-g-PKA signal transduction in endothelial cells [116]. Correspondingly, the present study demonstrated that the addition of a PKA inhibitor attenuated the iloprost-induced vWF/COL1 expression in the dental pulp tissue. These

results confirmed that in human pulp tissue, the upregulation of vWF/ COL1 expression by iloprost was also mediated via PKA signaling. Inactivation of PKA by some pharmacologic inhibitors might inhibit neovascularization by iloprost.

Regenerative endodontic procedures have advantages over conventional therapy for long-term tooth preservation [117]. Angiogenesis is crucial for recruiting and mobilizing stem cells to the injured site. Co-implantation of mesenchymal stem cells with endothelial cells accelerated dentin bridge formation *in vivo* [118]. As a result, the application of angiogenic biomolecules is beneficial to cell-free based tissue regeneration such as vital pulp therapy [21].Recently, we reported that iloprost also increased VEGF expression in human periodontal ligament cells, which is another candidate stem cell for tooth revitalization [119, 120]. Previous research demonstrated that iloprost enhanced dental pulp blood flow and tertiary dentin formation in a rat tooth mechanical exposure model [27]. These findings suggest the potential of iloprost as a dental pulp-capping material and/or scaffold in the root canal for pulp-dentin regeneration.

When dental pulp exposure occurs, many cytokines and mediators were released to promote the formation of a reparative dentinal bridge to preserve tooth vitality. Precise mechanism for pulp cells or stem/progenitor cells during pulp wound-healing process remains unclear. Wound healing process is associated with migratory and remodeling events that require the action of dissolving matrix [121]. Scratch-test assay or wound healing assay can be used to study cell migration *in vitro* [122]. In this study, the application of PGI_2 accelerated the cell migration in wound scratch

assay, suggesting the possible role of PGI_2 in enhancing tissue healing in the dental pulp.

Cell migration into surrounding wound field during wound healing requires the coordinate regulation of multiple cellular activities such as alteration of cell adhesion, locomotion, and proteolytic degradation of the ECM [87, 123]. MMPs play a crucial role in wound repairing process by modifying the wound matrix, cells migration and tissue remodeling. When the dental pulp injury occurs, pulp fibroblasts/odontoblast-like cells produced various protein families, MMPs, and vascular and nerve mediators in order to make a reparative dentinal bridge, thus closing the pulp exposure [110]. Previous study reported that the *in vivo* disruption of MMP-9 displayed compromise reepithelialization, disordered collagen fibrillogenesis, and impaired cells migration [124]. MMP-2, MMP-9 and TIMPs were also previously observed in both sound and inflamed dental pulp [125]. These informations imply the involvement of MMP on dental pulp tissue healing. Our finding demonstrated that MMP-9 mRNA and protein expression could be upregulated by PGI_2. It was noted that PGI_2 did not affect the expression of TIMP in this study. PGI_2 signaling is mediated through the activation of IP-receptors and the PKA pathway [114]. PGI_2 regulated vessels remodeling at the laminin basement membrane of the endothelial cells via PKA signaling [115]. Activation of PKA and its downstream effectors play an important role in signaling event initiated by IP-receptor [126]. A very recent study from our group, we demonstrated that iloprost promoted angiogenesis and collagen synthesis in human dental pulp tissue in the tooth slice organ culture was mediated *via* the PKA-pathway [127]. Correspondingly, the present study demonstrated that the addition of a PKA inhibitor attenuated the iloprost-induced MMP-9 expression in the hDPCs. Those

enhanced effect was also similarly observed by the direct activation of PKA by forskolin. These results confirmed that in human pulp tissue, the upregulation of MMP-9 expression by PGI_2 was mediated via PKA signaling. Further investigation of the mechanism involved in the pathway is required.

Cell migration is a complex phenomenon that requires the coordination of numerous cellular processes. MMP-9 has been implicated in various physiologic and pathologic conditions via the proteolytic activity of the homodimer of MMP-9 [128]. It was reported that PGI_2-IP signaling was responsible for inducing pro MMP-9 and cell migration in dendritic cells [129]. In contrast, some reports showed that PGI_2 inhibited MMPs expression in tumorigenesis and inflamed tissue [130, 131]. However, under physiological and pathological conditions, the regulations may have an overlapping signaling between the remodeling and tissue invasion [19, 110]. It was also reported that many MMPs shared the same substrates, such as dentin sialoprotein [132]. Thus, further study should focus on the reciprocal effect of other MMPs and wound healing by PGI_2.

In conclusion, human tooth slices provide a valuable model to investigate therapeutic approaches to improve dentin-pulp regeneration at the cellular and tissue level. To avoid confounding factors from growth factors in serum, this study showed that the tooth slices can be kept viable under serum-free conditions for up to 3 days. We also found that iloprost promoted angiogenesis, increased new blood vessel formation, and induced collagen deposition in the organ culture system. In addition to its function in promoting angiogenesis, we also demonstrated that iloprost promote hDPCs migration and upregulated MMP-9 expression. Taken together from the

results these studies, iloprost may be a promising biomolecule to enhance angiogenesis and increase the success rate of vital pulp therapy. We propose that iloprost can be used as a dental pulp capping material in clinical situation.

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APPENDIX

VITA

Mr. Sonntana Seang was born in Phnom Penh on October 17, 1985. He graduated the degree of Doctor of Dental Surgery (D.D.S) from Faculty of Dentistry, University of Health Science, Cambodia in 2009. Succeeding after government qualifying exam as government official with license practice; in 2010 he worked in Dental Department at Takeo Referral Hospital, Takeo, Cambodia as a minor oral surgeon. In 2012, he was transferred to, Khmer-Soviet Friendship Hospital, Phnom Penh, Cambodia and worked as an oral and maxillofacial surgeon at Odontostomatology Department. His work primarily involved in oral and maxillofacial surgeries that deal with a variety of conditions associated with surgery involving in both hard and soft with anatomical specialization in face, mouth, and jaws.

Mr. Sonntana Seang has a strong interest in the basic oral health science, as he recognizes that these provide an important basis for oral medicine, oral pathology and oral and maxillofacial surgery. In 2013, under ASEAN Scholarship funding program by Chulalongkorn University, he received a grateful opportunity to enroll in the Doctor of Philosophy Program in Oral Biology, Faculty of Dentistry, Chulalongkorn University. His research work was performed at Research Unit of Mineralized Tissue, Faculty of Dentistry, Chulalongkorn University.

Research fields: Angiogenesis and dental pulp biology.

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