ANTI-INFLAMMATORY AND REGENERATION PROPERTIES OF ILOPROST ON INFLAMED HUMAN DENTAL PULP CELLS MODEL: AN IN VITRO STUDY

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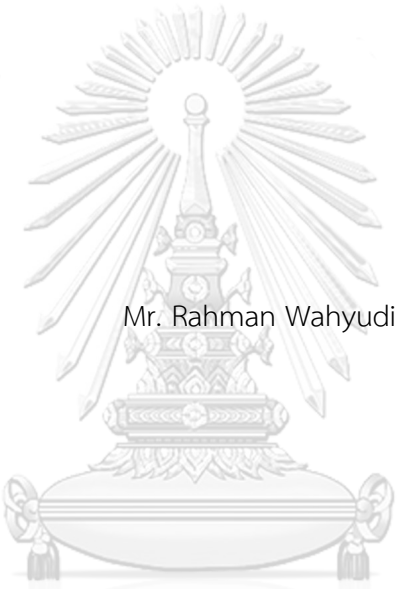

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คุณสมบัติการต้านการอักเสบและการฟื้นสภาพของยาไอโลพรอสต่อเซลล์เนื้อเยื่อในฟันใน สภาวะอักเสบของมนุษย์;การศึกษาในห้องปฏิบัติการ

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รามัน วายูดิ : คุณสมบัติการต้านการอักเสบและการฟื้นสภาพของยาไอโลพรอสต่อเซลล์เนื้อเยื่อในฟันใน สภาวะอักเสบของมนุษย์;การศึกษาในห้องปฏิบัติการ. (ANTI-INFLAMMATORY AND REGENERATION PROPERTIES OF ILOPROST ON INFLAMED HUMAN DENTAL PULP CELLS MODEL: AN IN VITRO STUDY) อ.ที่ปรึกษาหลัก : รศ.ทญ.ดร.ชลิดา ลิ้มจีระจรัส, อ.ที่ปรึกษาร่วม : ศ. ทพ. ดร.ธนภูมิ โอสถานนท์

การทะลุของโพรงประสาทฟันอาจเกิดขึ้นได้ในระหว่างขั้นตอนการกำจัดฟันผุ ก่อให้เกิดอาการอักเสบของเนื้อเยื่อในฟันในภายหลังได้ เพื่อป้องกันการอักเสบและทำให้เนื้อเยื่อในฟันอักเสบในภายหลัง การใช้สารปิดรอยทะลุฟัน เช่น การปิดทับเนื้อเยื่อในโพรงฟันโดยตรง นิยมนำมาใช้ในการรักษาทางคลินิกเพื่อรักษาสภาวะความมีชีวิตของฟันเอาไว้ สารเหล่านี้ควรมีคุณสมบัติในการลดการอักเสบ ต้านการเจริญเติบโตของเชื้อแบคทีเรีย และกระตุ้นการสร้างเนื้อฟันใหม่ ในการศึกษาก่อนหน้า พบว่าไอโลพรอสซึ่งเป็นอนุพันธ์ของพรอสตาซัยคลิน เป็นสารที่สามารถกระตุ้นการสร้างเนื้อฟันทดแทนภูมิใหม่ได้ในสัตว์ทดลอง และกระตุ้นการสร้างหลอดเลือดใหม่ในเนื้อเยื่อในฟันในห้องปฏิบัติการ อย่างไรก็ตามคุณสมบัติต้านการอักเสบของไอโลพรอสยังไม่เคยถูกนำมาศึกษา นอกจากนี้ การศึกษาในห้องปฏิบัติการเป็นการใช้เซลล์เนื้อเยื่อฟันที่ได้จากฟันที่มีสุขภาพดีของผู้ป่วย ซึ่งไม่ได้จำลองสถานการณ์ที่เกิดขึ้นจริงในคลินิกการศึกษานี้จึงมีวัตถุประสงค์เพื่อพัฒนาแบบจำลองเซลล์เนื้อเยื่อฟันของมนุษย์ที่อักเสบในห้องทดลอง และใช้แบบจำลองนี้เพื่อศึกษาคุณสมบัติการต้านการอักเสบและส่งเสริมผลการสร้างเนื้อเยื่อใหม่ของไอโลพรอส การกระตุ้นการอักเสบในห้องทดลองทำโดยการให้สารกระตุ้นการอักเสบที่มาจากค็อกเทลไซโตไคน์ส่วนผสมของอินเตอร์ลูคิน 1 เบต้า (IL-1β), อินเตอร์เฟียรอนแกมม่า (IFN γ) และทูเมอร์เนโครสิสแฟกเตอร์แอลฟ่า (TNF α)ในอัตราส่วน 1:10:100 (IL-1β:1ng/ml, TNF α:10 ng/ml และ IFN γ: 100 ng/ml) ความมีชีวิตของเซลล์จะถูกตรวจสอบ โดยการทดสอบ MTT และระดับของ IL-6 และ IL-12 จะได้รับการประเมินโดย RT-qPCR หลังจาก 6 ชั่วโมงและ 24 ชั่วโมง การแสดงออกของโปรตีนอินเตอร์ลูคิน 6 (IL-6) จะถูกประเมินโดยการสอบวิเคราะห์ด้วย ELISA การวิเคราะห์ผลการทดลองจะนำผลมาเปรียบเทียบกับกลุ่มควบคุมที่ให้ไซโตไคน์ค็อกเทลเพียงอย่างเดียวด้วยโปรแกรม GraphPad Prism 9 โดยสถิติ one-way ANOVA หรือ Kruskal-Wallis ผลการศึกษานี้แสดงให้เห็นว่าค็อกเทลไซโตไคน์กระตุ้นการแสดงออกของ IL-6และ IL-12 เมื่อให้การกระตุ้นด้วยค็อกเทลไซโตไคน์ที่ส่วนผสม คือ IL-1β:1ng/ml, TNF α:10 ng/ml, and IFN γ: 100 ng/ml ไม่ก่อให้เกิดความเป็นพิษต่อเซลล์และเมื่อให้ไอโลพรอส สามารถลดการแสดงออกของ IL-6และ IL-12 อย่างมีนัยสำคัญ อย่างไรก็ตามงานวิจัยพบว่าไอโลพรอสไม่สามารถลดการอักเสบก่อนการกระตุ้น ในลักษณะของการปกป้องเซลล์ได้ โดยสรุปคือการจำลองการอักเสบของเนื้อเยื่อฟันในห้องทดลองด้วยการใช้ค็อกเทลไซโตไคน์สามารถกระตุ้นไซโตไคน์การอักเสบเฉียบพลันได้สูงกว่าการใช้LPS และไอโลพรอสสามารถลดการอักเสบที่เกิดขึ้นจากการกระตุ้นด้วยค็อกเทลไซโตไคน์ได้ แสดงให้เห็นถึงแนวโน้มการนำมาใช้ของไอโลพรอสเพื่อลดอาการอักเสบในเนื้อเยื่อในฟันได้ในอนาคต

สาขาวิชา	ชีววิทยาช่องปาก	ลายมือชื่อนิสิต .....
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Rahman Wahyudi : ANTI-INFLAMMATORY AND REGENERATION PROPERTIES OF ILOPROST ON INFLAMED HUMAN DENTAL PULP CELLS MODEL: AN IN VITRO STUDY. Advisor: Assoc. Prof. Chalida Limjeerajarus, D.D.S., Ph.D Co-advisor: Prof. Thanaphum Osathanoon, D.D.S., Ph.D.

In the clinical situation, pulp exposure can happen during caries removal procedures. Inflammation can occur on the pulp of the exposed area because of infiltration of bacteria from caries or as a result of pulp reaction to caries removal drilling. When the pulp was exposed during the caries removal procedure, traditional methods to preserve the pulp such as pulp capping and pulpotomy were performed. The ideal treatment outcome of the pulp exposure is to regain the primary structure of tubular dentin as well as maintain the vitality and healthiness of the dental pulp Thus, good dental materials or drugs used for vital tooth therapy should have properties of antibacterial, anti-inflammatory, and dentin-pulp tissue regenerative capability. Previous studies suggested that iloprost, the analog of prostacyclin, could induce tertiary dentin, boost angiogenesis, increase blood flow, and promote neovascularization, both on healthy human dental pulp cells, and in rat models. However, the role of iloprost on inflammatory control in dental pulp has not been investigated before.This study aimed to develop an inflamed human dental pulp cell model *in vitro* and employ the model to investigate the anti-inflammatory and promoting tissue regeneration effect of iloprost. To create the inflammation model *in vitro*, HDPCs were treated by the cocktail of three cytokines; IL-1β, IFN γ, and TNF α. With three different doses of these cytokines in the ratio of 1:10:100 (IL-1β:1ng/ml, TNF α:10 ng/ml, and IFN γ: 100 ng/ml), the viability of the cells was examined by MTT assay and the level of IL-6 and IL-12 was assessed by RT-qPCR. After 6h and 24h, the expression of IL-6 protein was assessed by ELISA assay. After the mimicked inflamed HDPCs model was obtained, iloprost treatment was performed, using iloprost solution with concentrations of 10^{-6} mol for 6h and 24h. Then, the viability of the cells was examined by MTT assay and the expression level of IL-6 and IL-12 was measured by RT-qPCR, and IL-6 protein expression was assessed by ELISA assay. The results were compared to the control sample which was treated by cytokines cocktail only. The comparison was analyzed using GraphPad Prism 9, either with one-way ANOVA or Kruskal-Wallis method. The results of this study showed that cytokine cocktail that consist of IL-1β:1ng/ml, TNF α:10 ng/ml, and IFN γ: 100 ng/ml, was not toxic, and could induces the expression of IL-6 robustly and IL-12 on HDPCs. Iloprost treatment was capable to downregulate the expression of IL-6 and IL-12 mRNA but had no significant impact as a cytoprotective agent on HDPCs. In conclusion: This study suggested that cytokine cocktail creates a better acute inflammation model than LPS induced model, and iloprost was capable as an anti-inflammatory agent on inflamed HDPCs. Hence, open the possibility of iloprost to be used as additional vital pulp therapy material.

Field of Study:	Oral Biology	Student's Signature .....
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CHAPTER I

INTRODUCTION

Pulp inflammation (pulpitis) is an interesting topic in dentistry due to its high prevalence in dental diseases. Worldwide, more than 2.3 billion people suffer from permanent cavities and more than 530 million children suffer from deciduous cavities. (1). Studies tried to investigate the possibility to regulate the inflammation which can lead to the preservation of dental pulp. For example; genes that can downregulate the expression of some pro-inflammatory cytokines in dental pulp; and that of micro RNA can induce an anti-inflammatory response (2, 3). There are also *in vitro* and/or *in vivo* studies of pathways that are involved in the inflammatory processes, and many inflammatory factors and cytokines are found to play roles in pathways that eventually lead to the inflammation, such as, interleukin (IL)-17 which plays role in dental pulp inflammation via regulation of WNT5A, IL-1β which can stimulate IL-8 and Upa expression and is secreted in dental pulp cells through the TAK1 or MEK/ERK signal transduction pathways, Pannexin 3 (Panx3) participated in human and rat dental pulp inflammation in an NF-Kb-dependent manner (4-6).

Well innervated and vascularized, the dental pulp is a soft connective tissue formed by collagen fibers, fibroblasts, and dental stem cells. At its periphery, the odontoblast layer is located and can be differentiated to produce dentine. The odontoblast processes lengthwise along the dentine tubule can act as a sensor. With that, the pulp can sense the slightest mechanical, chemical, and thermal stimulation (7). The dental pulp can defend itself from various oral aggressions and microorganisms up to a certain point. After significant aggression, like other connective tissue, the pulp responds with an inflammatory process to eliminate pathogens and initiate the repair processes. However, the low-compliance nature of dental pulp can transform the inflammatory process into tissue destruction. The vitality of the pulp is always associated with the vitality of the tooth as a whole, and tooth vitality is essential for the functional life of the tooth itself. Hence the vitality of the pulp is critical to preserve (8).

The vertebrate immune system consists of two subsystems—the innate immune system and the adaptive immune system. The first to respond to initial infection and disease is the innate immune system. However, it doesn't have the memory of previous responses. The parts of the innate immune system include physical barriers such as the skin; cellular processes such as phagocytosis; and humoral components such as soluble proteins. If a pathogen persists, despite the innate immune defenses, the adaptive immune system is recruited. The adaptive immune system is highly specific to a particular antigen and can provide long-lasting immunity(9).

Those inflammatory factors reported to be increased in the caries-affected dental pulp and/or odontoblasts including pro-inflammatory biomarkers such as interleukin 8 (IL8), IL-1β, IL-2, IL-4, IL-6, IL-10, IL-11, interferon-γ (IFN-γ), C-C chemokine ligand 2 (CCL2), CCL20, CXC chemokine ligand 10 (CXCL10), epithelial cell-derived neutrophil attractant 78 (ENA78), and tumor necrotic factor-α (TNF-α). Also, some biomarkers related to the regeneration process such as transforming growth factor-β1 (TGFβ1), vascular endothelial cell growth factor (VEGF) has been reported (10).

The identification of the bacterial profile of deep dentinal caries and its correlation with the inflammation status of caries-induced pulpitis suggested that *Lactobacillus* had the highest relative abundance at the genus level among all the inflammation status groups (11). Another study showed that *Lactobacillus* induced early proinflammatory cytokines such as IL-8, TNF-α, IL-12, IL-1β, and IL-6 (12). When the pro-inflammatory cytokines increase, they will trigger the series of signaling cascades that will lead to inflammatory reaction patterns, such as edema, fever, pain, and hyperemia. If the pathogen persists and the pro-inflammatory cytokines are maintained at a high level, the inflammatory reaction will start to damage healthy cells, tissues, and organs. If this chronic condition continues, it can lead to DNA damage, necrosis, and internal scarring (13). Inflammation is a natural and important process for healing, however, for the regeneration process to occur, the balance between pro-inflammatory and anti-inflammatory cytokines is crucial to maintain the homeostatic conditions. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is the common method to control most of the chronic inflammation and can prevent the tissue from undergoing necrosis (14). There are several ways to create an inflammation model on cultured cells *in vitro*. Inflammation biomarkers can be used as triggers to create an inflammation model. The choice of the compounds usually depends on the purpose of the experiment the researcher conducts. Cytokines as bio-markers of the inflammation have been used to create the mimicked inflamed model on cultured cells of *in vitro* study. However, since the parameter of the *in vitro* inflamed condition itself is still a topic of study, there are no strong rules regarding cytokine choices or doses.

The pulp exposure during the caries removal procedures is a risk that could happen, especially in deep dentinal caries. An ideal treatment outcome of the pulp exposure is to regain the primary structure of tubular dentin as well as maintain the vitality and healthiness of the dental pulp. To achieve this condition, it requires pulp capping materials with antibacterial, anti-inflammatory, and dentin-pulp tissue regenerative properties (17). Iloprost is an analog of prostacyclin and share similar pharmacodynamic properties of this molecule, for example, the ability to create vasodilatation and inhibition of platelet aggregation. Because of those properties, iloprost has been used for peripheral vascular diseases such as Reynaud's disease, thromboangiitis obliterans, and diabetic angiopathy. Iloprost is also used as a therapeutic drug in

cardiac conditions such as myocardial ischemia and infarction, and prevention of heparin-induced platelet activation (18). A study has proved that iloprost has the potential to be a candidate agent to promote neovascularization in dental pulp tissue by upregulating the expression of VEGF, FGF-2, and PDGF (19). In line with that, another study proved that iloprost could boost angiogenesis by increasing new vessel formation and inducing collagen deposition in the pulp tissue in a tooth slice culture (20). Angiogenesis is important to maintain the vitality of the pulp, hence iloprost seems to have the potential to be used as vital pulp therapy additional material to help maintain the vitality of pulp during vital pulp therapy procedure.

Iloprost has been proved capable of hampering the production of proinflammatory cytokines TNF-α and GM-CSF and upregulating the production of an anti-inflammatory cytokine IL-10 by human peripheral mononuclear cells *in vitro* (21). Treprostinil, another prostacyclin analog, also has been shown to block NF-Kb nuclear translocation, hence inhibiting the production of IL-6, TNF-α, IL-1, and GM-CSF by human alveolar macrophages (22). However, the anti-inflammatory properties of prostacyclin analog have never been tested on dental pulp.

In this study, we aim to create an *in vitro* model mimicking inflamed human dental pulp and use the model to investigate the anti-inflammatory effect of iloprost on inflamed human dental pulp cells. The study also examines if the regeneration properties of iloprost (neovascularization, and angiogenesis) will also be found in this *in vitro* model.

CHAPTER II

LITERATURE REVIEW

I. Innate immune response in dentin-pulp complex

a. Inflamed dentin-pulp complex due to caries

The dental pulp is confined to hard tissue. The dentin that surrounds the pulp provides mechanical protection from noxious stimuli that affect the tooth. When the dentin that protects the pulp, damaged, the pulp vitality will be disturbed. The immune reaction that occurs will increase the volume of the pulp, but since the pulp is surrounded by hard tissue, the swelling in the pulp becomes limited, this condition will trigger the pain. There are four types of noxious stimuli that can cause inflammation of the pulp (pulpitis), namely; mechanical damage, thermal injury, chemical irritation, and bacterial effects (23).

Residual products produced by bacteria in dental caries can cause demineralization of enamel and dentine of the teeth. If this condition continues, it can cause pulp necrosis and eventually tooth loss. The three basic reactions that the pulp will experience against caries are; reduced dentin permeability, tertiary dentin formation, and immune-inflammatory reactions(24). Anatomically, the dental pulp consists of the odontoblast (which is in direct contact with the dentin), a cell-free zone, and a cell-rich zone beneath it (That consists of fibroblast, dendritic cells, and immune cells). The odontoblasts have processes that extend along the dentinal tubules. These processes act as the coordinator of the formation of peritubular dentin and also the first cells to be exposed to noxious stimuli from bacterial products of dental caries (25).

As the caries lesion deepens from enamel and reaches the dentin, bacterial metabolites, such as acids, become the initiators of the pulp reaction, however, the buffering ability of the dentin fluid will balance the pH before it can cause a continued pulp reaction. This at least happens until the damage is profound and causes the dentin thickness to be greatly reduced. In the early to intermediate stages of caries lesion in dentin, the acid products produced by bacteria will degrade the dentin matrix, during this degradation process, the bioactive molecules such as transforming growth factor (TGF) -β1, TGF-β3, insulin-like growth factors (IGF) -1 and -2, and fibroblast growth factor 2 (FGF2) that reside in the dentin matrix are released, and when free, these molecules will start the process of forming dentin back. This dentin formation is called tertiary dentinogenesis (24, 26, 27).

On early pulp inflammation where the caries is shallow, the outward flow of dentinal fluid increases, which will cause an increase in pulp tissue pressure. The increase of pulp tissue pressure will upregulate the neuropeptides, such as Substance P (SP), and Calcitonin-Gene-Related-Peptide (CGRP). These neuropeptides will cause the vascular effect, which will invite macrophages and monocytes to the pulp (28). The pathogens that invade the pulp tissue will activate the immune process. In humans, the immune system is consists of two subsystems—the innate immune system and the adaptive immune system (9). The first defense mechanism activated by the invading pathogen is the innate immune system (15). Innate immunity is a nonspecific defense mechanism that is activated promptly or within hours after antigen's emergence in the body. The innate immune system includes physical barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body (29). In a broad sense, innate immunity is not antigen-specific, however, it is capable to recognize the molecular pattern that is common to the pathogen. This molecular pattern is recognized by receptors called host pattern recognition receptors (PRRs), for example, mannose receptors that are usually present on neutrophils and macrophages, when activated can initiate phagocytosis (30). Some others groups of receptors such as Toll-Like Receptors can induce cytokines, chemokines, and T cell stimulatory molecules, when binding to lipopolysaccharide (LPS) or lipoteichoic acid (LTA). All those molecules will trigger some signaling pathway that leads to the elimination of the pathogen or if the infection persists, activation of adaptive immunity (31).

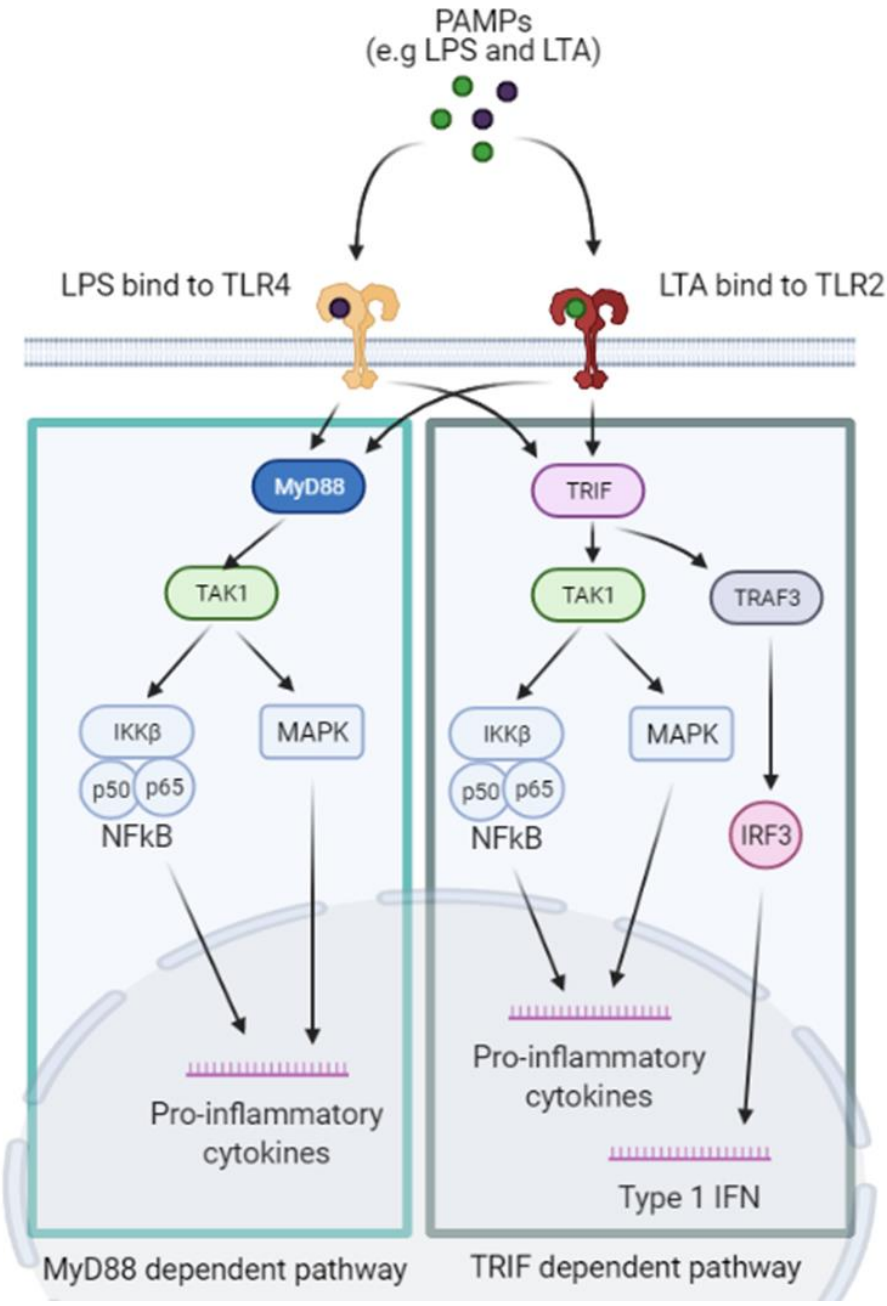

Figure 1. Immune inflammatory reaction on dental pulp due to caries. LPS from gram (-) bacteria will bind to TLR4, meanwhile, LTA from gram (+) bacteria will bind to TLR2. separately they will activate the cascade of signaling to produce cytokines (Lu, Yeh et al. 2008) .

The recognition of the pathogen is an important part of innate immunity. Pathogen-associated molecular patterns (PAMPs) are one of the main targets of the recognition of the innate immune (32). The term PAMPs is used to describe molecules produced only by microbial pathogens and not host organisms. Examples of PAMPs include LPS, which is the molecule produced by gram-negative bacteria, and LTA which is produced by

gram-positive bacteria (33). In the dental caries case, the microbiome of the deep dentinal caries from reversible pulpitis to irreversible pulpitis, showed the mixture of gram-negative and positive bacteria, with more predominant gram-positive bacteria (11). The LPS and LTA produced by bacteria in caries will bind to their respective receptors. Toll-Like Receptors-4 (TLR4) will recognize LPS, meanwhile, Toll-Like Receptors-2 (TLR2) will be the binding site of LTA. The activation of TLR-2 and TLR-4 will activate myeloid differentiation primary response 88 (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) which will provide some signaling cascade to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-**K**B), mitogen-activated protein kinase (MAPK), and Interferon regulatory factor 3 (IRF3) signaling axis (Figure.1) (34).

One of the reactions produced by the signaling axis of NF-**K**B is the inducement of the cytokines production that is related to the inflammation, such as IL-6, IL-12, and TNF-α (35). Meanwhile, MAPK also can regulate the expression of certain pro-inflammatory cytokines, such as IL-10, TNF-α, IL-4, and IFN-γ. The signaling axis of IRF3, however, only focuses on the production of IFN type 1, which plays a major role in the maturation of IL-1β that is produced on the NF-**K**B signaling axis (36).

b. Expression of cytokines in pulpitis

In *in vitro* and animal studies, acute pulpitis in rats showed the upregulation of IL-1β, TNF-α, and also TLR4 and NF-**K**B (37). Another study also showed an increase of IL-6, IL-1β, and TNF-α expression in mouse reversible pulpitis model induced by LPS. The increased expression of these cytokines became one of the factors for triggering inflammation on mouse pulpitis models (38). A different study, it showed the upregulation of IFN-γ and TNF-α mRNA expression on acute and chronic caries lesion of mouse dental pulp tissue. This study reveals that the expression of IFN-γ and TNF-α were downregulated in the chronic phase, due to the increase of IL-10 and TGF-β that acted as anti-inflammatory cytokines (39).

In human studies, IL-1β and TNF-α are predominant cytokines in the inflamed dental pulp. Another study revealed that IL-6, IL8, IL-10, IFN-γ, and TNF-α were increased in irreversible pulpitis. Meanwhile, other investigations showed that the level of IFN-γ and also IL-4 can increase in human dental pulp early pulpitis model induced by *P. Endodontalis* (40-42). Interestingly, the acute dental pain caused by pulp inflammation was linked to the increased level of IL-1β and IL-6 in saliva. A study conducted by Giones et.al proves that IL-6 presents more intense in acute pulpitis than chronic and healthy

human dental pulp tissue (43). This finding is aligned with the others that reveal the upregulation of anti-inflammatory cytokines in the chronic phase. Gram-negative bacteria such as *S. mutant,* known to induce high titer of IL-12, IFN-γ, and TNF-α (44, 45).

c. Roles of pro-Inflammatory cytokines

Cytokines mediate the body's immune response to microbial infections. These molecules are produced by living cells to provide signals, either paracrine or autocrine. Depending on the condition that triggers it, their functions can vary to recruit other cytokines that can function as pro-inflammatory (produce inflammation) or anti-inflammatory (reduce or balance the inflammation) (10). Previous study reported that several cytokines are produced by the signaling cascade triggered by LPS and LTA from bacteria of dental caries, such as IL-1β, IL-6, IL-12, IFN-γ, and TNF-α (35, 36).

Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine that has been known for the induction of pain, inflammation, and autoimmune conditions. This cytokine can be produced by fibroblast, endothelial, neural cells, and inflammatory cells such as macrophage and mast cells. Under normal physiological conditions, IL-1β beta is produced by the body to play a role in regulating hunger, sleep, and body temperature (46). During inflammation, IL-1β is produced via MAPK or NF-κB signaling cascade which can be activated by binding of LPS to TLR4. However, these signaling only produce the immature form of IL-1β (pro- IL-1β). The maturation of pro-IL-1β to be IL-1β p17 (the mature form) is done by the caspase-1 molecule which is activated by the activation of the inflammasome. The activation of the inflammasome structure can be induced by LTA, and enhanced by robust production of the immature form of caspase-1 triggered by IFN type 1 induced by the binding of LPS to TLR4 (47). In a mice study, upon the inflammation-induced condition, the deficiency effect of IL-1β included prohibited acute phase, anorexia, the production of IL-6, and fever (48).

In Inflammation conditions, the mature IL-1β will enhance the production of IL-6. This cytokine plays a major role in the acute response. When IL-6 is produced in macrophages on the infection area, it will be delivered to the liver via the bloodstream, and in that response, the body will produce C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, haptoglobin, and α1-antichymotrypsin. All of these protein molecules are considered acute phase modulators and cause acute conditions such as edema. IL6 also increases hepcidin production, which will hamper the iron transporter ferroprotein 1

that will result in the reduction of serum iron level. It means that IL-6 can cause hypoferremia and anemia, which are related to chronic inflammation (49, 50).

Streptococcus mutans, the gram-positive bacteria that mainly present in on the shallow lesion of caries and known as the main causative in the pathogenesis of dental caries, upregulated the expression of IFN-γ (51). This cytokine modulates the production of IL-6 and TNF-α. Rapid upregulation of IFN-γ from T cells and NK cells will increase the expression of human beta defensin-3 (HBD3) which has microbicidal properties. IFN-γ also affects the production of nitric oxide (NO) and MHC class II. These two molecules are known as major effectors of macrophages. The activated macrophages by these two molecules, induce the production of IL-12, while IL-12 can also induce the IFN-γ vice versa (52, 53). IL-12 is capable to trigger hematopoiesis on bone marrow and regulate innate resistance and adaptive immunity (53). Together with IFN type 1, IFN-γ will activate the JAK/STAT signaling pathway. This signaling cascade will induce the production of chemokines, orchestrating the differentiation and death of hematopoietic cells, and the production of nitric oxide (NO) (54).

Tumor necrosis factor α (TNF-α) is produced by various cells in the dental pulp when they detect the antigen. It is considered a strong pro-inflammatory cytokine that plays a major role in the immune system during inflammation. It also has an important role in cell proliferation, differentiation, and apoptosis (16). Macrophages produce TNF-α during inflammation, this cytokine plays several important roles, such as; promote vasodilatation via induction of nitric oxide (NO); creating a change of the structure of the endothelium of the blood vessel, hence promoting edema; also, TNF-α can facilitate the adhesion of leukocytes on the blood vessel, these promote the extravasation of leukocytes in the endothelial vascular area of the infection. During inflammation, TNF-α also induces the expression of tissue factor (TF) on monocytes and endothelial cells. The induced expression of TF will crosstalk to factor VII, resulting in the thrombin activation which will regulate the blood coagulation (16, 30).

d. Inflammation models *in vitro*

There are some methods to create the inflammation model on cultured cells *in vitro*. The biomarkers of the inflammation can be used as the triggering agent to create an inflammation model. However, the choice usually depends on the purpose of the experiments conducted by the researchers. For example, if the experiments aim to investigate the expression of TNF-α via the NF-κB signaling pathway, the use of LPS or

ATP is probably enough as an inducer. Meanwhile, if the experiments aim to investigate the expression of IL-1β, the use of LPS and LTA either as a cocktail or separate reagent is better since the maturation of IL-1β involving not only NF-κB signaling pathway, but also type 1 IFN signaling axis, and activation of caspase-1 via inflammasome(15, 16).

Cytokines as bio-markers of the inflammation have been used to create the mimicked inflamed model on cultured cells of *in vitro* study. However, since the parameter of inflamed condition *in vitro* itself is still experimental, there are no solid rules regarding the cytokine's choices or doses. The cocktail of cytokines is used as an attempt to mimic the clinical pathologic condition, and in the realization that biomarkers of the immune response are activated complex web of pathways that cross-talk to each other. The balance of pro-inflammatory and anti-inflammatory cytokines creates the chance for tissue repair while eliminating the invading pathogen. Many cytokines play the role in this process; hence researchers can use cocktails in their experimental design to study the crosstalk between them. For example, the study conducted by Zhang et al to investigate the synchronization effect of pro-inflammatory and anti-inflammatory cytokines on the regenerative ability of stem cells. This study uses gingival stem cells and treats them on the cytokine cocktail of 1 ng/ml IL-1β, 10 ng/ml TNF-α, and 100 ng/ml IFN-γ to mimic the natural inflammatory environment. They investigate the mRNA expression of alkaline phosphatase (ALP), type I collagen (Col-I), osteopontin (OPN), and osteonectin (ON)(55).

The mixture of cytokine and PAMPS molecules also can be conducted in *in vitro* study. To investigate the angiogenesis pathway, Shin et al used the mixture of 1μg/mL of LPS and 10μg/mL of TNF-α simultaneously on free serum media in human dental pulp cells to induce the expression of VEGF and MMP. Since there is still no exact rule for how many doses or concentrations should be used in *in vitro* study, scientists rely on the toxicity assay and compared the results from each dose, or use time-depended method to determine the exact dose that they will be going to use on their study (2, 56).

Table 1 Some examples of the use of cytokines in vitro studies

No	Reference	Cytokine used	Dosage/ concentration	Aim of the study
1	Shin, et al (56)	TNF-α, LPS (*P. Gingivalis*)	LPS (1 μg/ mL) and TNF-α (10 ng/ mL)	To investigate whether SIRT1 and VEGF are responsible for tumor necrosis factor-a (TNF- α).
2	Zhang, et al (55)	IL-1β, TNF-α, and IFN-γ	IL-1β (1 ng/ml), TNF-α (10 ng/ml), and IFN-γ (100 ng/ml)	Investigates proliferative and osteogenic differentiation potentials of gingival mesenchymal stem/progenitor cells (G-MSCs) by cytokine cocktail.
3	Liu, et al (5)	TNF-α, WNT5A or IL-17	IL-17 (10 ng/ml), rhWNT5A, (500 ng/ml), rhTNF-α (10 ng/ml)	To investigate the role of interleukin IL-17 in dental pulp inflammation and the relationship between WNT5A and IL-17.

4	Lin, et al (6)	IL-1β	IL-1β (5 ng/ml)	To study whether IL-1β can stimulate IL-8 and uPA expression and secretion in dental pulp cells through the TAK1 or MEK/ERK signal transduction pathways.
5	Fan, et al (57)	TGF-β1	TGF-β1 (10 ng/ml)	Aimed to evaluate the effect of TGF-β1 on human PDL SCs.
6	Liou, et al (58)	IL-17	IL17(0.5, 5, or 50 ng/ml)	Explored the effects of osteocytes and osteoblasts on the osteogenesis of MSCs with and without IL-17 supplementation.
7	Adams, et al (59)	IFN-γ	IFN-γ (0, 100, or 1000 U/ml)	To investigate epithelial barrier function on IFN-γ modulation.
8	Song, et al (4)	TNF-α	TNF-α (10 ng/ml)	To study the role of Panx3 in the inflammatory response and its potential mechanism.
9	Sakaguchi, et al (60)	VEGF, IGF-1, TGF-β1	IGF-1 (1400 pg/ml), VEGF (500 pg/ml), TGF-β1 (350 pg/ml)	To investigate the regeneration properties of the cytokine cocktail on periodontal tissue.
10	Kaka, et al (61)	IL-1β, IL-6, TNF-α	L-1β (10 ng/ml), IL-6 (100 ng/ml), TNF-α (10 ng/ml)	To augment DC stimulatory capacity by optimizing DC phenotype and IL-12 production using cytokine cocktail.

II. Vital pulp therapy

a. Indication

The classification of vital teeth is divided into; asymptomatic teeth without objective disease, are called normal teeth; second. Teeth with a reversible sensitivity to cold and/or osmotic changes such as sour or sweet called reversible pulpitis; and teeth with high sensitivity, intense, and long duration to temperature changes are called irreversible pulpitis (24). When removing deep caries, extra care must be taken to ensure that the dentin covering the pulp remains intact. However, pulp exposure during the caries removal is a risk that could occur, especially in the deep dentinal caries removal procedures. An ideal treatment outcome of the pulp exposure is to regain the primary structure of tubular dentin as well as maintain the vitality and healthiness of the dental pulp (17, 62). For this, traditional methods to preserve the pulp using pulp capping and pulpotomy, are carried out. Over time, the classification of reversible and irreversible pulpitis, and the therapies for treating both conditions, have become a subject of discussion. Pulp preservation vs pulp extirpation therapy is under question, and this makes the indication for pulpotomy broader(62).

Reversible pulpitis is characterized by a positive vitality test and pain associated with the stimuli (pain does not last long when the stimuli are removed). Irreversible pulpitis is characterized by a positive vitality test with constant and diffuse pain. Vital pulp therapy (VPT) consists of pulp capping and pulpotomy and it is used to treat reversible pulpitis. According to current scientific opinion, in the case of irreversible pulpitis, tissue healing cannot be achieved after eliminating the trigger stimulus. In this case, irreversible pulpitis

requires root canal treatment. Vital pulp therapy is excluded in some conditions such as the tooth that does not react to sensitivity testing, the tooth that shows positive remark to percussion or occlusal loads, the tooth which has spontaneous or persistent pain, as well as radiographic signs of periapical osteolysis. Another thing is if there is bleeding that cannot be stopped after exposure to the pulp, and if the pus or serous exudate is found (20, 24, 62).

Pulp capping is classified into ; indirect pulp capping and direct pulp capping. Indirect pulp capping refers to the treatment of a thin layer of dentine close to the pulp. When caries is deep, rather than risk pulp exposure, the dentin is allowed to remain adjacent to the vital pulp, and covered with a cavity sealer and restored. Direct pulp capping is done when the pulp is slightly exposed. A material is placed directly over the exposed pulp tissue, this way has been suggested to induce pulp healing and generate reparative dentin (62).

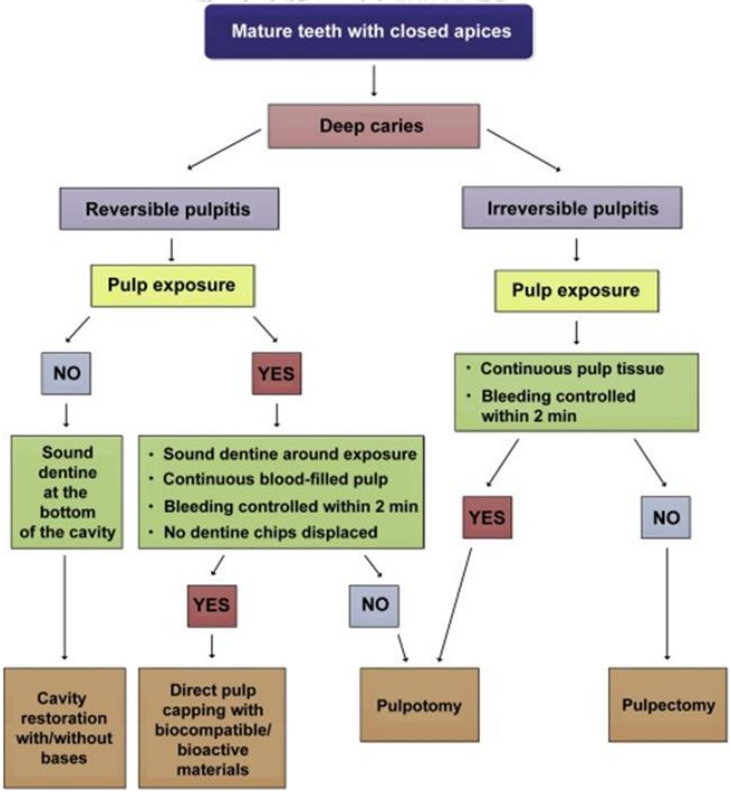

Figure 2. Treatment map of vital tooth. Figure taken from Vital pulp therapy: histopathology and histobacteriology-based guidelines to treat teeth with deep caries and pulp exposure. Journal of dentistry, 2019

For many years, the objective of VPT was to preserve the pulp of mature teeth with incomplete apex. The idea was by preserving the dental pulp, the apexogenesis can still occur. The current statement of the American Association of Endodontists (AAE) regarding VPT is that it can be applied to mature teeth with complete apex. The idea is that inflammation is a temporary condition that can be resolved when the pathogen is eliminated. Hence the removal of dental pulp that undergoes inflammation is unnecessary unless it has a bad prognosis that will lead to necrosis eventually (63).

b. Current disadvantages of VPT

Vital tooth therapy has a higher success rate when the tooth has the following conditions; an uninflamed pulp, the optimal time is 24 hours after trauma or infected when the inflamed pulp area is still superficial; bacterial tight seal, this will determine the success of VPT because the presence of bacteria can interfere with the healing process; pulp dressing, the choice of pulp dressing is very important to regain the primary structure of tubular dentin as well as maintain the vitality and healthiness of the dental pulp, the gold standard is, of course, it has to be able to act as antibacterial, anti-inflammatory, and dentin-pulp tissue regenerative properties(17, 24).

For decades, calcium hydroxide has been the gold standard of pulp capping materials. It has good anti-bacterial properties and a long track record of vital pulp therapy success. Calcium hydroxide also has several shortcomings, including easily dissolved, a poor adhesive ability so it is prone to leakage, and causing necrosis to a depth of about 1.5 mm from the area of contact with the pulp tissue. The toxicity of calcium hydroxide further neutralizes as the affected pulp deepens, creating coagulative necrosis in vital pulp junctions and areas of necrosis(64). Other materials such as zinc oxide eugenol (ZOE) or glass ionomer (GI) are known to have cytotoxic properties when place in direct contact with the pulp (24). However, the glass ionomer cement has anti-bacterial properties when incorporated with chlorhexidine or antibiotics. The versatility of GI to be combined with other materials makes GI a widely studied modified cement. For example, *in vitro* study of GIC that incorporated with an alcoholic extract from plants *(Salvadora persica, Olea europaea, and Ficus carcia*) can enhance the anti-microbial effect to *S. mutans* and also against *M. luteus* in a concentration-dependent manner (65-67). In recent years Mineral Trioxide Aggregate (MTA) has become more favorable among the dentist because it has good anti-bacterial properties like calcium hydroxide, but with better seal properties(24). However, MTA still has high solubility and is known to cause discoloration of the tooth (68).

Besides the materials factor, the operator factor also contributes greatly to the successful outcome of VPT. The ability to overcome leakage with proper technique is crucial, since providing well-sealed restoration as soon as possible after pulp capping will provide protection against leakage hence bacterial contamination that can lower the success chance of the pulp cap. The ability to control the bleeding is also important, saline or water is the most pulp friendly, but sodium hypochlorite is the best at controlling bleeding and it also has to disinfect properties(62).

III. Prostacyclin analog

a. Pharmacodynamic of prostacyclin analog

Prostacyclin (PGI_2) a prostaglandin member derived from the 20-carbon, omega-6 fatty acid, arachidonic acid (AA or 5,8,11,14-eicosatetraenoic acid). Cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2) will convert AA into the prostaglandin precursor PGH2, which will eventually be converted into PGI_2 via prostacyclin synthase (Figure.3A) (69). At physiological pH, PGI_2 is unstable hence it has a very short working span *in vivo* (<2 min). PGI_2 is known for its ability to act as a regulator within the cardiovascular system, where it promotes VSMC relaxation (vasodilatation) and inhibits platelet aggregation (anti-thrombotic). Prostacyclin is also known to be an inflammatory modulator. *In vivo* study showed that mice that lack the prostacyclin receptor had a reduced inflammatory response, such as lack of edema and hyperemia caused by the absence of vascular permeability during inflammation (70, 71).

Iloprost is a prostacyclin analog, a strong short-lived prostanoid mostly produced in the vascular endothelium, and shares the same pharmacodynamic properties of this compound (72). Prostacyclin analog is a universal and strong inhibitor of platelet activation. Significant inhibition of platelet aggregation on the patient with the peripheral vascular ischemic disease who undergoes the infusion of iloprost up to 2 ng/kg/ min has been recorded. The effect decreases significantly when the infusion stops. The investigation in the animal model of bleeding and vascular injury, affirms the dose-related decline in thrombogenesis. The mechanism of the anti-aggregatory effect of iloprost requires platelet receptor-mediated activation of adenyl cyclase which will upregulate levels of cyclic adenosine monophosphate (cAMP), which will be affecting phospholipase activity and cytosolic calcium levels (Figure. 3B) (18).

The upregulated cAMP in smooth muscle will also increase the activation of its respective receptors. This mechanism is believed made Iloprost also an arterial vasodilator. Iloprost is capable to inhibit vasoconstriction triggered by arachidonic acid, thromboxane

A2 analog, angiotensin II, and phenylephrine. This also made Iloprost capable to decrease peripheral vascular resistance and average arterial blood pressure in humans. It was also recorded to increase renal blood flow (18, 73). Another important feature of iloprost's pharmacodynamic profile is its cytoprotective action. Study in mice has proved that iloprost has the cytoprotecting ability of toxic from paracetamol (74).

Recently, prostacyclin has been shown to have anti-inflammatory properties. Iloprost has been proved capable of hampering the production of proinflammatory cytokine TNF-α and GM-CSF and upregulating the production of an anti-inflammatory cytokine IL-10 by human peripheral mononuclear cells *in vitro*. Treprostinil, another prostacyclin analog, also has been shown to block NF-kB nuclear translocation, hence inhibiting the production of IL-6, TNF-α, IL-1, and GM-CSF by humans alveolar macrophages. An *in vitro* study using bone marrow-derived DC (BMDC) induced using LPS, prostacyclin analog decreases the production of several pro-inflammatory cytokines (IL-12, TNF-α, IL-1 α, and IL-6) and chemokines (MCP-1 and MIP-1α). Also, it upregulates the expression of anti-inflammatory cytokines (IL-10). This effect is modulated by IP-dependent upregulation of intracellular cAMP and also hampering the signaling axis of NF-kB activity (22, 75).

Iloprost has the potential to be used as a candidate agent to promote neovascularization in dental pulp tissue by upregulating the expression of VEGF, FGF-2, and PDGF *in vitro* and mice models. This opens the potential of iloprost to be used for vital pulp therapy. Another study also found that angiogenesis increased new vessel formation, and induced collagen deposition in tooth slice organs treated by iloprost (19, 20).

b. Clinical use of prostacyclin analog

Prostacyclin can eliminate undesirable blocking or narrowing of blood vessels and allow more blood to flow through the arteries. It can help the healing process of ulceration which occurs because of ischemia. Because of its properties, iloprost has been used for peripheral vascular diseases such as Reynaud's disease, thromboangiitis obliterans, and diabetic angiopathy or peripheral atherosclerotic obliterative disease (PAOD). Thromboangiitis obliterans is an inflammatory disease of the small and medium-sized arteries and veins of the extremities. The symptoms which usually occur are pain in the feet or hands area triggered by insufficient blood flow during a workout. Raynaud's disease is a condition on finger blood vessels as a result of excessive stimulation of the sympathetic nervous system. In Raynaud's disease due to poor blood circulation, the blood flow vanishes, and fingers often become blueish appearance. PAOD is a condition of constricted blood vessels in the peripheral arteries. This can cause reduced oxygen

transport into these areas. Symptoms can be fast tiring, for example, leg muscle easily tired during walking. Permanent pain at rest and leg ulcers may occur in the advanced stage (18, 22, 76).

Iloprost is also used for the therapeutic drug in cardiac indication such as; myocardial ischemia and infarction, and prevention of heparin-induced platelet activation. Intermittent use of iloprost in patients with ischemia secondary to peripheral atherosclerotic obliterative disease or angiopathic diabetic with thromboangiitis obliterans may reduce the patient's chances of undergoing amputation (18). Iloprost in aerosol form is used for Primary pulmonary hypertension (PPH). The infusion of prostacyclin was reported the first therapy shown to be lifesaving. PPH is a lung condition in which the blood vessels in the lungs constrict and the pressure in the pulmonary artery is upregulated above normal levels. The pulmonary arteries carry blood from the body to the lungs, where carbon dioxide is traded for oxygen (77).

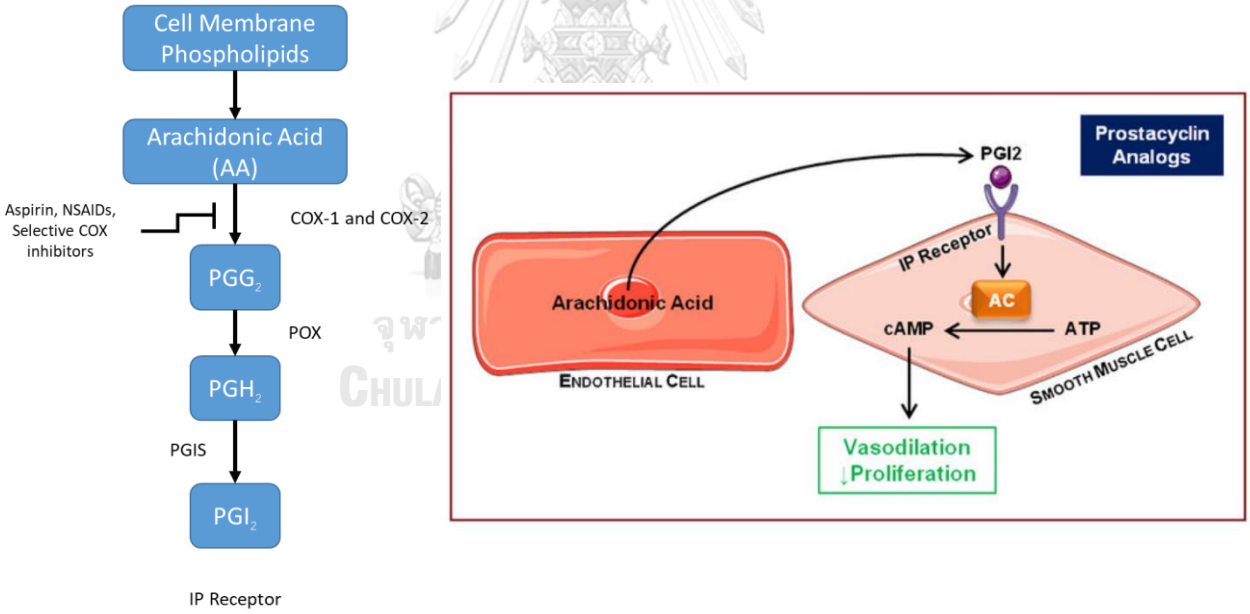

Figure 3. *Prostanoid biosynthesis pathway(Figure taken from Prostacyclin: an inflammatory paradox. Frontiers in pharmacology, 2011). (A). After secreted on endothelial cell, prostacyclin will bind to its receptor and increase expression of cAMP, which lead to the biological effects such as vasodilation (Figure taken from Pulmonary arterial hypertension: Basic knowledge for clinicians, 2016). (B).*

CHAPTER III

MATERIALS AND METHODS

I. Methods

a. Sample donor

Four patients who have dental surgery treatment for healthy third molar or premolar extraction due to orthodontics treatment at Department of Oral Surgery, Faculty of Dentistry, Chulalongkorn University (Ethic committee approval code: HREC-DCU 2021-069).

b. Sample extraction

After extraction, the third molars were placed into the general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% antibiotic, and antimycotic) and transferred into the laboratory at 4^0C. Under the sterile condition, each tooth was placed in a 25 mm culture dish and cleaned by PBS. The tooth was kept by dental forceps and a scalpel blade was used for cleaning off debris and periodontal ligaments on the root surface. Cemento-enamel junction was cut slowly used the sterilized dental diamond disc to make a cleft between the crown and the root. The cutting process was performed slowly to prevent overheating of the dental tissue. Then, the tooth was spitted by a hammer and a chisel. Pulp tissue was separated from the crown and the root with tweezer and cut into small pieces by using a scalpel blade.

c. Isolation and culture of primary Human Dental Pulp Cells

The small piece of pulp tissue was cut off into 1-2 mm fragments. Each fragment was placed in a 25 mm culture dish with the general medium. Then incubated at 37^0 C in 5% CO_2. It was estimated that the total volume of the general medium for the outgrowth of cells, can support the attachment of primary dental pulp tissue for further cells growth. The medium was changed every 2 – 3 days. The primary dental pulp cells at the third to fifth passage was used in these experiments.

d. Cytokine cocktail and iloprost preparation

Master stocks of 5µg/ml rhIL-1β (R&D, Minneapolis, USA), and 20µg/ml rhTNF-α (R&D, Minneapolis, USA) were diluted in 0.1% PBS. Meanwhile, master stocks of 100µg/ml rhIL-1β (R&D, Minneapolis, USA) were diluted in DI. For working concentration, all of these cytokines

(IL-1β, TNF-α, and IFN-γ) were diluted in serum-free media in the ratio of 1:10:100 with concentrations of cytokine cocktail 1 (CC1) consist of IL1β: 0,5ng/mL, TNFα: 5 ng/ml, and IFNγ: 50 ng/mL, or cytokine cocktail 2 (CC2) consist of IL1β: 1ng/mL, TNFα: 10 ng/ml, and IFNγ: 100 ng/mL (78). Porphyromonas gingivalis LPS (GIBCO/Invivogen, SanDiego, CA) in a master stock of 1mg/ml was diluted in serum-free media into 1µg/ml (LPS1) and 10µg/ml (LPS2). Iloprost (Ilomedin; Bayer AG, Leverkusen, Germany) 's master stock at 20µg/ml diluted in serum-free media into 10^{-6} M concentration.

e. Cytotoxic Assay

Cell viability of hDPCS was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT; #M6494, Invitrogen, USA) assay. MTT powder was dissolved in the serum-free culture media without phenol red to the concentration of 0.5 mg/ml. After 24 hours, the culture medium was removed and rinsed once by PBS. 0.5 mg/ml MTT solution was added to each culture well and incubated at 37°C in a culture incubator for 30 minutes. MTT solution was removed and formazan crystals were dissolved after adding 100 µl of the mixture of glycine buffer/DMSO (dimethyl sulfoxide). The absorbance was read at OD=570 nm within 1 hour and a standard curve was used to quantify the cell number.

f. RNA Extraction

The total RNA was collected by using a Trizol reagent to lyse the cell. After adding 1 ml of Trizol, the cell was scraped and mixed by using the pipette tip. The lysed cells were incubated at room temperature for 5 minutes then replaced into a 1.5 ml tube. 200 µl chloroform was added into the tube then centrifuged for 15 minutes at 14.000 rpm, 4^0C. After centrifugation, the mixture was separated into three layers. The aqueous phase was transferred into a new 1.5 ml tube. Isopropanol was added to the aqueous phase (with the amount of 1:1 of isopropanol: aqueous layer), then incubated overnight at -20^0C. Then, the sample was centrifuged for 15 minutes at 4^0C at 14.000 rpm. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. The supernatant was removed gently with a micropipette. The pellet was resuspended in 1 ml of 70% ethanol. Then, the sample was centrifuged at 14,000 rpm for 5 minutes at 4° C. After removing the supernatant, the RNA pellet was dried at room temperature until completely dry. Then, the RNA pellet was dissolved in Nuclease free-water (10µl) by passing the solution through a pipette tip a few times. The concentration of RNA was quantified by Nanodrop.

g. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription of total RNA sample was done by using ImProm-II Reverse Transcription System (Promega, WI, USA) and real-time PCR performed using a MiniOpticon real-time PCR system (Bio-Rad, CA, USA) and FastStart Essential DNA Green Master (Roche Diagnostics, Mannhem, Germany) as manufacturer's instructions. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as the housekeeping gene. All reactions will be triplicated.

h. Enzyme-linked immunosorbent assay (ELISA)

The ELISA (DuoSet ELISA human IL-6) protocol consists of two parts, the plate preparation, and assay procedure. The plate preparation begins with coating a 96-well microplate with 100 µl of capture antibody, then incubated overnight. Washed with wash buffer three times, then blocked the plates with 300 µl of reagent diluent, incubated for 1 hour, then washed with wash buffer. After the plate was ready, the assay procedure proceeds by adding 100 µl of samples. The samples were the conditioned media from HDPCs control group, CC treated group, or LPS treated group. After samples were added, they were incubated for 2 hours. The plate was washed again with wash buffer, then 100 µl of detection antibody was added to the plate followed by 2 hours incubation. After washed by wash buffer. 100 µl of streptavidin-HRP was added to each well. Continued by adding 100 µl of the substrate solution, then 50 µl of stop solution. The optical density was immediately measured by using a microplate reader which was set at 450 nm wavelength.

i. Data analysis

All data was reported in mean with SD. Statistical analysis was performed by one-way ANOVA or non-parametric (Kruskal-Wallis test). Statistical significance level at p-value <0,05. Data were analyzed using GraphPad Prism 9 software.

II. Research workflow

a. **Objective 1**: To determine the optimal concentration and time point after treatment of cytokine cocktail that consist of IL-1β, IFN γ, and TNF α, to create an inflammation model in human dental pulp cells by observing the viability level of cells.

Rationale: The previous study used a cocktail of IL-1β, IFN γ, and TNF α, with a dose ratio of 1:10:100 on gingival mesenchymal stem cell and mouse primary osteoblast has been tested and proved to mimic the natural inflammatory environment, with the increase of Nitrite and PGE_2 concentration (55, 78). However,

the effect of cytokines cocktail that consists of IL-1β, TNF-α, and IFN-γ with the ratio of 1: 10:100, on dental pulp cells was not yet known. On gingival mesenchymal stem cells, when induced by cytokines cocktail of IL-1β, TNF-α, and IFN-γ of 1: 10:100, MTT result showed decrease significantly after 72 hours of cocktail treatment (55). Hence, the maximum time point for this study is less than 72 hours. Since one of the hallmarks of acute inflammation is the high infiltrate of neutrophils that last until 24 to 48 hours post-infection (79), the exact maximum time point was 24 hours for this current study. From the maximum time point (24 hours), a quarter of it (6 hours) will be picked as the initial time point.

Experimental Design: Human dental pulp cells seeded at the density 0.05 x 10^6 cells/well in 24-wells-plate, in general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% of antibiotic, and antimycotic) and incubated for 24 hours to allow cell attachment. The medium changed to serum-free medium for 24 hours before being exposed with a cocktail of IL-1β, TNF α, and IFN γ, in different concentrations with the ratio of 1:10:100 such as; IL-1β (0.5, and 1 ng/mL), TNF α (5, and 10 ng/mL), and IFN γ (50, and 100 ng/mL) in general medium Dulbecco's modified Eagle's medium (DMEM) supplemented with, 1% L-glutamine, 1% of antibiotic and antimycotic for further 6 hours or 24 hours. The cytotoxicity and cell viability were observed by performing an MTT assay. The absorbance was measured at 570 nm of wavelength. The negative control group was cultured in a general medium without any cytokines. The positive control group was treated with LPS *P. Gingivalis* (concentration: 1μg/ml or 10 μg/ml) instead of a cytokines cocktail.

b. **Objective 2**: To investigate the expression of pro-inflammatory related genes and protein, and angiogenesis-related genes, on human dental pulp cells that are treated by the cocktail of IL-1β, IFN γ, or TNF α.

Rationale: The effect of cytokines cocktail that consists of IL-1β, TNF-α, and IFN-γ with the ratio of 1: 10:100, on dental pulp cells has never been done before. However, these cocktails can induce inflammation on gingival mesenchymal stem cells and mouse primary osteoblast (55, 78). The study conducted by Giones et.al proves that IL-6 presents more intense acute pulpitis than chronic and healthy human dental pulp tissue (43). Streptococcus mutans, the gram-positive bacteria that mostly appears on the shallow lesion of caries and is known as the main causative in the pathogenesis of dental caries, upregulated the expression of IFN-γ,

and high expression of IFN-γ can induce IL-12 (45). For that reason, IL-6 and IL-12 are selected as biomarkers of inflammation in this study.

Experiment Design: Human dental pulp cells seeded at the density of 0.3 x 10^6 cells in 6- wells plates, in general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% of antibiotic, and antimycotic) and incubated for 24 hours to allowed cell attachment. The medium changed to serum-free medium for 24 hours, before being exposed to a cocktail of IL-1β, TNF α, and IFN γ, in different concentrations such as; IL-1β (0.5, and 1 ng/mL), TNF α (5, and 10 ng/mL), and IFN γ (50, and 100 ng/mL) in general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with, 1% L-glutamine, 1% of antibiotic and antimycotic) for further 6 hours or 24 hours. The total RNA was extracted with Trizol reagent and converted into cDNA by using a reverse transcriptase system. Real Time-PCR was performed to analyze the mRNA expression levels of IL-6, IL-12, FGF, and VEGF that regulated during the inflammation process in pro-inflammatory cytokines treated human dental pulp cells. GAPDH was used as the housekeeping gene. The negative control group was cultured in a general medium without any cytokines. The positive control group was treated with LPS *P. Gingivalis* (concentration: 1μg/ml or 10 μg/ml) instead of a cytokines cocktail. The ELISA was used to measure the expression of IL-6 protein.

c. **Objective 3**: To investigate the expression of factors related to inflammation and neovascularization on human dental pulp cells that are treated by pro-inflammatory cytokines and then iloprost.

Rationale: Iloprost can promote neovascularization in dental pulp tissue by upregulating the expression of VEGF and FGF-2 *in vitro* and mice models (19). Another study also reveals that prostacyclin analog has been shown to block NF-kB nuclear translocation, hence inhibiting the production of IL-6 and IL-12 (22, 75).

Experiment Design: Human dental pulp cells seeded at the density of 0.3 x 10^6 cells in 6- wells plates, in general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% of antibiotic, and antimycotic) and incubated for 24 hours to allow cell attachment. The medium changed to serum-free medium for 6 hours before being exposed with a cocktail of IL-1β, IFN γ, and TNF α (In concentration that established from the previous experiment (Result of Objective 1 and 2)) in general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1%

L-glutamine, 1% of antibiotic and antimycotic) for further 6 hours. The prostacyclin analog was then added with concentrations of 10^{-6} mol for 6 and 24 hours. The total RNA was extracted with Trizol reagent and converted into cDNA by using a reverse transcriptase system. Real Time-PCR was performed to analyze the mRNA expression levels of IL-6, IL12, VEGF, and FGF2 on mimicked inflamed human dental pulp cells model. The negative control group was cultured in a general medium without any cytokines and iloprost. Positive control group cultured in general medium with cytokines cocktail only.

d. **Objective 4**: To investigate the expression of factors related to inflammation and neovascularization on human dental pulp cells that are treated by iloprost and then pro-inflammatory cytokines.

Rationale: One study showed that iloprost has cytoprotective properties against paracetamol-induced toxicity in hamster isolated hepatocytes(80). Iloprost can promote neovascularization in dental pulp tissue by upregulating the expression of VEGF and FGF-2 *in vitro* and mice models (19). Another study also reveals that prostacyclin analog has been shown to block NF-kB nuclear translocation, hence inhibiting the production of IL-6 and IL-12 (22, 75).

Experiment Design: Human dental pulp cells seeded at the density of 0.3 x 10^6 cells in 6- wells plates, in general medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% of antibiotic, and antimycotic) and incubated for 24 hours to allow cell attachment. The medium changed to serum-free medium for 6 hours before being exposed with prostacyclin analog then with concentration 10^{-6} mol for 6 hours. After 6 hours, a cocktail of IL-1β, IFN γ, and TNF α (In a concentration that was established from the previous experiment (Result of Objective 1 and 2)) was added for a further 6 hours or 24 hours. The total RNA was extracted with Trizol reagent and converted into cDNA by using a reverse transcriptase system. Real Time-PCR was performed to analyze the mRNA expression levels of IL-6, IL12, VEGF, and FGF2. The negative control group was cultured in a general medium without any cytokines and iloprost. Positive control group cultured in general medium with iloprost only.

III. Materials

a. Instruments

a. 60-mm tissue culture dishes; Nunc; Thermo Scientific, Waltman, MA, USA
b. 6-wells plates; Nunc; Thermo Scientific, Waltman, MA, USA
c. 12-wells plates; Nunc; Thermo Scientific, Waltman, MA, USA
d. 24-wells plates; Nunc; Thermo Scientific, Waltman, MA, USA
e. Microplate reader; Molecular Devices, Palo Alto, CA, USA
f. MiniOpticon real-time PCR system; Bio-Rad, USA
g. Nanodrop spectrophotometer; Thermo Scientific, USA

b. Chemicals

a. Dulbecco's modified Eagle's medium (DMEM); Sigma, Saint Louis, USA
b. Fetal bovine serum (FBS); Gibco, Grand Island, NY, USA
c. L-glutamine; Gibco, Grand Island, NY, USA
d. Antibiotic-antimycotic solution; Gibco, Grand Island, NY, USA
e. Phosphate buffered saline (PBS); Gibco, Grand Island, NY, USA
f. 0,25% Trypsin-EDTA; Gibco, Grand Island, NY, USA
g. FastStart SYBR Green Master; Roche Diagnostics, Mannhem, Germany
h. TRIzol reagent; Invitrogen, Carlsbad, CA, USA
i. Reverse transcriptase ImPromII kit; Promega, Madison, WI, USA
j. Human IL-6 DuoSet ELISA; R&D Systems, Minneapolis, USA
k. MTT + solution; USB Corporation, USA
l. Primers for GAPDH; Wardmedic, Thailand
m. Primers for IL-6; Wardmedic, Thailand
n. Primers for IL-12; Wardmedic, Thailand
o. Primers for FGF2; Wardmedic, Thailand
p. Primers for VEGF; Wardmedic, Thailand

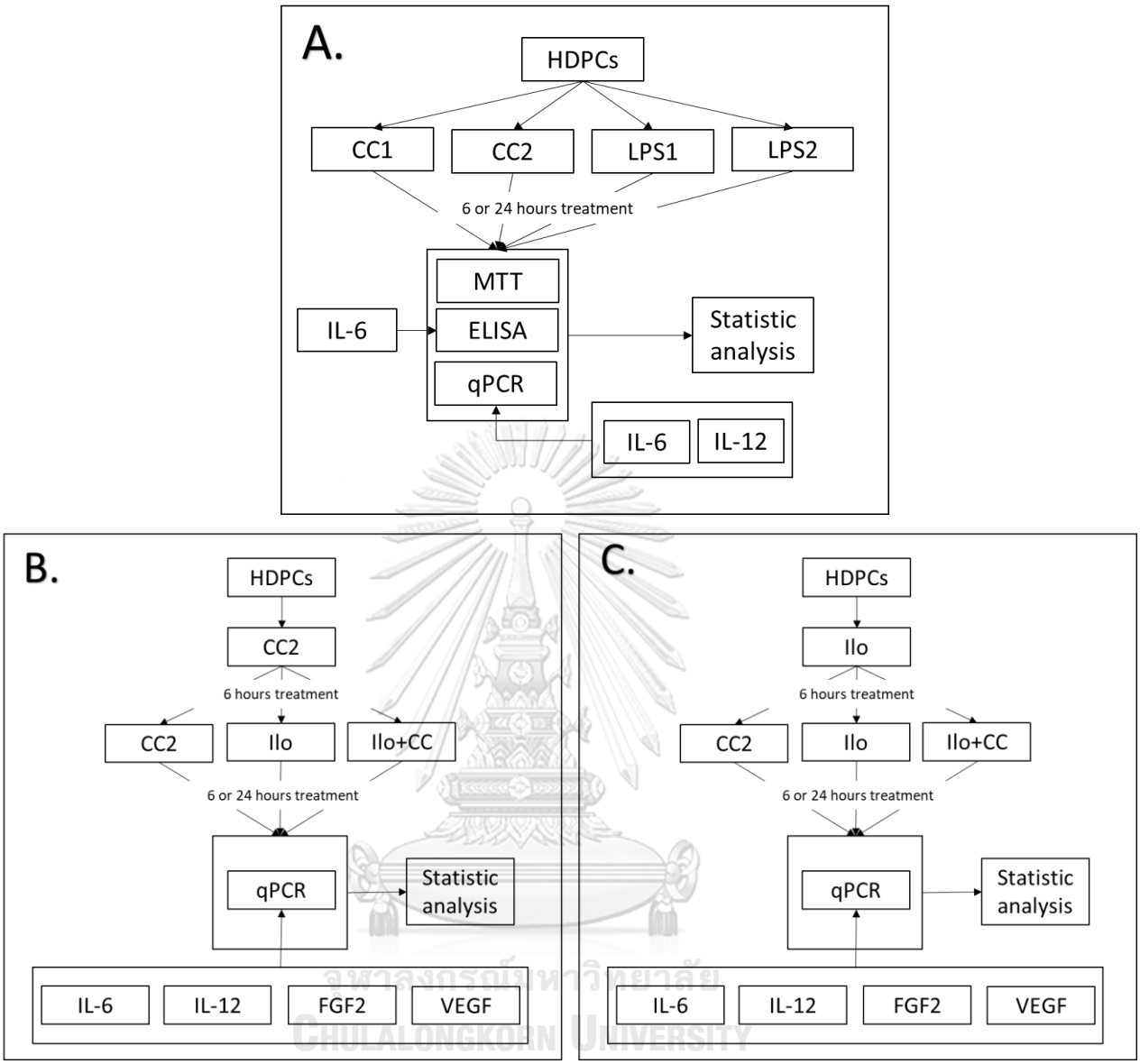

Figure 4 Research workflow schematic. To create an mimicked acute inflammation model, expression of IL-6 and IL-12 from two doses of CC and LPS was compared in two different time points treatment (A). After the working CC dosage established, HDPCs was treated by the proposed model for 6h, then the conditioned media was changed to either continue with CC, changed to iloprost, or change to mixture of CC+Iloprost. The expression of pro-inflammatory cytokines and regenerative genes maesured by q-PCR and compared among groups (B). To investigate whether iloprost can act as cytoprotective agent, HDPCs was swaped treated by iloprost instead of CC (C).

CHAPTER IV

RESULTS

a. Effect of CC and LPS treatment on HDPCs

To evaluate the effect of CC or LPS treatment on HDPCs viability, the MTT assay was performed on HDPCs in SFM containing IL-1β, TNF-α, and IFN-γ, or LPS. Two doses of concentration and two-time points from each kind were selected for comparison. MTT results showed that cell numbers in all group treatments were almost similar with no significant differences (Figure 5. A, and figure 6. A).

b. The expression of IL-6 and IL-12 post CC and LPS treatment on HDPCs

Expression of IL-6 and IL-12 mRNA in HDPCs induced by CC or LPS were investigated with RT-qPCR. Both CC1 and CC2 treatment could upregulate the expression of IL-6 robustly at both 6 hours and 24 hours of treatment. Statistically, CC2 induced IL-6 mRNA higher than CC1 at both 6 and 24 hours time points. Both CC concentrations are capable to upregulate IL-12 mRNA expression, however only CC2 could upregulate significantly compare to the control on both 6 and 24 hours treatment (Figure 5. B). Because of that, CC2 was selected as the working concentration. The upregulation of IL-6 and IL-12 treatment of single cytokine (IL1β or TNFα) on HDPCs weren't as high as the cocktail treatment (Figure 5. C).

The result of HDPCs treated by LPS1 or LPS2, showed that the expression of IL-6 was higher on HDPCs treated by LPS 2 compare to LPS 1 treatment on 6 hours time point. The result also showed a much higher expression of IL-6 mRNA on 24 hours treatment in LPS 2. HDPCs treated by LPS 2 also showed significant expression of IL-12 mRNA compared to the control group in 24 hours of treatment. However, statistically, both LPS1 and LPS2 could upregulate IL-12 significantly compare to the control group in 6 hours of treatment (Figure 6. B). Because LPS2 could induce IL-6 and IL-12 higher than LPS1, LPS 2 concentration was used to be compared with the CC2 results. The comparison between CC and LPS showed that the cytokine cocktail consists of IL1β (1ng/mL), TNFα (10 ng/ml), and IFNγ (100 ng/mL), induced the mRNA expression of IL-6 and IL-12, and IL-6 protein excessively compared to the LPS (10ug/mL) treatment (Figure 6. C, D).

c. Effect of Iloprost treatment on HDPCs after 6 hours CC treatment on the expression of IL-6 and IL-12

The results of iloprost treatment post CC incubation showed that the sample group treated by iloprost was able to downregulate the expression of IL-6, that induced by 6 hours CC incubation, significantly compared to the group that was treated with prolonged CC on both 6 hours and 24 hours treatment time. Meanwhile, the combination of iloprost and CC significantly decreases the expression of IL-6 on 6 hours iloprost treatment time point. The IL-12 mRNA was also downregulated significantly on the iloprost treated group compared to the prolonged CC treated group. But, the combination of iloprost and CC couldn't downregulate the IL-12 expression significantly when compared to the prolonged CC treated group (Figure 7. A, B).

To investigate whether iloprost still has regeneration properties on the inflammatory environment on HDPCs, the expression of FGF2 and VEGF were measured by RT qPCR. The results showed that both iloprost treated group, the prolonged CC treated group, and the combination of the iloprost and CC group were able to upregulate the expression of FGF2 significantly compare to the control group on both times points. However, there were no significant expression differences among those groups that were treated by iloprost, CC, or the combination of iloprost and CC on both 6 hours and 24 hours treatment (Figure 7. C, D). The VEGF mRNA expression showed no significant change on all groups in 6 hours treatment time point. There was significant expression of VEGF mRNA on the iloprost and CC combination group at 24 hours treatment time points. However, in the iloprost group, there was no significant change noted.

d. Effect of Iloprost pre-treatment before CC treatment on expression of IL-6 and IL-12 on HDPCs

HDPCs pre-treated by iloprost (10^{-6} M), before the treatment of cocktail of IL1β (1ng/mL), TNFα (10 ng/ml), and IFNγ (100 ng/mL) began. The qPCR results showed significant upregulation of the IL-6 gene on both time points on CC-treated groups. As well as the upregulation of IL-12 gene expression on 6 hours time point compared to the control group and iloprost-only treated group (Figure 8 A, B). Significant upregulation of FGF2 can be seen on the cocktail group compared to the control group and iloprost only treated group (Figure 8. C, D).

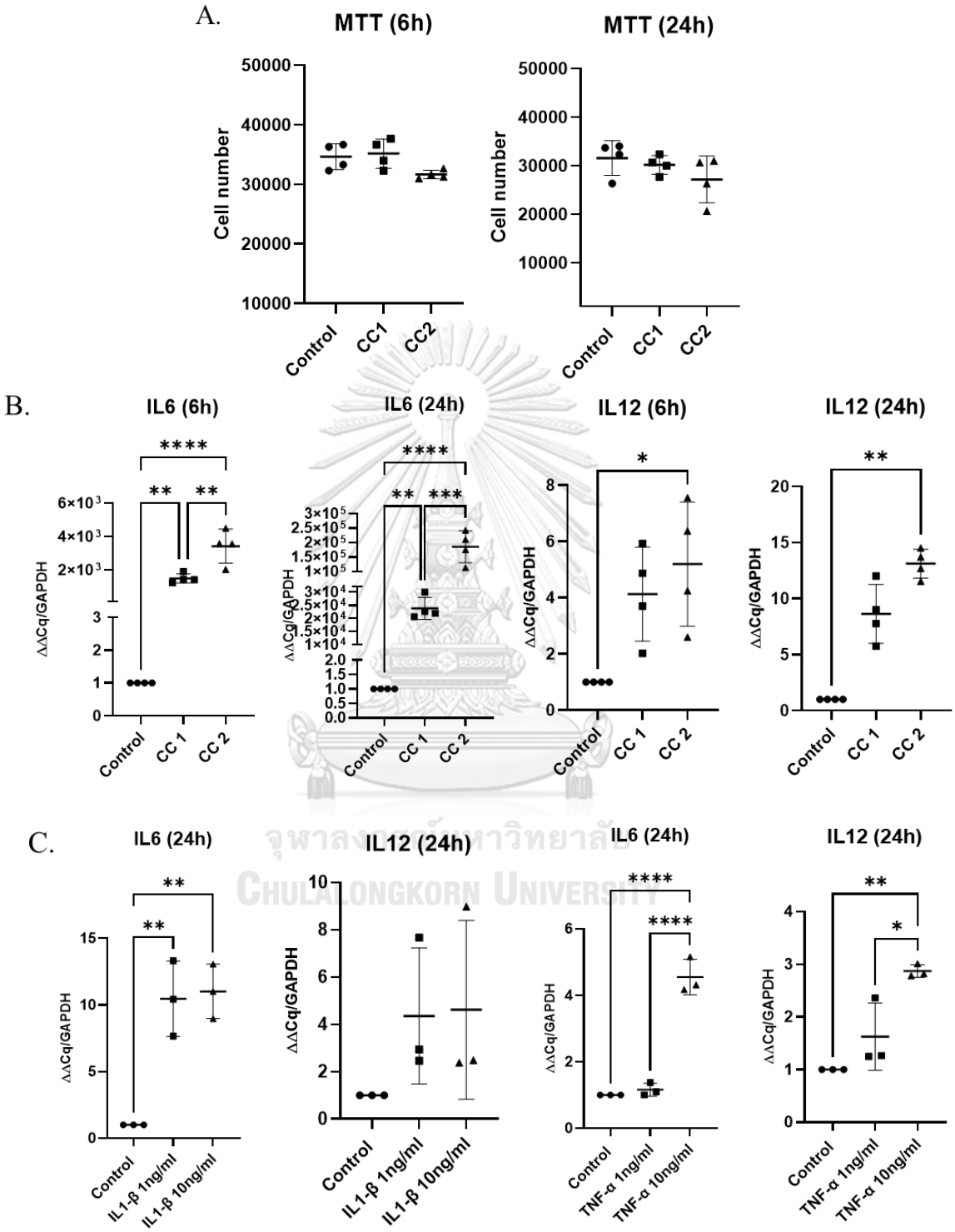

Figure 5 Figure 4. Combination of three cytokines can upregulate IL-6 and IL-12 in HDPCs. MTT assay result of HDPCs treated by CC1 or CC2 performed to investigated the cells viability, n=4(A). Expression of IL-6 and IL-12 mRNA after treatment of two concentration of CC assessed by RT-qPCR, n=4(B). The expression of IL-6 and IL-12 mRNA after treatment of two concentration of IL1β or TNFα was measured by RT-qPCR,n=3 (C.)

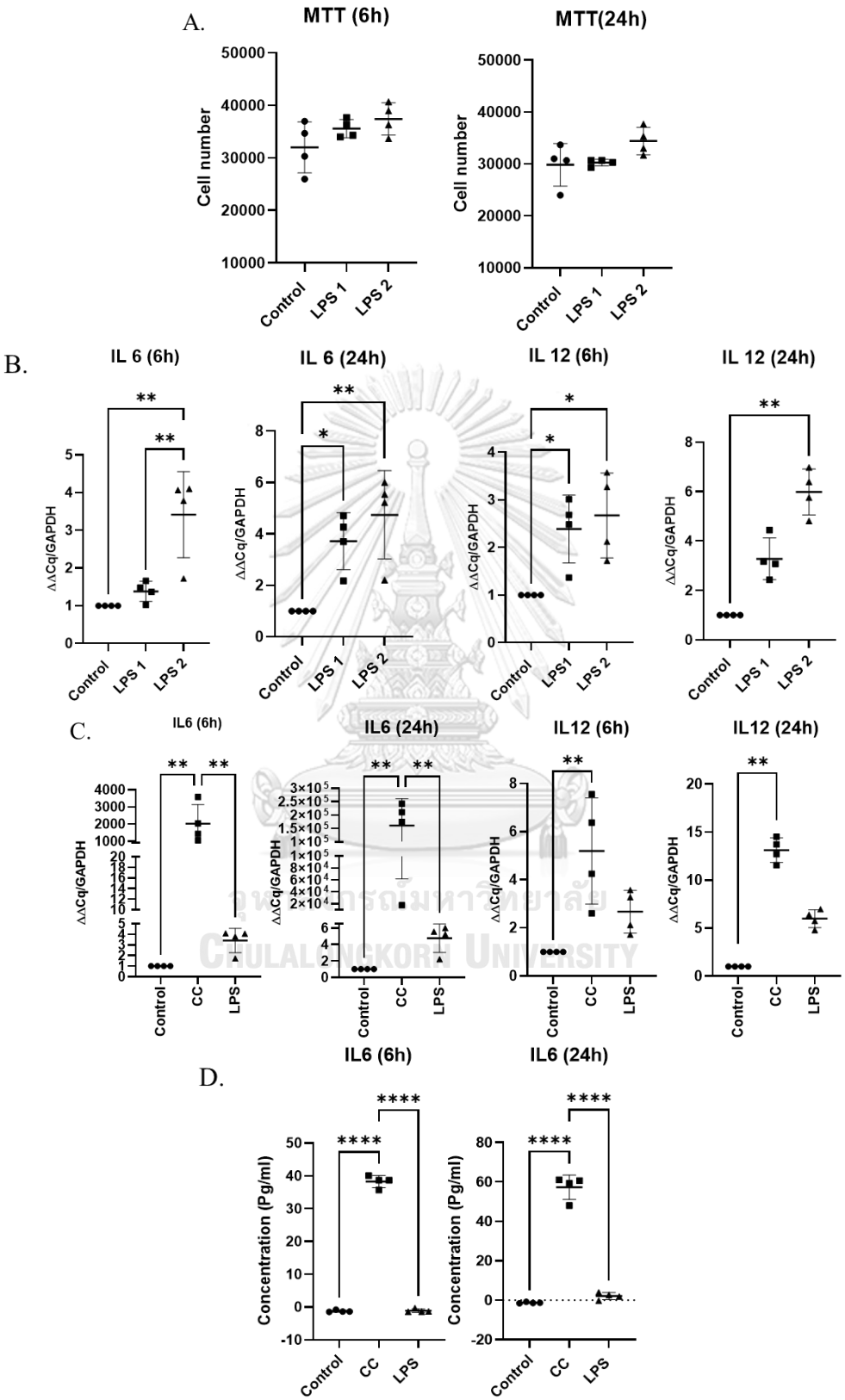

Figure 6. *CC treatment can upregulate more expression of IL-6 and IL-12 mRNA and IL-6 protein than LPS treatment on HDPCs. MTT assay result of HDPCs treated by LPS1 or LPS2 for 6 hours or 24 hours to assessed viability of the cells(A). RT-qPCR performed to measured the expression of IL-6 and IL-12 mRNA after treatment of two concentration of LPS (B). Comparison of expression of IL-6 and IL-12 mRNA after treatment of CC2 or LPS2 (C). Protein level of IL-6 after treatment of CC2 or LPS2 measured by ELISA then compared (D) , n=4*

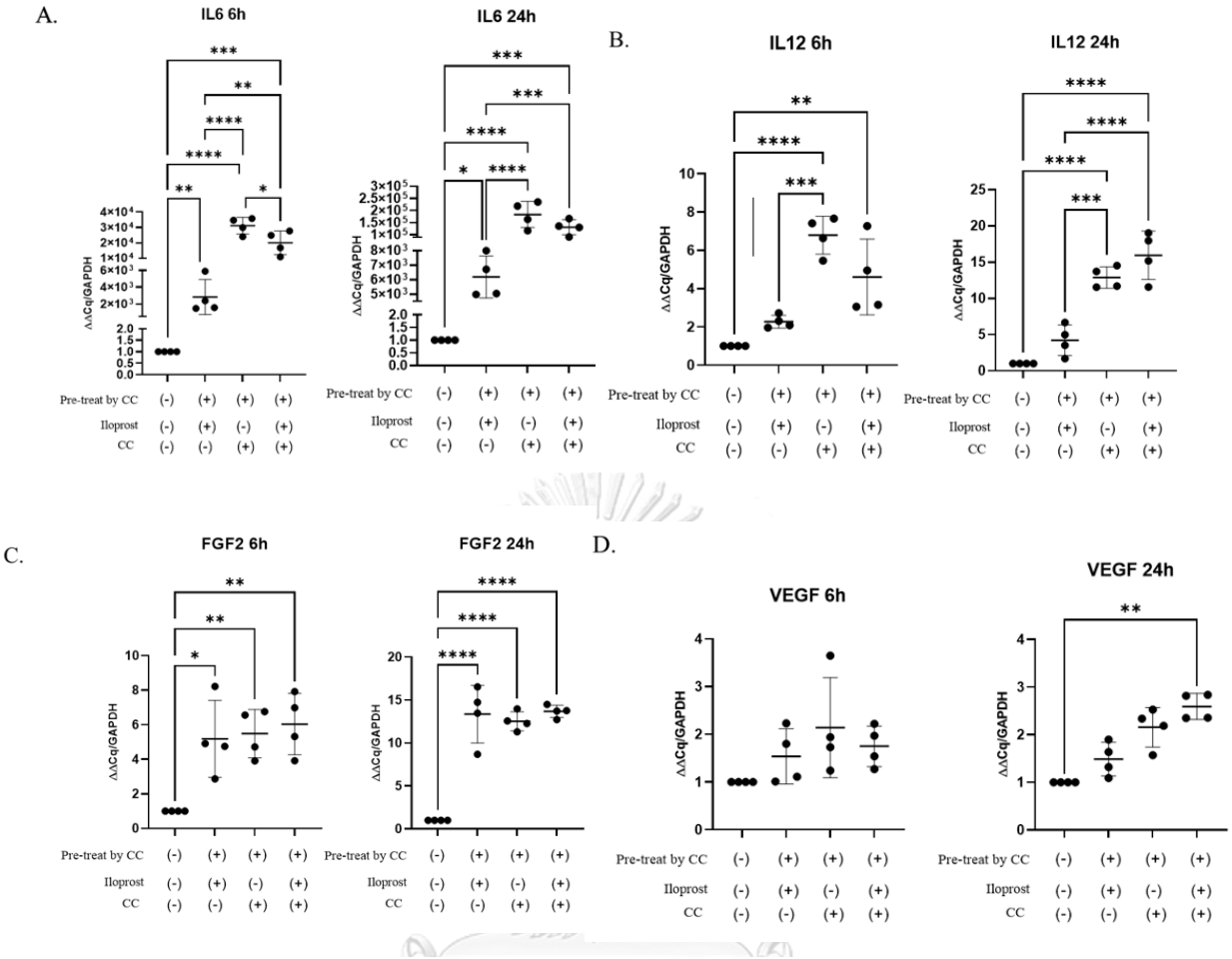

Figure 7. Iloprost treatment on inflamed model of HDPCs induced by CC can downregulate IL-6 and IL-12 gene. Inflamed HDPCs model was induced by CC treatment for 6 hours. Then, the conditioned media was changed to SFM with iloprost (10^{-6} M), or SFM with CC, or combination of iloprost and CC for 6 hours or 24 hours treatment. The expression of IL-6 and IL-12 mRNA were measured by RT qPCR (A,B). Also, expression of FGF2 and VEGF were measured by RT qPCR (C,D), n=4.

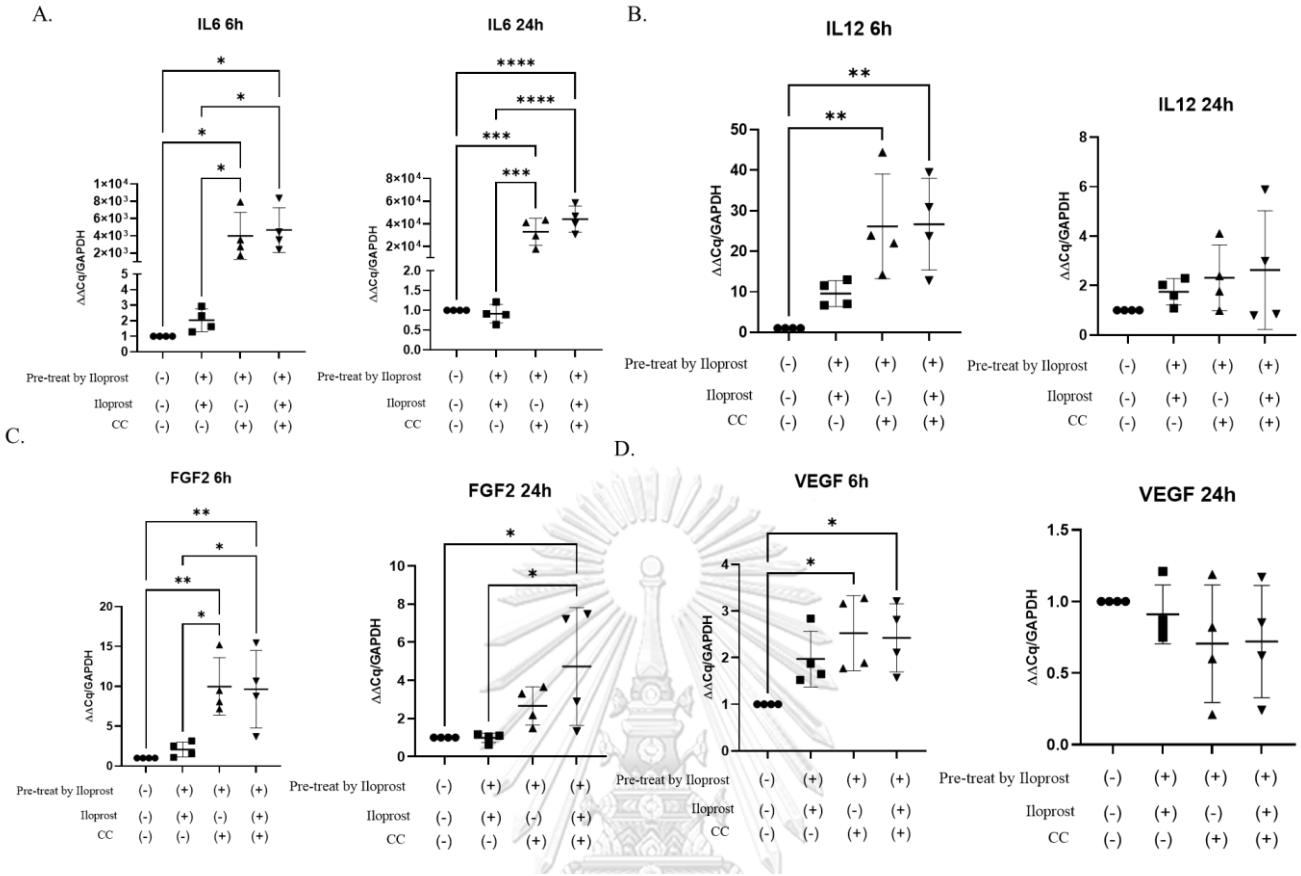

Figure 8. Iloprost pre-treatment before CC couldn't downregulate IL-6 and IL-12 on HDPCs. HDPCs was pre-treated with iloprost for 6 hours. Then, the conditioned media were changed to SFM with iloprost, or SFM with CC or combination of iloprost and CC for 6 or 24 hours treatment. The expression of IL-6 and IL-12 mRNA were measured by RT qPCR (A,B). Also, expression of FGF2 and VEGF were measured by RT qPCR (C,D), n=4.

CHAPTER V

DISCUSSION AND CONCLUSION

This study investigated the use of pro-inflammatory cytokines to induce acute inflammation biomarkers in HDPCs, and the anti-inflammatory properties of iloprost in the proposed inflamed model. The MTT results showed the cell number of the HDPCs was not altered significantly post-treatment with either cytokine cocktail or LPS in two kinds of concentration. Cytokine cocktail with concentrations of IL1β (1ng/mL), TNFα (10 ng/ml), and IFNγ (100 ng/mL) could upregulate IL-6 and IL-12 significantly on both time points of treatment. Because of that, CC2 was selected as the working concentration to create mimicked acute inflammation pulp cells. The comparison result between CC treated vs LPS treated showed that the upregulation of IL-6 and IL-12 mRNA expression was higher in CC treated HDPCs than LPS treated. In healthy pulp, the IL-6 expression protein is around 0.01 pg/mg, and in pulpitis is around 39 pg/mg (81). Our study showed CC could upregulate the IL-6 protein around 35-40 pg/ml in 6 hours. This finding, when compared to the clinical situation, suggests that the inflammation model induced by cytokine cocktail was better as mimicked acute pulpitis model *in vitro* than the one treated by LPS P.Gingivalis. In the clinical situation, both gram-negative and gram-positive bacteria on caries produce pathogens (LPS or LTA) that will bind to their receptors on the cytoplasm and activate the Nf-kB signaling pathway that leads to the production of pro-inflammatory cytokines including IL-6 and IL-12 (35, 36, 82). IL-6 expressed more intensity in acute pulpitis than chronic and healthy human dental pulp tissue (43). Meanwhile, Streptococcus mutant, the gram-positive bacteria are known as the main causative in the pathogenesis of dental caries, upregulated the expression of IFN-γ, and high expression of IFN-γ can induce IL-12 (45). For these reasons, IL-6 and IL-12 are selected as biomarkers of acute inflammation in this study.

The previous *in vitro* study used a cocktail of IL-1β, IFN-γ, and TNF-α, with a concentration ratio of 1:10:100 on gingival mesenchymal stem cells and mouse primary osteoblast, increased expression of NO and PGE2 concentration (55, 78). On gingival mesenchymal stem cells, when induced by cytokines cocktail of IL-1β, TNF-α, and IFN-γ of 1: 10:100 ratio, MTT result showed a decrease of cell number significantly on 72 hours post cocktail treatment (78). Hence, the maximum time point for this study is less than 72 hours. Since one of the hallmarks of acute inflammation is the high infiltrate of neutrophils that last until 24 to 48 hours post-infection, for that reason, 6 and 24 hours were chosen as time points on this study (From the maximum time point (24 hours), a quarter of it (6 hours) picked as the initial time point to mimicked the acute period (79). The induction of IL-6 *in vitro* has been done before in many ways. Since the

expression of IL-6 is related to the activation of Nf-kB, scientists used many reagents to stimulate this pathway. For example, previous studies used a single pro-inflammatory cytokine, LPS, or substance P to induce IL-6 expression in HDPCs (82-84). However, the effect of cytokines cocktail that consists of IL-1β, TNF-α, and IFN-γ with the ratio of 1: 10:100, on IL-6 and IL-12 expression on dental pulp cells has not been proposed before. Cytokines produce and maintain the body's immune response to microbial infections. these molecules are produced by living cells to provide necessary signals. When the pro-inflammatory cytokines increase, they will trigger a series of signaling cascades that will lead to inflammatory reaction patterns, such as edema, fever, pain, and hyperemia. If the pathogen persists and the pro-inflammatory cytokines are maintained at a high level, the inflammatory reaction will start to damage healthy cells, tissues, and organs. If this chronic condition continues, it can lead to necrosis (10, 13). Inflammation is an important process for healing, however, for the regeneration process to occur, the balance between pro-inflammatory and anti-inflammatory cytokines is crucial to maintain the homeostatic conditions (14). From all of those reasons, it is important to control the inflammation.

Iloprost can upregulate the expression of IL-10, which acted as an anti-inflammatory cytokine by inhibiting the Nf-kB (21, 22, 75, 85). The increase of IL-10 induced by iloprost was probably the reason why IL-6 and IL-12 expression induced by CC, was downregulated in this study. We also investigated if iloprost has cytoprotective properties by incubating the HDPCs with iloprost 10^{-6} M for 6 hours before CC treatment. The results showed significant upregulation of the IL-6 gene on both time points on CC-treated groups and also IL-12 gene expression on 6 hours time point compared to the iloprost-treated only group. Suggesting that 10^{-6} M of iloprost couldn't act as a cytoprotective agent. Nonetheless, Iloprost's anti-inflammatory properties on inflamed HDPCs induced by CC open the possibility of iloprost to be used as a candidate agent for pulp capping materials in reversible pulpitis.

Iloprost is shown to be able to induce angiogenesis, and upregulate expression of FGF2 and VEGF in previous studies. These two genes are important for angiogenesis. Another study also found that angiogenesis increased new vessel formation, and induced collagen deposition in tooth slice organs treated by iloprost (19, 20). However, in this study, iloprost showed no significant impact on the expression of FGF2 and VEGF mRNA post CC treatment. The expression of FGF2 showed no significant difference between those samples treated by CC+iloprost and CC only. Whereas, the mixture of CC+iloprost was capable to increase the expression of VEGF significantly on 24 hours time point. Iloprost can block NFkB nuclear translocation by inducing IL-10 (22, 75). The study by Wu et. Al suggested that IL-10 expression can dampen the expression of VEGF which is produced by macrophages 1 in inflammation (86). Moreover, IFN-γ and IL-12 are known to act as an anti-angiogenic factors (87). Our study used a cocktail of cytokines that

contain IFN-γ, and the CC treatment induced the expression of IL-12 significantly. All of these factors can interfere with the VEGF expression in this study.

An ideal treatment outcome of the pulp exposure is to regain the primary structure of tubular dentin as well as maintain the vitality of the dental pulp. Achieving this condition requires dental materials with antibacterial, anti-inflammatory, and dentin-pulp tissue regenerative properties (17). Iloprost could promote neovascularization in dental pulp tissue(88). Iloprost also could block NF-kB nuclear translocation by inducing IL-10 that will be modulated by IP-dependent upregulation of intracellular cAMP and also hampering the signaling axis of NF-kB activity, hence inhibiting the production of IL-6 and IL-12 (22, 75). The finding on this study added by the literature review showed that the proposed cytokine cocktail on this study offered a better model to mimic acute inflammation in HDPCs *in vitro*.

In conclusion, the novel mimicked inflammation model in HDPCs by cocktail of IL-1β, TNF-α, and IFN-γ in ratio of 1:10:100, that we proposed on this study can upregulated expression of two biomarkers of acute inflammation (IL-6 and IL-12) higher than LPS treated HDPCs. Using our proposed mimicked acute inflammation model, iloprost treatment can downregulate the expression of IL-6 and IL-12. This finding open the potential of iloprost to be used as vital pulp therapy additional material to help maintain the vitality of pulp during the vital pulp therapy procedure. We proposed the pathway that involve including Nf-kB signaling pathway that induced by the cytokine cocktail and become major regulator of increased IL-6 and IL-12. The binding of iloprost to IP receptor will increase the expression of cAMP that lead to upregulation of IL-10 which act as inhibitor of Nf-KB. Hence created anti-inflammatory effect from iloprost (Figure 9). However, this study was performed in primary dental pulp cell culture whereas the real clinical situation is composed of a mixed population of cells and blood circulations. With a low-compliance environment, thus the present acute inflammation model *in vitro* may not represent the clinical acute inflammation. Also, the pathway that we proposed was only supported by the literature review and not experimental report in this study. For all those reasons, further investigations *in vivo* models and clinical experiments are needed.

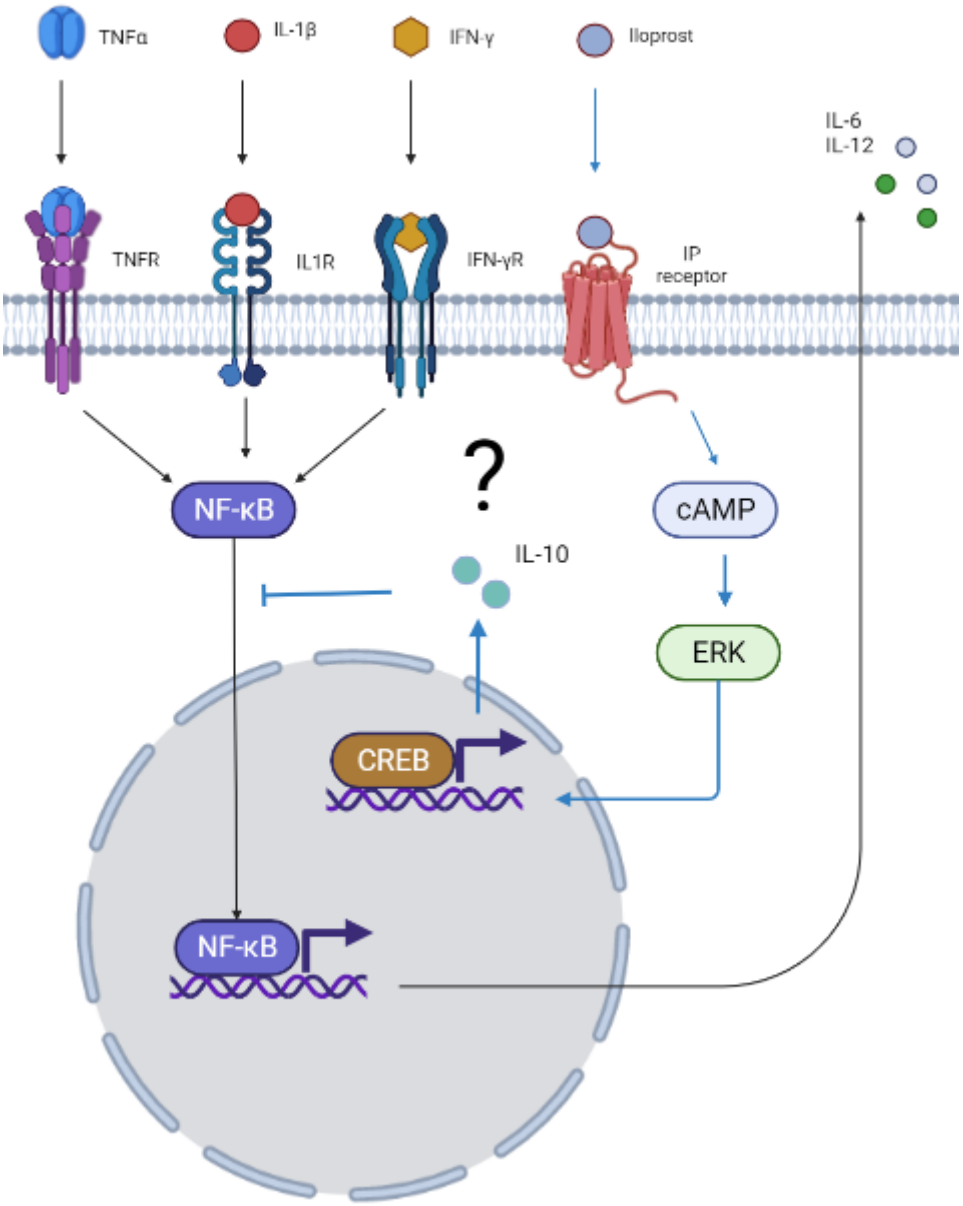

Figure 9. The proposed pathway of cytokines cocktail and iloprost. The cytokine cocktail that contain IL-1β, TNF-α, and IFN-γ will upregulate expression of IL-6 and IL-12 via NF-kB signaling pathway. Meanwhile iloprost will bind to PGI2 receptor which will activate ERK via cAMP. Activation of ERK will upregulate expression of IL-10 that act as inhibitor for Nf-kB signaling pathway.

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