การประยุกต์ใช้เพลทเลท ริช ไฟบรินที่ได้จากเลือดของตัวเองในการแก้ไขภาวะโรคปริทันต์ในสุนัข

นางสาวฉัตรวดี กรสุทธิโสภณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาศัลยศาสตร์ทางสัตวแพทย์ ภาควิชาศัลยศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

Application of autologous blood-derived platelet-rich fibrin for periodontitis treatment in dogs

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Surgery
Department of Veterinary Surgery
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2017

Thesis Title	Application of autologous blood-derived platelet-rich fibrin for periodontitis treatment in dogs
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ฉัตรวดี กรสุทธิโสภณ : การประยุกต์ใช้เพลทเลท ริช ไฟบรินที่ได้จากเลือดของตัวเองในการแก้ไขภาวะโรคปริทันต์ในสุนัข (Application of autologous blood-derived platelet-rich fibrin for periodontitis treatment in dogs) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ชนินทร์ กัลล์ประวิทธ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร.นพดล พิฟ้ารัตน์, 80 หน้า.

โรคปริทันต์ ถือเป็นหนึ่งในโรคการอักเสบของช่องปากที่พบได้บ่อยในสุนัข ในระยะที่มีการอักเสบเรื้อรัง พบว่ามีความจำเป็นที่จำต้องได้รับการผ่าตัดแก้ไขทางด้านปริทันต์ รวมถึงการถอนฟัน ซึ่งก่อให้เกิดผลข้างเคียงต่างๆ อาทิเช่น การมีเลือดออก การเกิดบาดแผล ระยะเวลาในการวางยาสลบและการพักฟื้นที่นานขึ้น รวมถึงทำให้ขบวนการหายของบาดแผลเกิดขึ้นได้ช้า ดังนั้น เพื่อหลีกเลี่ยงผลข้างเคียงดังที่กล่าวมา ในการศึกษาครั้งนี้ จึงได้ทำการศึกษาการใช้เพลทเลทริชไฟบรินเพื่อนำมาใช้เป็นวิธีรักษาโรคปริทันต์ โดยมีวัตถุประสงค์เพื่อวัดประสิทธิภาพของเพลทเลทริชไฟบรินที่ได้จากเลือดของตัวเองในการแก้ไขภาวะโรคปริทันต์ในสุนัข โดยทำการศึกษาในฟันกรามน้อยซี่ที่ 4 ของฟันกรามบน และฟันกรามใหญ่ซี่ที่ 1 ของฟันกรามล่าง ในการทดลองครั้งนี้ สุนัขที่มีสุขภาพช่องปากปกติ ถูกนำมาใช้เป็นกลุ่มควบคุมทั้งหมด 2 ตัว จำนวนซี่ฟันที่ทำการทดลองทั้งหมด 5 ซี่ (n=5) ส่วนกลุ่มทดลองนั้นประกอบด้วย สุนัขที่มีภาวะโรคปริทันต์ทั่วทั้งช่องปากจำนวน 10 ตัว ประกอบไปด้วยจำนวนซี่ฟันที่ทำการทดลองทั้งหมด 40 ซี่ โดยแบ่งออกเป็นสองกลุ่มโดยใช้การทดลองแบบสุ่มแบ่งครึ่งช่องปาก กลุ่มทดลองประกอบไปด้วย กลุ่มที่ทำการรักษาแบบการผ่าตัดเปิดแผ่นเหงือกเพื่อขูดทำความสะอาด (n=20) และกลุ่มที่ทำการรักษาแบบการผ่าตัดเปิดแผ่นเหงือกเพื่อขูดทำความสะอาดร่วมกับการใช้เพลทเลทริชไฟบริน (n=20) ตัวแปรในการศึกษาทางคลีนิก ได้แก่ ดรรชนีคราบหินปูน (PI) ดรรชนีสภาพเหงือก (GI) ร่องลึกปริทันต์ (PPD) และดรรชนีการคลอนของฟัน (MI) ได้ถูกตรวจสอบก่อนการทำการทดลองและหลังทำการทดลอง 7 วัน 14 วัน 21 วัน และ 56 วัน ในการวิเคราะห์กระดูกขากรรไกร ทำการถ่ายภาพรังสีในช่องปากในช่วงก่อนทำการทดลอง 21 วัน และ56วันหลังการทดลอง การวิเคราะห์ทางจุลพยาธิวิทยา ซึ่งประกอบไปด้วยคะแนนการอักเสบ และคะแนนการสร้างเนื้อเยื่อ จะถูกตรวจสอบก่อนทำการทดลอง และหลังทำการทดลอง 14 วัน นอกจากนี้ ยังมีการทำการทดสอบการแสดงออกของยีนส์ไซคายน์กลุ่มที่ออกฤทธิ์ต้านการอักเสบ และออกฤทธิ์กระตุ้นการอักเสบ โดยทำการทดสอบก่อนทำการทดลอง 7 วันและ 14 วันหลังทำการทดลอง

จากผลการทดลอง พบว่า กลุ่มที่ทำการรักษาแบบการผ่าตัดเปิดแผ่นเหงือกเพื่อขูดทำความสะอาดร่วมกับการใช้เพลทเลทริชไฟบริน มีการลดลงของดรรชนีสภาพเหงือกอย่างมีนัยสำคัญในวันที่ 14 หลังทำการทดลอง มีร่องลึกปริทันต์ลดลงอย่างมีนัยสำคัญในวันที่ 21 และ 56 หลังทำการทดลอง จากการวิเคราะห์ทางจุลพยาธิวิทยา พบว่าคะแนนการอักเสบลดลงอย่างมีนัยสำคัญในวันที่ 14 หลังทำการทดลอง รวมถึงมีการเพิ่มระดับของไซโตคายน์ลดการอักเสบ และการลดระดับของไซโตคายน์เพิ่มการอักเสบอย่างมีนัยสำคัญ จึงกล่าวโดยสรุปได้ว่า เพลทเลทริชไฟบรินที่ได้จากเลือดของตัวเองน่าจะเป็นหนึ่งในวัสดุทางเลือกเพื่อนำไปใช้ในการรักษาโรคปริทันต์ในสุนัขได้

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา ศัลยศาสตร์ทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5875306631 : MAJOR VETERINARY SURGERY

KEYWORDS: PERIODONTITIS / PLATELET-RICH FIBRIN / DOG

CHATVADEE KORNSUTHISOPON: Application of autologous blood-derived platelet-rich fibrin for periodontitis treatment in dogs. ADVISOR: ASSOC. PROF. CHANIN KALPRAVIDH, D.V.M., M.Sc., D.T.B.V.S., CO-ADVISOR: ASSOC. PROF. NOPADON PIRARAT, D.V.M., Ph.D., D.T.B.V.P., 80 pp.

Periodontitis is one of the most prevalent inflammatory oral diseases in dog. Non-surgical managements are conventionally performed to obtain healthy oral status. With progressive periodontitis, advanced periodontal surgery and also dental extraction become warranted with major disadvantages of trauma, bleeding, prolonged anesthetic time and hospitalization, and wound healing impairment. Hence, use of platelet-rich fibrin has been studied and considered one of the novel approaches to augment in periodontitis management. This study aimed to evaluate the efficacy of autologous blood-derived platelet-rich fibrin in an aspect of periodontitis treatment in a canine models. In this study, the experiment was done at maxillary 4th premolar and mandibular 1st molar. Dogs with healthy oral status were served as a control (C group, n =5). Another 10 systemically healthy subjects presenting equally bilateral periodontitis were randomized into split mouth clinical design. Experimental groups included periodontitis group with conventional open-flap debridement (PD group, n = 20) and periodontitis group with conventional open-flap debridement and platelet-rich fibrin treatment (PD^{+} group, n = 20). Clinical parameters included plaque index (PI), gingival index (GI), periodontal pocket depth (PPD), and mobility index (MI) were evaluated at baseline, 7, 14, 21, and 56 days post experiment (DPE). Intra-oral radiography was undergone to assess alveolar bone at baseline, 21 and 56 DPE. Histopathological analysis concerning inflammatory and fibrosis score was evaluated at baseline and 14 DPE. Cytokine expression analysis via anti-inflammatory (TGF-β1, PDGF-B, VEGF-A, TIMP-1, COL1A1, and COL3A1) and pro-inflammatory cytokines (TNF-α and IL-1β) were evaluated at baseline, 7 and 14 DPE.

The results revealed that PD^{+} group presented significant improvement in GI (at 14 DPE), PPD (at 21 and 56 DPE), and inflammatory reaction score (at 14 DPE) compared with PD group. Also, significant up-regulation of anti-inflammatory cytokines (except for COL1A1) together with down-regulation of pro-inflammatory cytokines were observed. In conclusion, platelet-rich fibrin might be an alternative novel approach for periodontitis treatment in dogs.

(n represented surgical site)

Department:	Veterinary Surgery	Student's Signature	.....
Field of Study:	Veterinary Surgery	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

I am grateful to the 90th Anniversary of Chulalongkorn University and the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej, Overseas Academic Presentation Scholarship for Graduate Students, and the Faculty of Veterinary Science, Chulalongkorn University, for supporting my graduated study.

I would like to express my sincere thanks to my thesis advisor, Assoc. Prof. Chanin Kalpravidh and also my thesis co-advisor, Assoc. Prof. Dr. Nopadon Pirarat for give an opportunity, confidence, encouragement, and kindness. Their encouragement, time, and assistance during the research period and thesis preparation cannot be describable. Their expertise and remarkable aptitude have made my academic achievable.

I would like to express my sincere appreciation to all my thesis committee, Assist. Prof. Dr. Kumpanart Sundaravibhata, Assist. Prof. Dr. Nan Choisunirachon, and Assoc. Prof. Dr. Thanaphum Osathanon for their useful comments. Also, I would like to thank Assoc. Prof. Dr. Somporn Techangamsuwan, department of Pathology, Ms. Siripen Komolvanich, department of Veterinary Physiology and Mrs. Pussadee Makum from Veterinary Diagnostic Laboratory for their laboratory technical supports. Appreciation is extended to all of instructors, clinicians and officers in the Department of Veterinary Surgery and Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University for their helpful assistance and generosity.

I would like to thank truly all of my friends and colleagues for their sincere friendship, assistance and support. A special thanks is given to all patient dogs and their owners in this experimental study for their co-operation and devote time. Finally, I wish to thank my family for their unconditional love, support and encouragement as always and all along.

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CHAPTER I

INTRODUCTION

1.1 Importance and Rationale

Periodontitis (PD) is one of the most common inflammatory oral diseases in companion animals, affecting utmost 80% of all breeds of animals (Booij-Vrieling et al., 2010; Pieri et al., 2012). Moreover, it is conferred to be one of the most prevalent clinical problems in domestic carnivores especially dogs (Kortegaard et al., 2008). The incidence and severity are epidemiological correlated with age (Riggio et al., 2011). It is believed to be a multi-factorial disease: microbiology, behavior, environment, systemic, genetic, etc. (Albuquerque et al., 2012). Among the aetiological factors, plaque accumulation on a dental surface is believed to be an initiation of the disease, leading to progressive dental tissue destruction and eventually dental loss (Riggio et al., 2011).

The hallmark clinical features of PD are not specifically occurred in oral cavity, but it furthermore affects systemic manner (Pavlica et al., 2008). The local consequences include gingival recession, gingivitis, periodontal pocket formation, oro-nasal fistula, tooth mobility, bone loss, pathological fracture, etc. Other potential systemic consequences are endocarditis, hepatitis, and nephritis (Hardham et al., 2005; Pavlica et al., 2008).

PD comprises of 4 stages. Stage 1 and 2 are reversible with proper dental prophylaxis, dental home care, and additionally with topical or systemic antibiotic in case of stage 2. On the other hand, stage 3 and 4 are considered irreversible, which surgical managements such as conservative dental extraction and alternative crown lengthening are frequently performed with majority of disadvantages. For example, excessive bleeding, trauma, risk of infection, prolonged hospitalization, and increased expense could be occurred (Davis et al., 2016). As a result, the concept of wound healing augmentation and regenerative periodontology is currently focused to be a novel therapeutic approach in order to cure or diminish periodontal disease and achieve optimal

periodontal tissue regeneration. The strategy of wound healing and periodontal tissue regeneration therapies is to stimulate periodontal tissue regeneration from progenitor cells together with augment wound healing and reduce inflammation. Another goal is to replace the lost of injured tissue with physiologically equivalent engineered tissues (Liu et al., 2008). However, severe periodontal conditions, particularly severe alveolar bone loss found in stage 4, result in impossibility for periodontal healing and regeneration, which dental extraction is solely done to resolve the problem (Li et al., 2013). Thus, wound healing and regenerative approaches could be beneficial interventions for preventing the periodontal condition of stage 2 to become irreversible and subsequently replacing the surgical managements in stage 3. Conventional regeneration therapies, such as guided tissue regeneration, topical application of enamel matrix derivative, and use of various growth factors, can partially regenerate periodontal tissues (Liu et al., 2008). Since the oral tissue harbors unique progenitor populations residing within specialized extracellular matrix frameworks, recent approaches favor conducting a research in an aspect of using platelet concentrates as a natural scaffold that allows repopulation with the patient's own cells, thereby producing an autologous tissue-engineered organ (Verma et al., 2017). Platelet-rich fibrin (PRF), a second generation platelet concentrate, predominantly consists of a fibrin matrix richly integrated with platelet and leukocyte cytokines, which together acts as a bioscaffold providing matrix for cell migration and growth factors for favoring wound healing process and tissue regeneration (Dohan et al., 2006c; Li et al., 2013). Since it was first described by Choukroun et al. in 2001, there have been many researches to evaluate its role and potency in human periodontal regeneration. Nonetheless, evaluation of its efficacy in terms of periodontitis treatment, periodontal regeneration and oral wound healing in dogs has not yet been fully described.

This study aimed to evaluate the efficacy of using autologous blood-derived PRF to treat PD and augment periodontal tissue regeneration in dogs. The result would be able to demonstrate the application of PRF as a novel modality in an aspect of PD treatment and periodontal regeneration.

1.2 Objectives of Study

To evaluate the efficacy of using autologous blood-derived PRF to favor PD management by diminishing inflammation, enhancing wound healing, and augmenting periodontal tissue regeneration in dogs through clinical, radiologic, histopathological, and inflammatory cytokine expression analysis.

1.3 Research question

Could the autologous blood-derived PRF be able to favor PD management by diminishing inflammation, enhancing wound healing, and augmenting periodontal tissue regeneration in dogs?

1.4 Hypothesis

Application of autologous blood-derived PRF could be able to favor PD management by diminishing inflammation, enhancing wound healing, and augmenting periodontal tissue regeneration in dogs.

Keywords: Dogs, Platelet-rich fibrin, Periodontitis

1.5 Advantages of study

The autologous platelet-rich fibrin could be a novel modality to favor PD treatment in the aspects of diminishing inflammation, enhancing wound healing process, and augmenting periodontal tissue regeneration in dogs.

CHAPTER II

LITERATURE REVIEW

2.1 Periodontitis

2.1.1 General consideration

PD is the progressive inflammatory disease of teeth and periodontium, which is comprised of alveolar bone, cementum, and periodontal ligament. It is one of the most prevalent oral diseases founded in dogs and cats. It is defined as a multifactorial disease resulted from many aetiologies: microbiology, systemic, behavior, environment, immuno-incompetence, allergy, genetic disorder, etc. (Albuquerque et al., 2012).

Plaque adhered to a dental surface is a primary cause of the disease. It is a microbial biofilm made up with *Actinomyces* spp., *Streptococcus* spp., etc. as the early colonizers, embedded in a matrix of salivary glycoproteins and extracellular polysaccharides. Fundamentally, plaque accumulates on the supragingival dental surface and later on extends to the area of subgingival sulcus. In this particular area, microenvironment changes to facultative anaerobe condition, which promotes the growth of Gram-negative, motile, anaerobic bacteria: *Actinobacillus actinomycetemcomitans*, *Porphyromonas* spp., *Prevotella* spp., *Tannerellar forsythia*, and *Treponema* spp. The multiplying of these bacteria results in gingivitis, which is the onset of the periodontitis (Albuquerque et al., 2012; Murai et al., 2012). With the progressive extension, plaque combines with saliva organic substances: calcium carbonate, calcium phosphate, etc., and forms a dental calculus (Niemiec, 2008a; Niemiec, 2008b).

2.1.2 Stages of periodontitis

In general, PD is referred to gingivitis and periodontitis. Based on clinical features, it can be categorized into four stages: stage 1 (gingivitis), stage 2 (early PD), stage 3 (moderate PD), and stage 4 (advanced PD) (Albuquerque et al., 2012).

PD stage 1 is characterized by mild to severe gingivitis. In this stage, gingivitis can be just mild degree (slight gingival margin erythema), moderate degree (gingival swelling and bleeding on probe), or severe degree (severe gingival erythema with spontaneous bleeding with or without probe). Plaque accumulation is seen without attachment loss. The gingival sulcus remain in normal range. This condition is reversible with dental prophylaxis and sustained good quality of dental home care (Albuquerque et al., 2012; Iida et al., 2014).

PD stage 2 is characterized by gingivitis with 0-25% attachment loss. Bleeding on probe is occurred. Dental prophylaxis together with local or systemic antibiotics are warranted to prevent this condition to become irreversible (Niemiec, 2008a).

PD stage 3 is characterized by 25-50% attachment loss with the deepened of the periodontal pocket. Tooth mobility and bifurcation exposure may be observed. Apart from normal dental prophylaxis, dental extraction, crown lengthening and apical reposition flap may be warranted to maintain quality of oral health (Niemiec, 2008b).

PD stage 4 is characterized by more than 50% attachment loss with obvious toot mobility. Dental extraction is appropriate choice for this advanced stage. Chronic bacterial infection resulted from severe accumulation of calculus may augment the bacterial translocation to vital organs: heart, liver, kidney, etc. (Niemiec, 2008a).

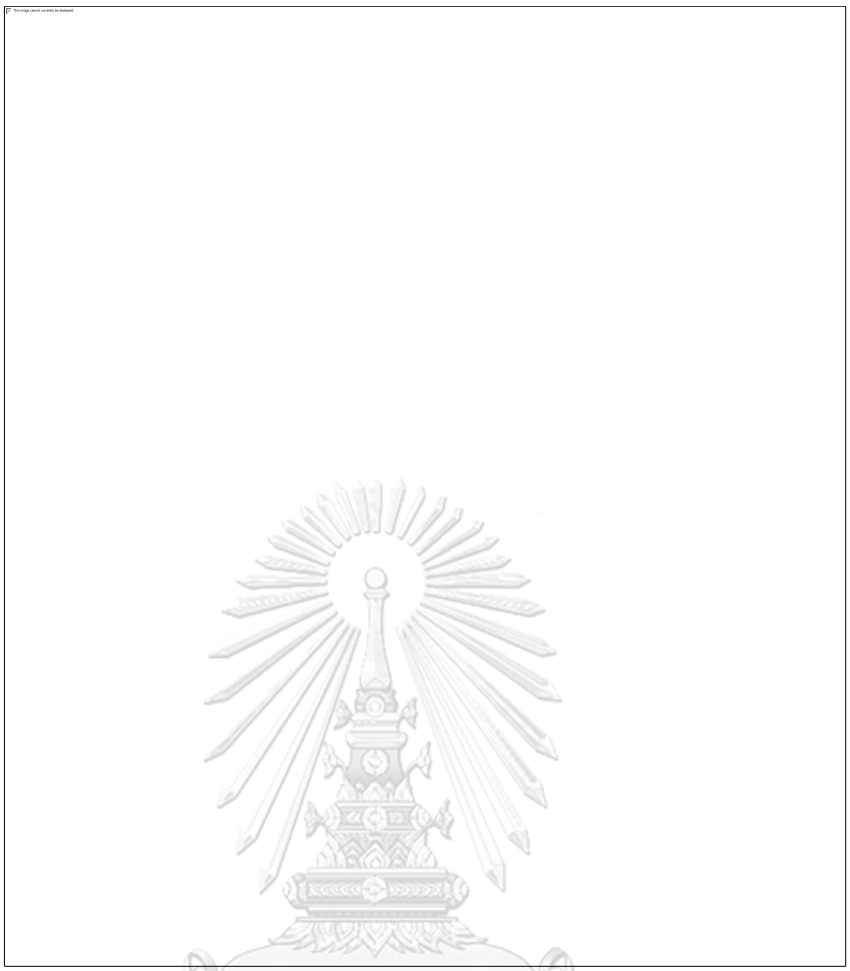

Figure 1 Stage of periodontitis in dogs and cats.

Available from: https://www.30avet.com/pet-dentistry.html

2.2 Oral wound healing

Wound healing is an integrated and complex cascade of biochemical events concerning cellular interactions, coagulation cascade, and large number of regulatory molecules including growth factors and cytokines. It is composed of sequential overlapping processes: hemostasis, inflammation, proliferation, and remodeling (Giuseppe et al., 2006). Initially, injury damages capillary and causes blood clot as a consequence of coagulation cascade. Blood clot induces hemostasis and functions as a temporary wound protection and also a matrix for inflammatory cells migration, which triggers inflammatory process. Platelets are activated and degranulated resulted in

releasing alpha granules (α-granules), which contains various growth factors such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) etc. PDGF together with other pro-inflammatory cytokines like interleukin-1 (IL-1) plays an important role in attracting neutrophils to the wound site to remove harmful pathogen and bacteria. TGF-β functions in converting monocytes to macrophages, which stimulates a release of pro-inflammatory cytokines and growth factors such as tumor necrotic factor-alpha (TNF-α), IL-1, interleukin-6 (IL-6), fibroblast growth factor (FGF), EGF, PDGF, and TGF-β itself. TNF-α and IL-1 are concerned as a major cytokines involved in pathogenesis of PD. TNF-α and IL-1 are associated with bone and tissue destruction by stimulating the release of prostaglandin E_2 (PGE_2) and MMPs (matrix metalloproteinases) (Guo and DiPietro, 2010; Smith et al., 2015). Proliferative phase normally occurs approximately 3days after inflammation. TGF-β, PDGF, VEGF, and FGF function as a stimulator for endothelial proliferation resulted in angiogenesis. This process is vital for synthesis, deposit, and organization of a new extracellular matrix (ECM) (Armstrong and Jude, 2002). Also, they stimulate fibroblastic infiltration and proliferation, which results in collagen and ECM formation. Collagen type III is primitively synthesized and then replaced by collagen type I in the remodeling phase (Lingen, 2001). Tissue remodeling involves in synthesis of collagen type I mediated by TGF-β incardinated with breakdown of old collagen mainly mediated by PDGF. Cellular activity also reduces and blood vessels are regressed by apoptosis (Braiman-Wiksman et al., 2007).

2.3 Platelet-rich fibrin

2.3.1 Development of platelet-rich fibrin

Platelet regenerative property was firstly introduced by Ross et al. in 1974, by describing a growth factor from platelets that stimulated the proliferation of monkey arterial smooth muscle cells in vitro (Naik et al., 2013). Since then, there have been many researchers studying on regenerative potency of platelet and platelet derivatives. The platelet concentrates are now focused regarding their regenerative and augmented wound healing properties. PRF is the second generation platelet concentrates offering

simplified and optimized production protocols compared with the first generation platelet concentrates (concentrated platelet-rich plasma, cPRP) (Dohan et al., 2006b). The concepts are derived from the fact that cPRP had been heavily used in various fields of medicine despite bearing the negative aspect of containing anticoagulants (Dohan et al., 2006c). PRF was first developed in France by Choukroun et al in 2001 for the use in the field of oral and maxillofacial surgery (Preeja and Arun, 2014). Since then, PRF has been extensively used in many dental procedures: extraction socket management, gingival recessions, intra bony defect regeneration, sinus elevation procedures, etc. (Dohan et al., 2006c).

2.3.2 Biologic features

Configuration

The 3-dimensional organization of a fibrin network affects the biologic and mechanical properties of the platelet concentrates. During gelling mode of these fibrin structures, the fibrin fibrillae can be assembled in 2 different biochemical structures: 1) condensed tetramolecular or bilateral junctions and 2) connected trimolecular or equilateral junctions. As fibrin glue as well as cPRP need an additional bovine thrombin to commence the last stage of coagulation, bilateral junctions of fibrin fibrillae are polymerized with strong thrombin concentrations, which in turn thicken the fibrin polymer. These thick rigid fibrin networks result in poor cytokine entrapment and cellular migration. On the contrary, characteristic of natural and slow polymerization during centrifugation as well as no need of additional bovine thrombin of PRF, the equilateral junctions of fibrin fibrillae are presented. These connected junction allows the forming of fine and flexible fibrin network, which favors cytokine entrapment and cellular migration (Dohan et al., 2006b; Preeja and Arun, 2014). In addition, glycosaminoglycan from blood and platelets incorporated within fibrin polymers has a strong affinity with cytokines and also have a great capacity to support cell migrations and healing processes. Thus, PRF is considered a better healing biomaterial compared with cPRP and other fibrin adhesives (Dohan et al., 2006c; Dohan et al., 2006d).

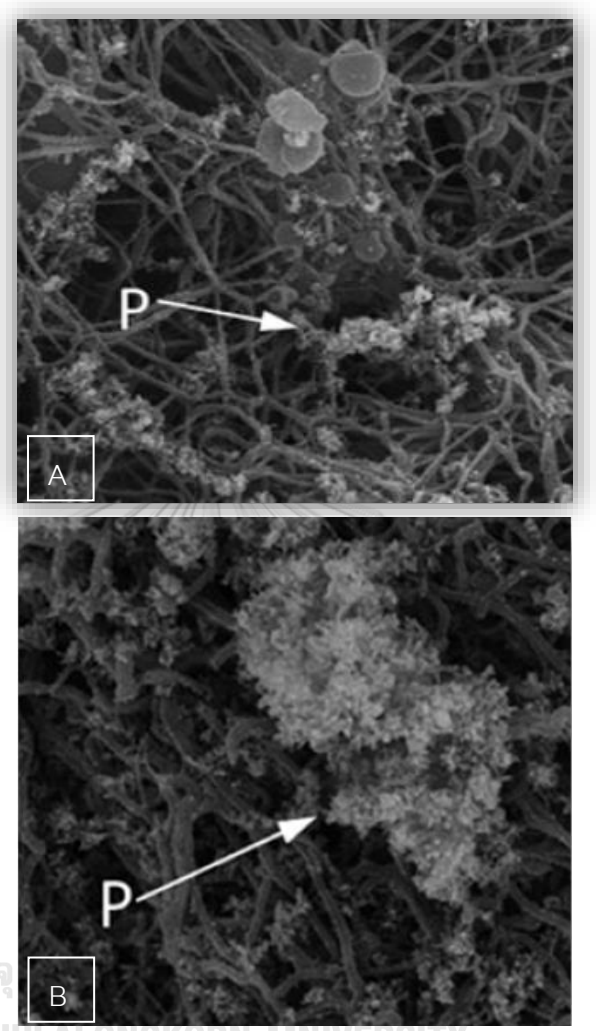

Figure 2 Theoretical computer modelling of fibrin branch. (A) Condensed tetramolecular or bilateral fibrin branch junctions. (B) Trimolecular or equilateral fibrin branch junctions (P represented platelets) (Dohan et al., 2006a).

Cytokines and growth factors

PRF predominantly consists of fibrin matrix rich in platelet and leukocyte cytokines owing to its tridimensional architecture fibrin matrix (Dohan et al., 2006b; Li et al., 2013). Cytokines and growth factors found in PRF play important roles in cell migration and proliferation. **α**-granules found in platelets act as a main reservoir for platelet cytokines.

During centrifugation, platelets are activated and their massive degranulation implies a very significant cytokine release leading to the first stage of healing (Dohan et al., 2006c). The combination of fibrin and cytokines within PRF becomes a powerful bioscaffold with an integrated reservoir of growth factors for tissue regeneration (Li et al., 2013).

Table 1 Key cytokines presented in platelet-rich fibrin and their functions (Khiste and Naik Tari, 2013)

Cytokines	Functions
Transforming growth factor-β (TGF-β1)	1) Stimulates proliferation of osteoblasts 2) Stimulates collagen type I and fibronectin synthesis 3) Enhances chemotaxis of osteoblast cells 4) Stimulates angiogenesis
Platelet-derived growth factor (PDGF)	1) Migration and proliferation of mesenchymal lineage cells 2) Angiogenic effect on endothelial cells
Vascular endothelial growth factor (VEGF)	1) Initiates angiogenesis
Insulin growth factor-1 (IGF-1)	1) Stimulates osteoblast proliferation

Cytokines	Functions
	2) Chemotactic effects towards human osteoblasts 3) Increased expression of osteocalcin 4) Enhances wound healing
Epidermal growth factor (EGF)	1) Stimulation of cell proliferation and extracellular matrix turnover 2) Chemotactic effect on periodontal fibroblast cells
Interleukin-1 (IL-1)	1) Key mediator for inflammation control 2) Stimulates T-helper lymphocytes
Interleukin-6 (IL-6)	1) Differentiation factor for B-lymphocytes 2) Activator for T-lymphocytes 3) Stimulates antibodies secretion

Cytokines	Functions
	4) Supports the chain reaction leading to inflammation, destruction and remodeling
Interleukin-4 (IL-4)	1) Supports activation and proliferation of active B cells 2) Supports healing by moderating inflammation 3) Increases fibrillary collagen synthesis by fibroblast
Tumor necrosis factor α (TNF-α)	1) Activates monocytes 2) Stimulates remodeling capacity of fibroblast 3) Increases phagocytosis and neutrophil cytotoxicity 4) Modulates IL-1 and IL-6 expression

2.3.3 Platelet-rich fibrin preparation protocol

PRF is a new generation of platelet concentrates developed with the concept of simplicity in preparation, biochemical blood handling avoidance and natural fibrin polymerization imitation (Dohan et al., 2006b). The production protocol requires neither

anticoagulants, bovine thrombin, nor any other geltifying agent, which makes it no more than centrifuged natural blood without additives (Choukroun et al., 2006). There are several preparation protocols with different rounds per minute (rpm), centrifugation speed, and tube angulation. According to Choukroun et al 2001, who firstly developed PRF, blood sample is taken in dry glass tube and centrifuged at 2700 rpm for 12 min with the 33 degrees angulation. This particular angulation is proved to be the best angulation allowed the red blood cell segment to move laterally to the tube's wall and form a rouloux pattern. The rouloux phenomena helps the sedimentation rate and leaves the platelets and leukocytes in the upper segment (Kobayashi et al., 2016). Since the potential of platelets as the sources of platelet cytokines, and white cells as the cell migration and proliferation stimulants has been proved, there have been many studies conducted to formulate a new protocol for platelet-rich fibrin with the goals of capturing more platelets and white cells in the fibrin matrix. In 2014, the new PRF, termed as advanced platelet-rich fibrin (1300 rpm-14 min; A-PRF), was firstly introduced by Ghanaati et al. (2014). By means of immunohistochemistry evaluation, A-PRF, produced by lower centrifugation force, was shown a better potential of capturing neutrophilic granulocytes, which play an important role in early inflammation and tissue regeneration (Ghanaati et al., 2014). Recently, the latest PRF, termed advanced platelet-rich fibrin plus (1300 rpm-8 min; A-PRF^{+}), was introduced by Masako et al. (2017) with the modification of centrifugation time and speed. The latest A-PRF^{+} preparation protocol was formulated with 2 proposed reasons: 1) reduction of centrifugation speed would achieve increased cell populations in the PRF collected from the top one-third layer, whereas the high centrifugation forces used in the previous platelet-rich fibrin preparation protocol would shift cell populations to the bottom of the tubes, 2) reduction of centrifugation time would reduce cell pull-down by centrifugation forces, which would increase cell populations in the platelet-rich fibrin matrix. According to Masako et al. (2017) and Fujioka-Kobayashi et al. (2016), A-PRF^{+} demonstrated a significant higher increase in platelet cell numbers, monocytes/macrophages behavior, and growth factor release compared with A-PRF. Also, in terms of tissue regeneration, the cultures of human gingival fibroblastic cells with

A-PRF and A-PRF^{+} were investigated and compared. Gingival fibroblastic cells cultured with A-PRF^{+} demonstrated a significantly higher levels of migration and proliferation compared with those cultured with A-PRF. Thus, A-PRF^{+} demonstrated an improved potential of cells capture and growth factor release, which in turn directly influenced tissue regeneration.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental animals

Experimental animals were recruited for the experiment from the clinical oral surgery cases presented at the oral section of surgery unit, the Small Animal Teaching Hospital of Chulalongkorn University. Breeds and genders were not specific. Inclusion criteria were as follows: 1) Mesocephalic dogs aged between 8 months to 5 years 2) Healthy dogs based on physical examination and laboratory test 3) Maxillary 4th premolars and mandibular 1st molar with periodontal pocket depth (PPD) between 3-5millimetres 4) No periodontal complications e.g. fractured teeth (Di Bello et al., 2014). Basic data (body weight, heart rate, respiratory rate, body temperature, history of previous dental procedure and dental homecare, etc.) were collected before starting the experiment. Other dogs under the condition of healthy oral status were served as controls. Dogs were considered ineligible for the study on the following criteria: 1) Systemic/metabolic/immunosuppressive disease 2) History of receiving anti-inflammatory drug within 30 days 3) The tooth that presented alveolar bone loss over 75% and mobility index (MI) grade 2 or higher (Sturgeon et al., 2014). An informed consent of the dogs' owners was required and all experiments were conducted in accordance with guidelines for animal welfare of experimental animals and approved by Chulalongkorn University Animal Care and Use Committee, Pathumwan, Bangkok, Thailand.

Firstly, maxillary 4th premolars and mandibular 1st molar from each dogs were scored individually following the modified Wiggs & Lobprise scoring system (Davis et al., 2016) and the American Veterinary Dental College classification of periodontitis stages (AVDC, 2009). Dental assessments and scoring were performed by only researcher to avoid variation. Any teeth matching with the inclusion criteria were included in the study. Control group was composed of a total of 5 surgical sites selected from dogs presented

under the condition of healthy oral status (Group 1, n=5). Another 40 PD sites were identified as mentioned in the inclusion criteria and divided into 2 groups using split mouth clinical design. They were categorized as Group 2 (n=20): Those to be treated with opened-flap debridement (OFD), and Group 3 (n=20): Those to be treated with OFD and PRF.

Group 1: healthy gingiva, no periodontitis (Control group, n=5)

Group 2: Periodontitis group treated with opened-flap debridement (PD group, n=20)

Group 3: Periodontitis treated with opened-flap debridement and platelet-rich fibrin (PD^{+} group, n=20)

(n = number of experimental surgical sites)

Figure 3 Maxillary 4th premolars (red arrow) and mandibular 1st molar (white arrow).

All studied sites in every group were investigated as follows:

i. Clinical evaluation was assessed on day 0, day 7, day 14, day 21, and day 56.

ii. Intra-oral radiograph was obtained on day 0, day 21, and day 56.

iii. Histopathological analysis was assessed on day 0 and day 14.

iv. Inflammatory cytokine expression analysis was assessed on day 0, day 7, and day 14

Data were recorded and compared with the other groups.

After categorization, full mouth dental scaling and polishing were undergone using air-driven dental unit. Root planning was performed using 11/12 Gracey curette (Hu-Friedy Mfg Co. Inc., Chicago).

3.2 Anesthetic protocol

Animals were given a premedication of 0.02 mg/kg acepromazine (2 mg/ml, Vetranquil; CEVA Sante Animal, France) and 0.3 mg/kg morphine (10 mg/ml) intramuscularly, then induced with 2-4 mg/kg propofol (10 mg/ml, Lipuro 2%; Braun, Germany) given intravenously. Then an endotracheal tube was placed using direct visualization of the trachea by a laryngoscope. Anesthesia was maintained with 2% inhalation isofurane. Local anesthesia of maxilla and mandible was obtained with 0.5% bupivacaine. Normal saline solution was administered intravenously throughout the operation. 22mg/kg cefazolin was given as a prophylaxis antibiotic (Dewhirst et al., 2012).

3.3 Surgical procedure

First of all, clinical evaluation and intra-oral radiograph were performed in each surgical site followed by full mouth dental scaling and polishing. After 0.5% bupivacaine administration as a regional nerve block, all studied sites were performed OFD via oft-modified Widman flap technique (“Modified Widman Flap,” MWF). The procedure was composed of internal bevel incision by means of scalpel blade no. 11, mucoperiosteal flap reflection using periosteal elevator, intrasulcular incision, and horizontal incision along the alveolar crest. Fine Gracey curettes were used to remove pocket epithelium and granulation tissue. Root planing was carried out using ultrasonic instruments and Grecey currette (Hu-Friedy Mfg Co. Inc., Chicago) without ostectomy and contouring osteoplasty. Then, mucoperiosteal flap was repositioned in place using a 4-0 monofilament absorbable suture material (monosyn®, B. Braun, Spain) in an interrupted interdental sutures pattern. The sutures were removed after a healing period of 7 days (Heitz-Mayfield et al., 2002).

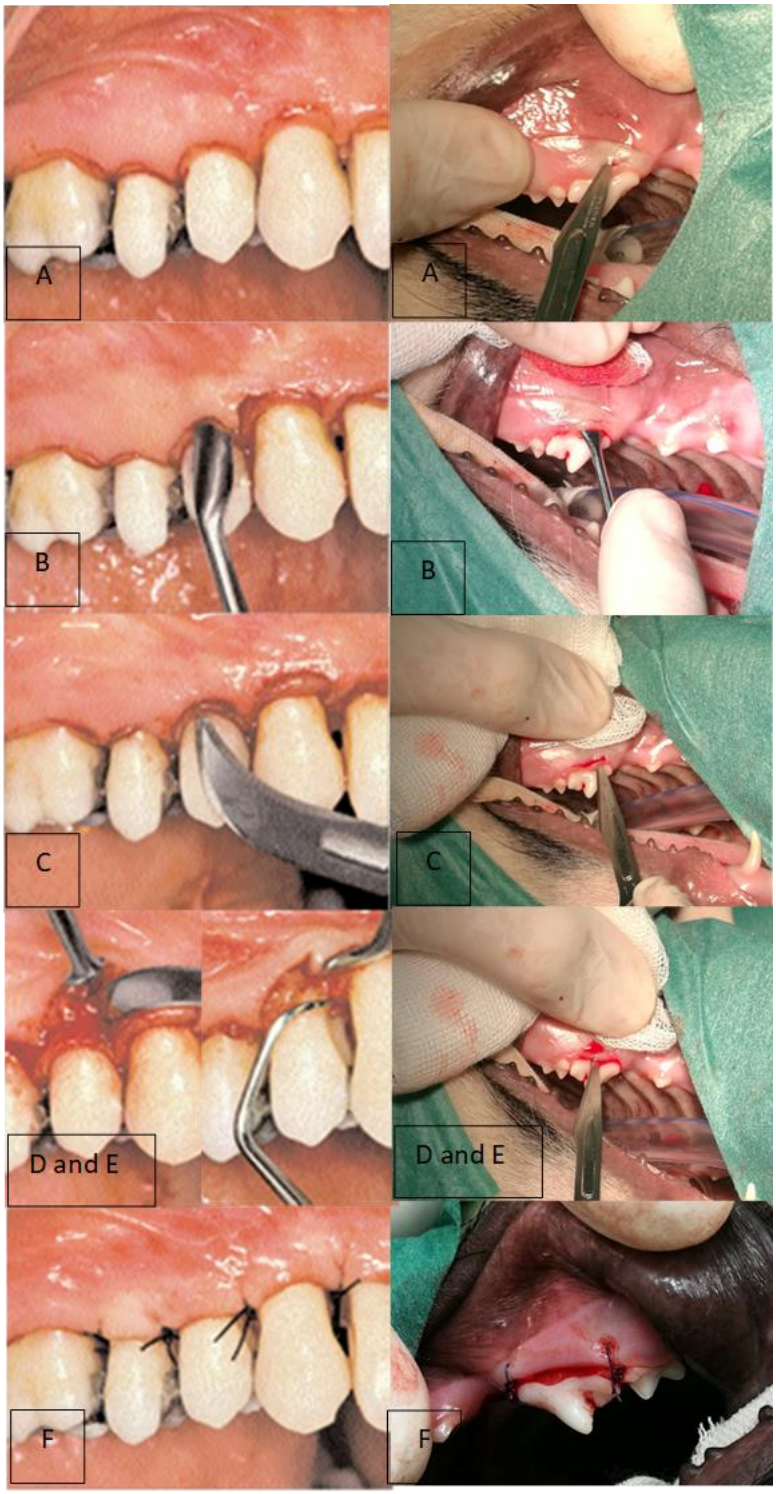

Figure 4 The Modified Widman Flap technique. (A) Internal bevel incision. (B) Mucoperiosteal flap reflection. (C) Intrasulcular incision. (D) Horizontal incision. (E) Pocket epithelium removal and root planing. (F) Interdental suture.

3.4 Platelet-rich fibrin preparation and administration

PRF was prepared according to the protocol of Masako et al. (2017) with slight modification. For each surgical sites of PD^{+} group (n=20), PRF harvested from 4ml of autologous venous blood collected from jugular vein was kept in dry glass tubes. Then, each tubes were immediately centrifuged for a period of 8 min at 1300rpm. After centrifugation, three layers were formed in the tube: a base of red blood cells at the bottom, acellular plasma on the top (supernatant), and a clot of PRF between the two layers (Dohan Ehrenfest et al., 2009a). The fibrin clot was picked up with forceps and scissors was used to separate fibrin clot from the lower part of the centrifuged blood at 1 mm below the apparent border between red blood cells fraction and fibrin clot in order to maximize the collection of platelet (Dohan Ehrenfest et al., 2009b; Watanabe et al., 2017). Then, the fibrin clot was compressed between two sterile gauzes to drive out the serum. After compression, PRF membrane was measured and cut in a 4x5mm membrane by means of scalpel blade. Also, the exudate serum collected from compression was used for graft material hydration, surgical site rinse, and autologous graft storage (Preeja and Arun, 2014). After the OFD procedure, prepared PRF was positioned over the denuded root surface just below the cemento-enamel junction (CEJ) (Aroca et al., 2008; Sharma and Pradeep, 2011). The mucoperiosteal flap closure was done in the same manner as the other groups.

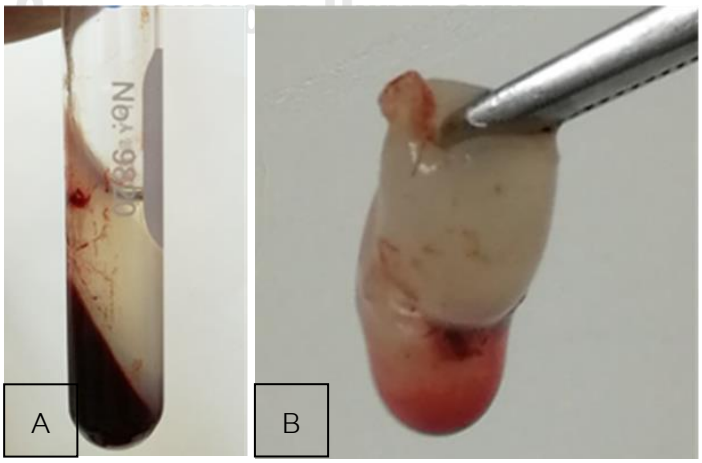

Figure 5 Platelet-rich fibrin preparation. (A) After at 1,300 rpm for 8 minutes. Modified from Masako et al. (2017) (B) PRF clot after separation from lower red blood cell layer.

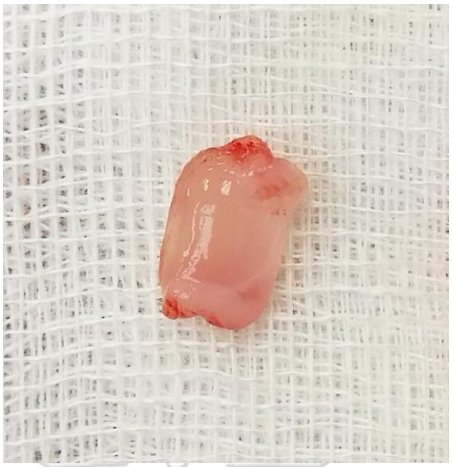

Figure 6 Platelet-rich fibrin membrane after gauze compression.

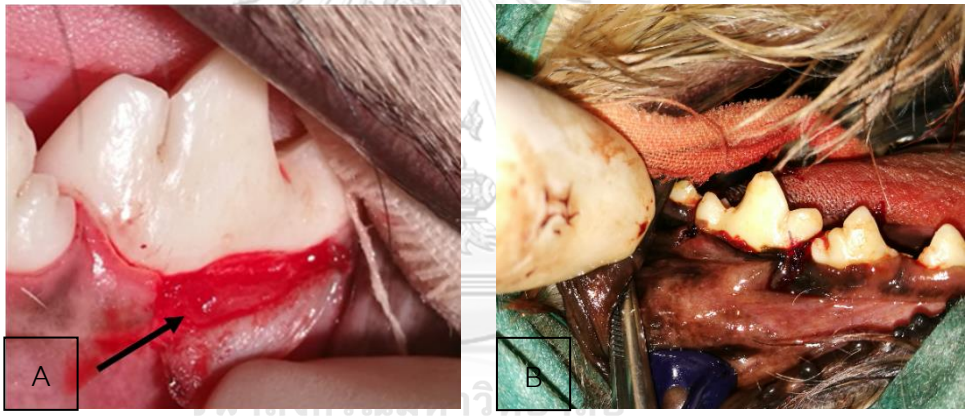

Figure 7 Platelet-rich membrane administration.

(A) Placement of platelet-rich fibrin over alveolar bone at cemento-enamel junction level.

(arrow: platelet-rich fibrin membrane)

(B) Mucoperiosteal flap was closed in an interdental suture pattern.

3.5 Post-operative management

Each dogs received 15mg/kg of amoxy-clavulanic acid and 4mg/kg of tramadol hydrochloride twice a day for 5 days. 0.12% chlorhexidine gluconate was used as a mouth wash and Hill's® Prescription Diet a/d® Canine/Feline was fed for a period of 7 days.

3.6 Clinical evaluation

Clinical evaluation was assessed as follows:

3.6.1 Plaque index (PI)

Plaque accumulation was clearly visualized by the use of disclosing solution (iC plaque®, iM3). The swab at the end of the applicator was rolled across the dental surface in order to stain plaque accumulation as a pink layer. PI was recorded as presence or absence of plaque on six surfaces of each tooth by using plaque index according to Silness and Loe (1964) and Zhang et al. (2013) as described below

Score 0: no plaque

Score 1: up to 25% of plaque accumulation on the surface of the tooth

Score 2: 25-50% of plaque accumulation on the surface of the tooth

Score 3: >50% of plaque accumulation on the surface of the tooth

Figure 8 iC plaque®, iM3.

Figure 9 Plaque index evaluation using iC plaque®, iM3.

3.6.2 Gingival index (GI)

Gingival inflammation was examined and scored on the mesial, distal, buccal and lingual surfaces using gingival index according to Silness and Loe (1964) and Zhang et al. (2013) as described below

Score 0: no inflammation and healthy periodontium

Score 1: mild inflammation, slight change in color, slight edema and no bleeding on probing

Score 2: moderate inflammation, moderate change in color and consistency and bleeding on probing

Score 3: severe inflammation, marked redness, hypertrophy, ulceration and spontaneous bleeding

3.6.3 Periodontal pocket depth (PPD)

PPD was measured on 6 different areas around each surgical sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) by means of William's probe. The probe was inserted until the tip encountered the resistance of junctional epithelium and positioned parallel to the long axis of the tooth. Mean value was calculated and recorded.

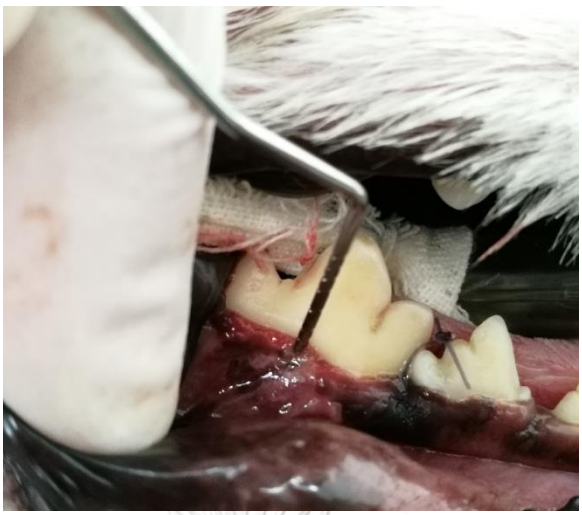

Figure 10 Periodontal pocket depth measurement using Wiliam's probe.

3.6.4 Mobility index (MI)

Tooth mobility was examined via the use of explorer. Mobility index was assessed according to Kerry et al. (1982) and Xu and Wei (2006) as described below

Score 0: normal physiology <0.2mm

Score 1: slightly mobile (bucco-lingual direction)

Socre 2: moderate mobility (bucco-lingual and mesio-distal direction)

Score 3: severe mobility (bucclingual, mesio-distal and vertical direction)

3.7 Intra-oral radiographic evaluation

The intra-oral radiographs were undergone for alveolar bone loss assessment. The intra-oral radiographs were taken with a Belmont DX 073 X-ray head at 70 kV, 10 mA (total filtration 2.1 mm AlEquiv) (Toschiba, Japan). Kodak Ektaspeed standard size film was used to obtain the radiograph with the technique of parallelism and developed in an automatic film processor (Du¨rr, XR42- II, Bieligheim, Germany). The intra-oral radiographs were digitized and processed using a custom made image analysis software program. All intra-radiographs were read by the same examiner throughout the experiment to prevent inter-examiner variation. Alveolar bone loss was assessed by direct measurements of the distance between the CEJ and the alveolar bone level (BL) as well

as the proportional values in relation to the root length (CEJ-BL/root length). The distance between the CEJ and the marginal BL was measured at three points on the mesial, middle, and distal aspects of each tooth to quantify the alveolar bone level. BL was defined as the most coronal location of the bone margin adjacent to a clearly visible periodontal ligament space. Similarly, the distance between the CEJ and the apex of the tooth was measured, and the proportional distance between CEJ and BL relative to the length of the root was calculated. A mean value for each tooth was recorded (Persson et al., 2003; Balci Yuce et al., 2014).

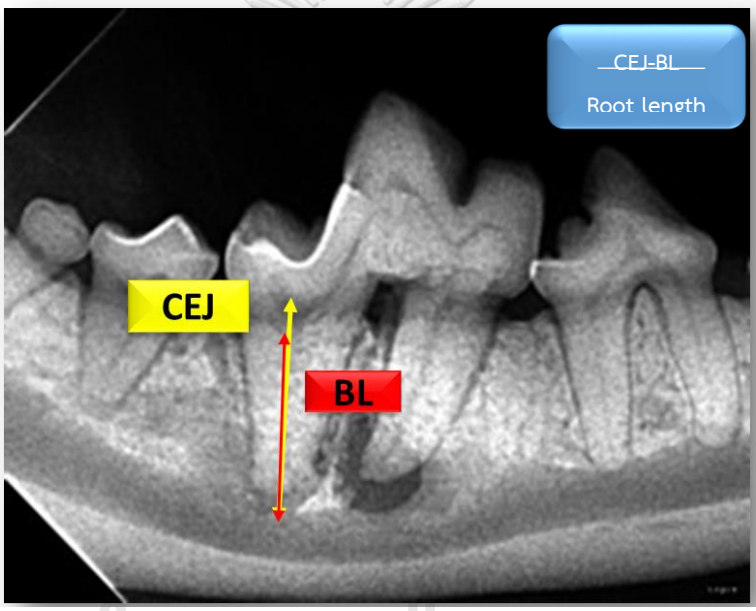

Figure 11 Intra-oral radiographic evaluation.

Abbreviations: CEJ: cemento-enamel junction; BL: alveolar bone level.

3.8 Histopathological analysis

Tissue samples measuring approximately 3 x 5 mm were collected from the middle buccal area via the three incision technique (Internal bevel, intrasulcular, and interdental incision) in the MWF in order to orient and present pocket epithelium, oral epithelium, and connective tissue in the same section (Takahashi et al., 1994). Samples were sent for histological analysis to assess the healing and inflammatory response. (de

Oliveira et al., 2011). The samples were fixed with 10% neutral buffered formalin for 48h and dehydrated through successive baths of Isopropyl alcohol (70%, 90%, 95%, and 100%). The samples were clarified in xylene and embedded in paraffin wax. Serial 5-μm-thick sections in mesio-distal plane were obtained via a rotary microtome (Leica RM2135). Afterwards, the samples were stained with hematoxylin-eosin (H&E) and Masson's trichrome, and examined for descriptive histology under Olympus BX41 microscope at 40X magnification. The bright field microscopic images were taken with an Olympus UC30 camera and processed using Olympus Stream Basic image analysis software. All histopathologic examinations were performed by the same pathologist (Ionel et al., 2015).

Regarding histopathological analysis, five areas were examined for inflammatory reaction and fibrosis. Mean value was calculated and recorded. The inflammatory infiltration and fibrosis were be evaluated as described below

3.8.1 Inflammatory reaction score

Firstly, inflammatory cells (neutrophils, lymphocytes, plasma cells, and eosinophils) were identified and individually counted as follows: Not present (0), Mild (1), Moderate (2), and Severe (3). Then, total scores of each classification were summed up and divided into three grades of inflammatory reaction (Furtado and Constantino-Casas, 2013).

Score 0-3: mild inflammation

Score 4-6: moderate inflammation

Score ≥7: severe inflammation

3.8.2 Fibrosis score

According to Balci Yuce et al. (2014), fibrosis score was classified as follows

Score 0: no reaction, no fibroblasts and fibrocytes

Score 1: fibroblasts and fibrocytes found between 1-35% of the fields

Score 2: fibroblasts and fibrocytes found between 36-70% of the fields

Score 3: fibroblasts and fibrocytes found between 71-100% of the fields

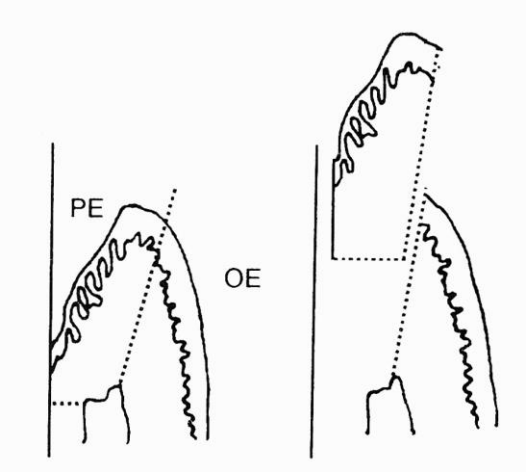

Figure 12 The schematic diagram of gingival tissue (Takahashi et al., 1994).

Abbreviations: PE: pocket epithelium; OE: oral epithelium.

3.9 Measurement of growth factor concentration in platelet-rich fibrin

The concentrations of TGF-β and VEGF-A in PRF were measured using commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA): TGF-β (Cat. No. DG100) and VEGF-A (Cat. No. DVE00). All kits used in the experiment were based on immunoenzymatic method and were produced by R&D Systems. TGF-β and VEGF-A from PRF were measured at baseline (immediately after preparation), 7 days after preservation at -20°C, and 14 days after preservation at -20°C.

After blood collection, 3 pieces of PRF were prepared from each dogs in experimental groups according to earlier mentioned protocol. After PRF preparation, 1 piece of fibrin clot was cut into small pieces by scalpel blade and then homogenized by disposable homogenizers (BioMasher II, Nippi, Inc., Tokyo, Japan). After centrifugation at 3000rpm for 10 min at 4°C temperature, the supernatant was collected and stored at -80°C until ELISA processing (Masuki et al., 2016). Other 2 pieces were preserved at -20°C until termination day. The measurements of the growth factor and cytokine levels were performed according to the manufacturer's instructions. The standard and sample solutions were pipetted on a 96-well plate. After the corresponding incubation time, the 96-well plate was washed with a prepared washing buffer. Afterwards, a solution of

corresponding polyclonal antibodies (conjugated to horseradish peroxidase) was added and incubated. After another washing procedure, the substrate solution was added to start the enzyme-catalysed reaction, which was stopped by the addition of a stop solution. The concentrations of the growth factors and cytokines were photometrically determined using the Tecan Sunrise photometer (Tecan Group Ltd.). Standard dilutions of isolated growth factor and cytokine solutions were used to create calibration curves. The actual growth factor and cytokine concentrations were calculated by the optical densities using the calibration curves and the Table Curve 2D software, version 4.0 (Systat Software Inc., Point Richmond, CA, USA) (Christgau et al., 2006).

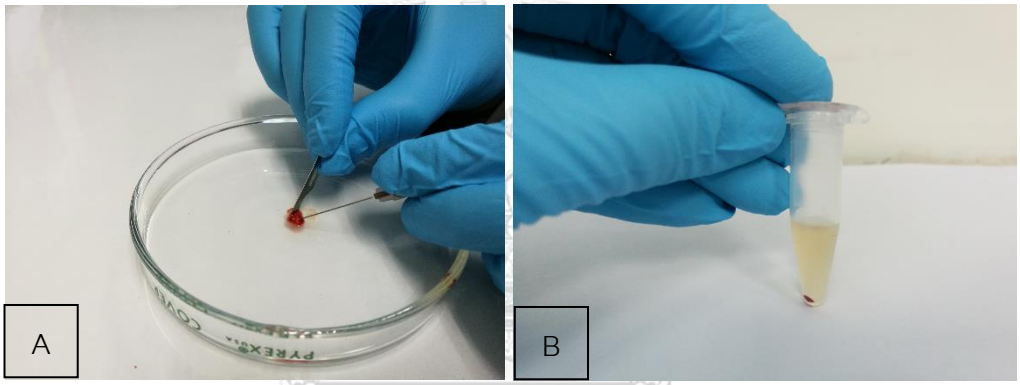

Figure 13 Preparation for cytokine concentration measurement. (A) Platelet-rich fibrin membrane was cut into pieces by scissors. (B) After homogenization and centrifugation, supernatant was collected to determine the cytokine concentration via ELISA technique.

3.10 Inflammatory cytokine expression analysis

3.10.1 Sample collection and processing

Gingival tissue samples collected from the edges of the defect sites were harvested from the mid-buccal area by means of scalpel blade and tissue scissors at baseline, 7 DPE, and 14 DPE to evaluate for transforming growth factor-beta 1 (TGF-β1), platelet-derived growth factor subunit B (PDGF-B), vascular endothelial growth factor variance A (VEGF-A), tissue inhibitor matrix metalloproteinase-1 (TIMP-1), collagen type I alpha 1 chains (COL1A1), collagen type III alpha 1 chains (COL3A1), tumor necrotic

factor-alpha (TNF-α), and interleukin 1-beta (IL-1β) gene expression level by quantitative real-time polymerase chain reaction (qPCR) technique. Samples were pooled in 1000µl of phosphate-buffered saline (PBS, 0.01 M, pH 7.2) solution and stored at -80°C until processed for RNA extraction.

3.10.2 RNA isolation

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the protocol provided by the manufacturer. RNA samples were treated with RNase-Free DNase I (Qiagen, Netherland) to remove contaminated genomic DNA. The RNA concentration and purity were measured by a Nanodrop spectrophotometer (Thermo Scientific, USA). Adequate purity with *A*260/*A*280 ratios ranging between 1.80 and 1.99 was acceptable. RNA samples were dissolved in RNA storage solution and stored at -80°C until reverse-transcription (RT) processing (Kirakodu et al., 2008).

3.10.3 Reverse transcription

100 ng of total RNA was used as a template to be reverse transcribed to first-strand complementary DNA (cDNA) using cDNA reverse transcription kit (superSCRIPT Reverse Transcriptase kit, Analytik-Jena, Germany) performed on a thermos-cycler. One µg of total RNA, 1 ml of 100 ng of random primers, 1 ml of dNTP mix (10 mM each), and 10 ml of distilled water were added to a nuclease-free microcentrifuge tube. The mixture was heated to 65°C for 5 min and quickly chilled on ice. The contents of the tube were collected by brief centrifugation. Four µl of 5'first-strand buffer (250 mM Tris-Cl, pH 8.3; 375 mM KCl; 15 mM MgCl2) and 2 ml of 0.1M dithiothreitol (DDT) wass added. This was incubated at 25°C for 10 min, and the contents of the tube were mixed gently and incubated at 42°C for 2 min. Finally, 1 ml (200 U) of Superscript II (Invitrogen, Breda, Netherlands) was added and the mixture was incubated at 42°C for 50 min. The reaction was inactivated by heating the mixture at 70°C for 15 min. The cDNA obtained from reverse transcription was used as a template in the qRT-PCR reaction. The specificity of the amplified DNA fragments was confirmed by electrophoresis on 2% (w/v) agarose gels

stained with ethidium bromide. The DNA bands were analyzed using the computer software Quantity One (Bio-Rad). All cDNA samples were stored at -20 °C for subsequent PCR amplification (Dolder et al., 2006).

3.10.4 Real time polymerase chain reaction (qPCR)

qPCR was used to determine the expression level of gene of interested (GOI). Quantification of GOI mRNA was performed on Rotor-gene Q using the SYBR-green fluorescence quantification system (Qiagen, Venlo, Netherlands). The amplification reaction was composed of an initial denaturation at 95°C for 3 min, followed by three-step cycling: denaturation at 95°C for 3 s, annealing at optimum temperature for each primer for 20 s, and extension at 72°C for 20 s. The conditions for amplification cycles and primers used for detection were as described in Table 2. The amplification reactions were carried out using 20-µL mixture of SYBR Green PCR Mix (Applied Biosytems) composed of 20 µl SYBR-green master mix, 1 µl template, 0.4 µl forward primer, 0.4 µl reward primer, and nuclease-free water. The analyses were performed in triplicate for three independent experiments to confirm reproducibility of the results. The relative level of gene expression in the sample was calculated using the cycle threshold (CT) method with normalization to the mRNA level of β-actin. The mean CT values from three measurements was used to calculate the GOI expression. Normal gingival tissue at baseline was used as a calibrator. Relative mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method and value was graphed as the mean expression level ± standard deviation (Mogi et al., 1999; Dolder et al., 2006).

Table 2 Primers used for quantitative real-time polymerase chain reaction. (*Sequences of primers designed using Primer3 software)

Gene of interested	Oligonucleotide sequence (5'-3')	Annealing temperature (°C)	Amplified product size (bp)	Accession no.	Reference
TGF-β1*	Fw: GGACTTCGAGCAGGAGATGG Rw: TTCCATGCCCAGGAAGGAAG	57	136	NM_001003309.1	-
VEGF-A*	Fw: CCGGTATAAACCCTGGAGCG Rw: GCAACGCGAGTCTGTGTTTT	55	115	NM_001003175.2	-
PDGF-B*	Fw: ACCGGAAGTTCAAGCACACA Rw: TGCCCTCAATCTCCTCCAGA	55	84	NM_001003383.1	-
COL1A1*	Fw: GGCAGGAGGGTTCAGCTAAG Rw: GCAACAAAGTCCGCGTATCC	57	160	AF153062.1	-
COL3A1*	Fw: TTCCTGGGAGAAATGGCGAC Rw: AGGACCAGTAGGGCAGGATT	59	98	HM775210.1	-
TIMP-1	Fw: GATGTTCAAGGGTTTCAGCG Rw: TGTCACTCTGCAGTTGCAG	55	294	AF077817_1	(Nganvongpanit et al., 2013)
TNF-α	Fw: TCTCGAACCCCAAGTGACAAG Rw: CAACCCATCTGACGGCACTA	59	152	NM_001003244	(Tamura et al., 2014)
IL1-β	Fw: CAAGTCTCCCACCAGCTCTGTA Rw: GGGCTTCTTCAGCTTCTCCAA	59	80	NM_001037971	(Tamura et al., 2014)
β-actin*	Fw: AGCTCCACGGAGAAGAACTG Rw: GGCTCCAAATGTAGGGGCAG	57	148	NM_001195845.2	-

3.11 Statistical Analysis

Data on cytokine concentration determined by ELISA method were evaluated using student's T test.

Data on clinical, intra-oral radiographic, and histological evaluation were expressed as mean ± S.D. The intergroup and intragroup comparisons on particular parameters were evaluated using two-way ANOVA followed by the Bonferroni-type multiple t-test.

Data on relative cytokine expression were showed as mean ± S.D. of fold expression value according to $2^{-\Delta\Delta Ct}$ method. The intergroup and intragroup

comparisons on particular parameters were evaluated using two-way ANOVA followed by the Bonferroni-type multiple t-test.

All data were quantitatively analyzed using SPSS version 22 for Windows program (Version 22, IBM, US). All tests used a significant difference level of $P<0.05$.

CHAPTER IV

RESULTS

4.1 Clinical evaluation

4.1.1 Plaque index (PI)

The mean value of plaque index was expressed as mean ± S.D. as showed in table 3. Plaque accumulation was not found in C group until 21 DPE. In PD and PD^+ group, PI could not be evaluated at baseline owing to calculus accumulation. After dental scaling on day 0, plaque was found accumulated on dental surface since 14 DPE onwards. There was no significant difference regarding intergroup comparison at any time point. Nonetheless, PI of PD group was slightly higher compared with PD^+ group.

Table 3 Plaque index (PI) assessed by using score 0-3 according to Silness and Loe (1964) and Zhang et al. (2013). The scores of plaque index for each groups were determined by the mean measurements of index teeth.

Group	Time of data collection				
	Baseline	7 DPE	14 DPE	21 DPE	56 DPE
C	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.15	0.21±0.10
PD	ND	0.00±0.00	0.30±0.48	0.50±0.71	0.80±1.57
PD^+	ND	0.00±0.00	0.30±0.48	0.40±0.52	0.70±0.67

ND = Not determined

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group.

Different small letters differ significantly among groups in each period.

4.1.2 Gingival index (GI)

The mean value of GI was expressed as mean ± S.D. as showed in table 4. C group presented an increase in GI from 0.00 at baseline to 1.00±0.00 (7 DPE) and then decreased to 0.00 throughout the experimental periods. In PD and PD^+ group, degree of gingivitis was found at the maximum at baseline and gradually declined. Regarding intergroup comparison, C group presented a statistical difference in GI from other groups at every experimental periods. There was no significant difference between PD and PD^+ group except at 14 DPE, which GI of PD^+ group decreased significantly compared with PD group. At 21 and 56 DPE, PD and PD^+ group presented same value of GI at both time points. Regarding intragroup comparison, value of C group at 7 DPE was statistically different from other trial periods. In PD group, there was statistically different among baseline, 7 and 14 DPE, and 21 and 56 DPE. In PD^+ group, there was statistically different among baseline, 7 DPE, and remaining DPE.

Table 4 Gingival index (GI) assessed by using score 0-3 according to Silness and Loe (1964) and Zhang et al. (2013). The scores of gingival index for each groups were determined by the mean measurements of index teeth.

Group	Time of data collection				
	Baseline	7 DPE	14 DPE	21 DPE	56 DPE
C	0.00 ± 0.00^{a}	1.00 ± 0.00^{Aa}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
PD	2.00 ± 0.00^{A}	1.70 ± 0.48^{B}	1.40 ± 0.52^{Bb}	1.00 ± 0.00^{C}	1.00 ± 0.00^{C}
PD^+	2.00 ± 0.00^{A}	1.50 ± 0.53^{B}	1.00 ± 0.00^{Cc}	1.00 ± 0.00^{C}	1.00 ± 0.00^{C}

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group.
Different small letters differ significantly among groups in each period.

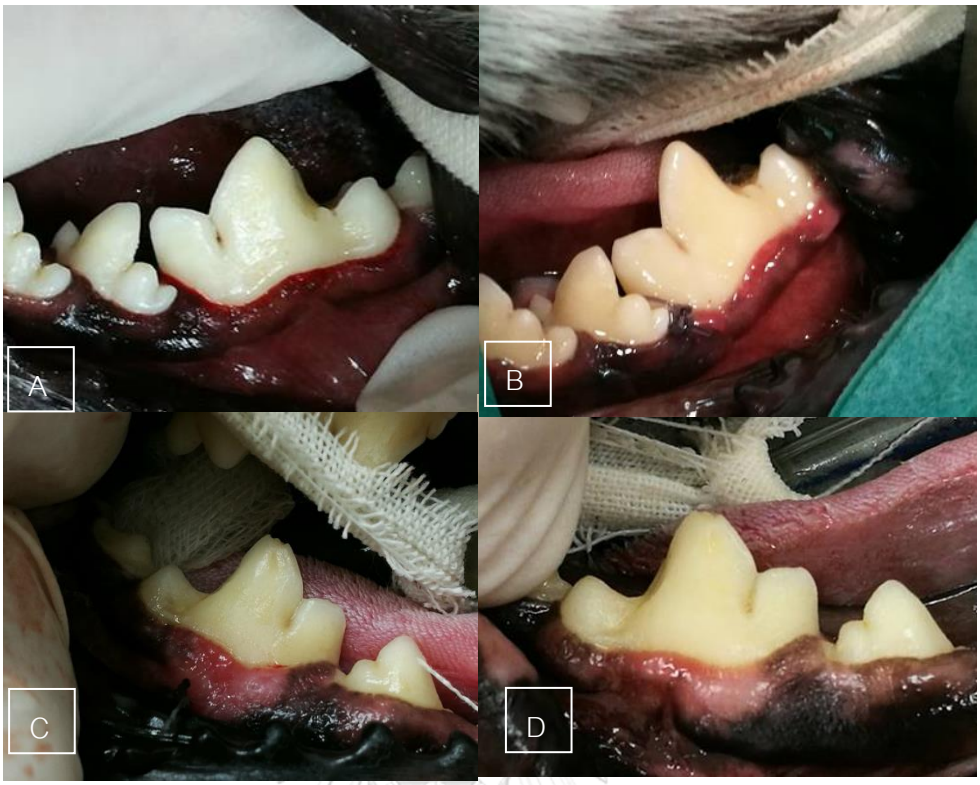

Figure 14 Gingival appearance represented in experimental groups.

(A) Gingival appearance of PD group at baseline. Gingivitis was presented with slight bleeding on probe.

(B) Gingival appearance of PD group at 14 DPE. Gingivitis was still presented.

(C) Gingival appearance of PD^{+} group at baseline. Gingivitis was presented with slight bleeding on probe. Ulcerative lesion was observed owing to calculus accumulation.

(D) Gingival appearance of PD^{+} group at 14 DPE. Gingivitis was decreased and ulcerative lesion was improved.

4.1.3 Periodontal pocket depth (PPD)

The mean value of periodontal pocket depth was expressed as mean ± S.D. as showed in table 5. Control group presented PPD value within normal range throughout the experimental periods. In PD and PD^+ group, PPD value was highest at baseline and gradually declined. Regarding intergroup comparison, C group presented a statistical difference in PPD from other groups at every time points of the experiment. At 21 and 56 DPE, PPD value of PD^+ group decreased significantly compared with PD group. Regarding intragroup comparison, C and PD presented no significant difference at any trial periods. PD^+ group presented significantly lower in PPD at 14, 21, and 56 DPE.

Table 5 Periodontal pocket depth (PPD) assessed by mean value measured on 6 different areas around each surgical sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual). The values of periodontal pocket depth for each groups were determined by the mean measurements of index teeth.

Group	Time of data collection				
	Baseline	7 DPE	14 DPE	21 DPE	56 DPE
C	0.83±0.23[a]	0.83±0.23[a]	0.87±0.19[a]	0.90±0.14[a]	0.95±0.08[a]
PD	3.33±0.33	3.28±0.27	3.22±0.21	3.05±0.11[b]	2.97±0.10[b]
PD^+	3.53±0.42	3.17±0.25	2.87±0.11[A]	2.57±0.27[Ac]	2.22±0.19[Ac]

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group.
Different small letters differ significantly among groups in each period.

4.1.4 Mobility index (MI)

The mean value of mobility index was expressed as mean ± S.D. as showed in table 6. There was no significant difference in the MI among all groups at any time points of the experiment.

Table 6 Mobility index (MI) assessed by using score 0-3 according to Kerry et al. (1982) and Xu and Wei (2006). The scores of mobility index for each groups were determined by the mean measurements of index teeth.

Group	Time of data collection				
	Baseline	7 DPE	14 DPE	21 DPE	56 DPE
C	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
PD	0.40±0.52	0.40±0.52	0.40±0.52	0.10±0.32	0.00±0.00
PD^{+}	0.40±0.52	0.40±0.52	0.20±0.42	0.00±0.00	0.00±0.00

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group.
Different small letters differ significantly among groups in each period.

4.2 Intra-radiographic evaluation

The mean proportion of the CEJ-BL/root length equation was expressed as mean ± S.D. as showed in table 7. There was no significant difference between experimental groups at any experimental periods.

Table 7 Intra-radiographic evaluation was assessed by CEJ-BL/root length equation according to Persson et al. (2003) and Balci Yuce et al. (2014). The values of alveolar bone loss as a proportion to root length for each groups were determined by the mean measurements of index teeth.

Abbreviations: CEJ: cemento-enamel junction; BL: alveolar bone level.

Group	Time of data collection		
	Baseline	21 DPE	56 DPE
C	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
PD	0.21±0.06	0.20±0.09	0.14±0.10
PD^{+}	0.20±0.09	0.18±0.10	0.11±0.04

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group.
Different small letters differ significantly among groups in each period.

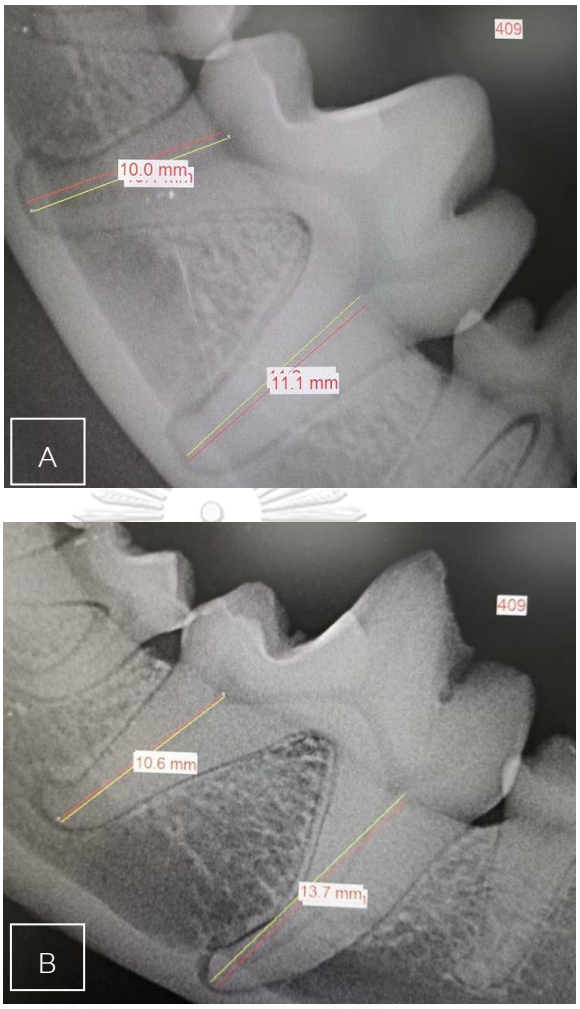

Figure 15 Radiological evaluation of control group.

(A-B) Radiological evaluation of C group at baseline and 56 DPE respectively. Alveolar bone loss was not observed. (Red line represented distance from alveolar bone level to the root, yellow line represented distance from cemento-enamel junction to the root)

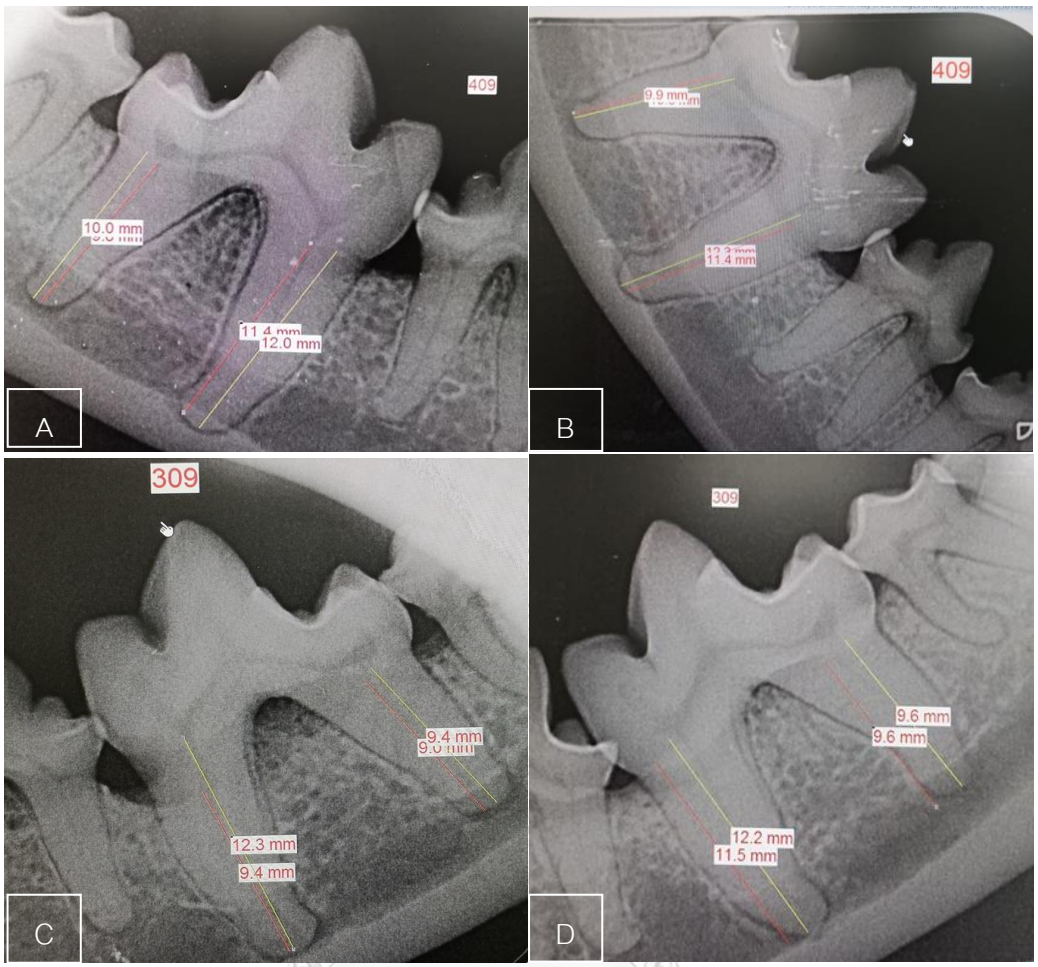

Figure 16 Radiological evaluation of experimental groups.

(A-B) Radiological evaluation of PD group at baseline and 56 DPE respectively. Bone filling was observed from the radiographs.

(C-D) Radiological evaluation of PD^{+} group at baseline and 56 DPE respectively. Bone filling was observed from the radiographs.

(Red line represented distance from alveolar bone level to the root, yellow line represented distance from cemento-enamel junction to the root)

4.3 Histopathological analysis

4.3.1 Inflammatory reaction score

The mean value of inflammatory reaction score was expressed as mean ± S.D. as showed in table 8. Regarding intergroup comparison, control group presented mild inflammation and also statistically lower value of inflammatory reaction score compared with other groups in both experimental periods. At 14 DPE, PD^{+} group presented a significant decrease in inflammatory score compared with PD group. Regarding intragroup comparison, value of PD^{+} group at 14 DPE significantly decreased compared with baseline.

Table 8 Inflammatory reaction score assessed according to Furtado and Constantino-Casas (2013). The scores of inflammatory reaction for each groups were determined by the mean measurements of each gingival tissue sections.

Group	Time of data collection	
	Baseline	14 DPE
C	1.60 ± 0.49^{a}	1.60 ± 0.50^{a}
PD	6.60 ± 1.71	5.60 ± 0.80^{b}
PD^{+}	7.4 ± 1.96	4.30 ± 0.50^{Ac}

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

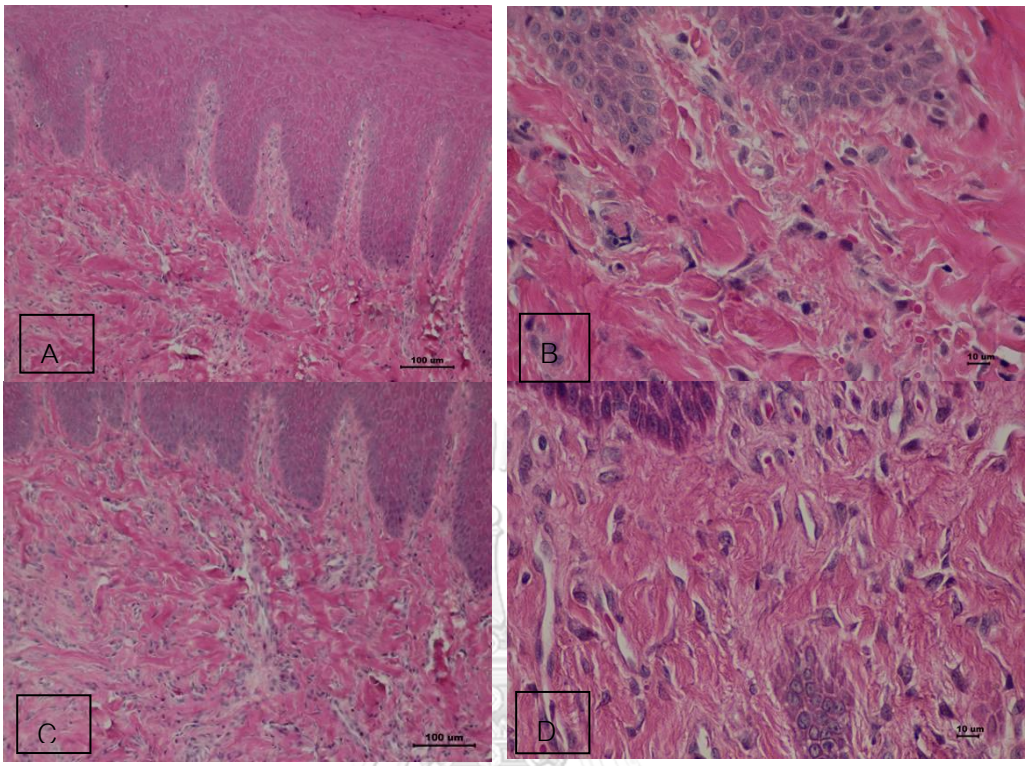

Figure 17 Histopathological observation concerning inflammatory reaction score. (H&E stain; magnification x10: scale bar = 100 μm, magnification x40: scale bar = 10μm)
(A-B) Histopathological observation of C group at baseline. Dense connective tissue were seen. Fibroblasts and fibrocytes were mainly found. Inflammatory cells were rarely seen owing to normal healthy condition.
(C-D) Histopathological observation of C group at 14 DPE. Less dense connective tissue were seen compared with baseline. Inflammatory cells were rarely seen due to resolution of inflammatory process. Fibroblasts and fibrocytes were found in response to proliferation but not that distinct from baseline.

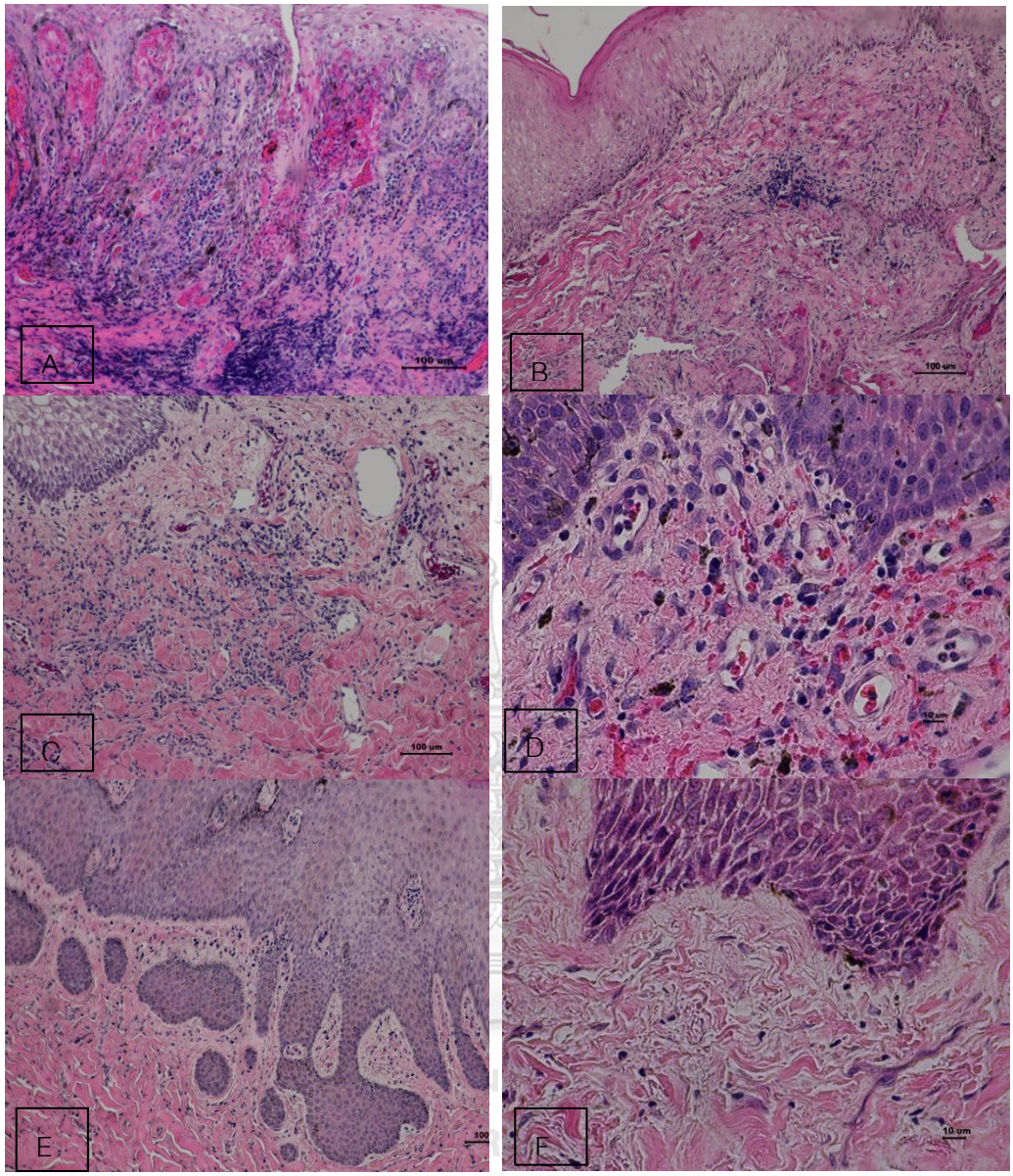

Figure 18 Histopathological observation concerning inflammatory reaction score. (H&E stain; magnification x10: scale bar = 100 μm, magnification x40: scale bar = 10μm)

(A-B) Histopathological observation of PD and PD^{+} group at baseline. Abundant of inflammatory cells especially plasma cells and lymphocytes were found due to chronic inflammation. Vacuolization of gingival epithelium was observed.

(C-D) Histopathological observation of PD group at 14 DPE. Large amount of inflammatory cells, mainly lymphocytes and plasma cells, were found.

(E-F) Histopathological observation of PD^{+} group at 14 DPE. Inflammatory cells were sill observed but in lower amount compared with PD group.

4.3.2 Fibrosis score

The mean value of fibrosis score was expressed as mean ± S.D. as showed in table 9. Regarding intergroup comparison, there was no significant difference in the fibrosis score among all groups at any experimental periods. However, PD^+ group presented more value of fibrosis score compared with other groups at 14 DPE. Regarding intragroup comparison, value of PD^+ group at 14 DPE was significantly higher compared with baseline.

Table 9 Fibrosis score assessed by using score 0-3 according to Yucel (2003). The scores of fibrosis for each groups were determined by the mean measurements of each gingival tissue sections.

Group	Time of data collection	
	Baseline	14 DPE
C	3.00±0.00	3.15±0.55
PD	1.80±0.42	2.10±0.47
PD^+	1.70±0.48	$2.40±0.48^A$

Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

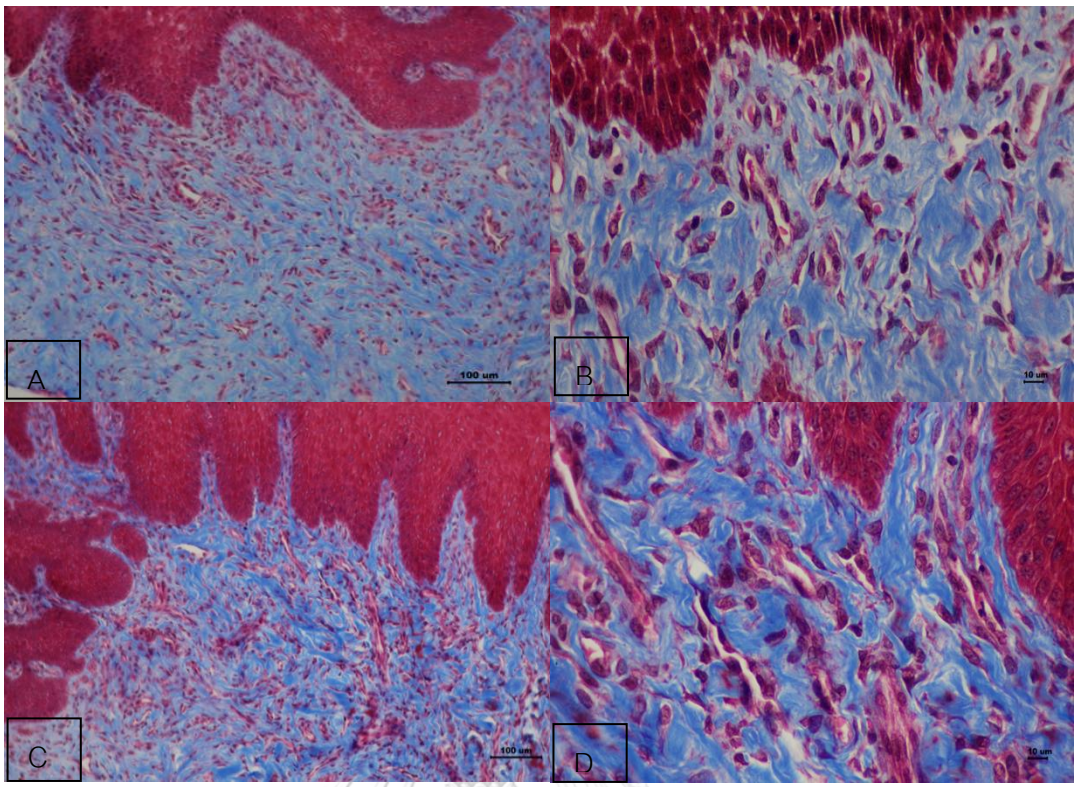

Figure 19 Histopathological observation concerning fibrosis score. (Masson's trichrome stain; magnification x10: scale bar = 100 μm, magnification x40: scale bar = 10μm) (A-B) Histopathological observation of C group at baseline. Dense and well-organized connective tissue were seen. Large amount of fibroblasts and fibrocytes were observed. (C-D) Histopathological observation of C group at 14 DPE. Plenty of fibroblast and fibrocyte were observed but connective tissue were less well-organized compared with baseline. Neovascularization was obviously seen due to active proliferative stage.

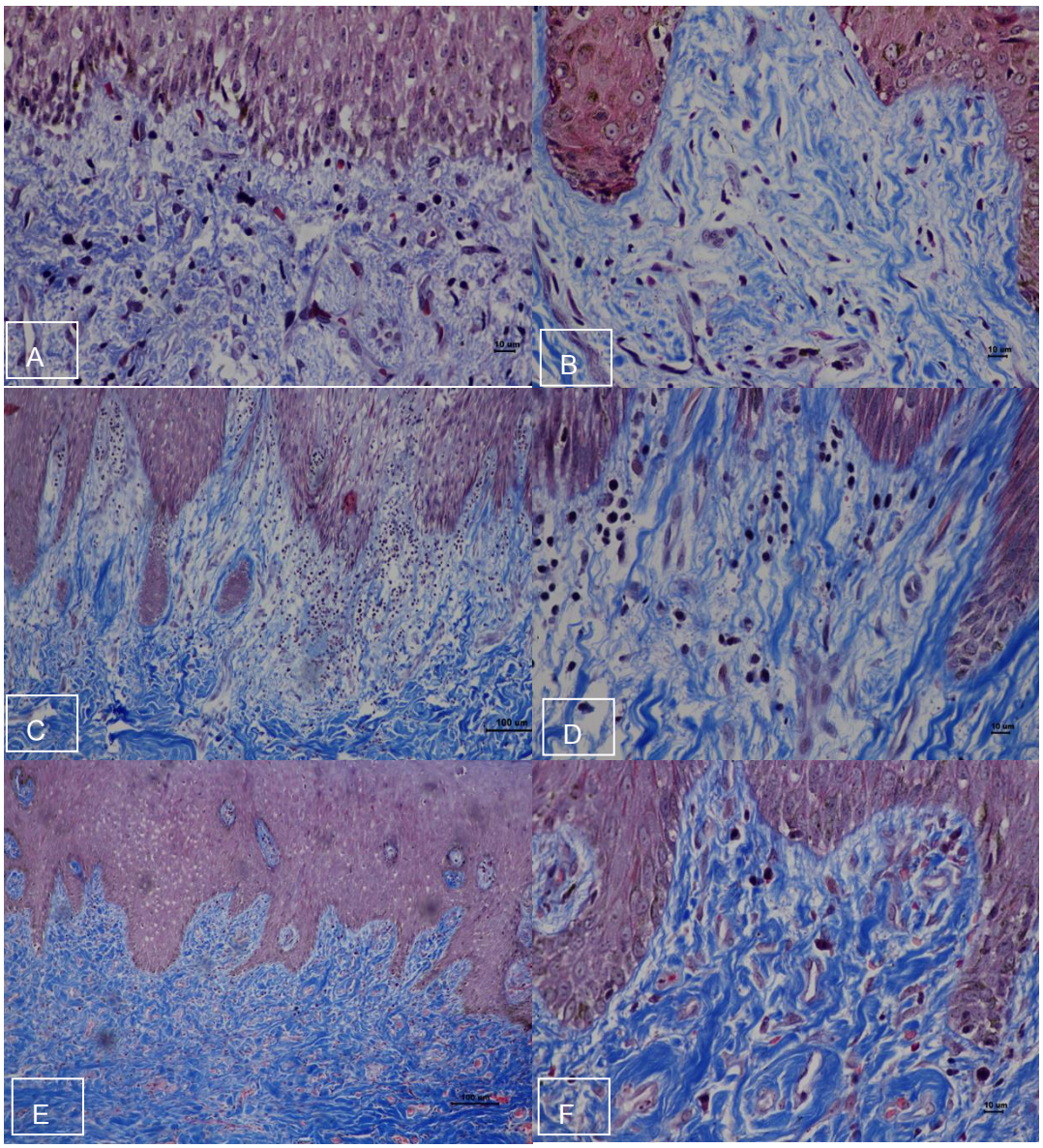

Figure 20 Histopathological observation concerning fibrosis score. (Masson's trichrome stain; magnification x10: scale bar = 100 μm, magnification x40: scale bar = 10μm)

(A-B) Histopathological observation of PD and PD^{+} group at baseline. Arrangement of connective tissue were loose and uncoordinated. Inflammatory cells were also observed.

(C-D) Histopathological observation of PD group at 14 DPE. Loose arrangement of connective tissue were still observed. Inflammatory cells mainly lymphocyte were also seen.

(E-F) Histopathological observation of PD^{+} group at 14 DPE. Connective tissue were relatively dense and well-organized. Fibroblasts and fibrocytes were observed in the field.

Small amount of inflammatory cells were observed. Also, neovascularization was seen due to active proliferative stage

4.4 Measurement of growth factor concentration in platelet-rich fibrin

4.4.1 TGF β-1 concentration

The mean concentrations of TGF β-1 in PRF utilized in PD^{+} group were expressed as mean ± S.D. as showed in figure 23. Level of TGF β-1 decreased from 171.50 ng/ml (at baseline) to 125.8 ng/ml (1.36-fold) on day 7, and to 88.9 ng/ml (1.93-fold) on day 14. Concentration at day 14 was significant lower compared with baseline. No significant difference was found between day 7 and other periods.

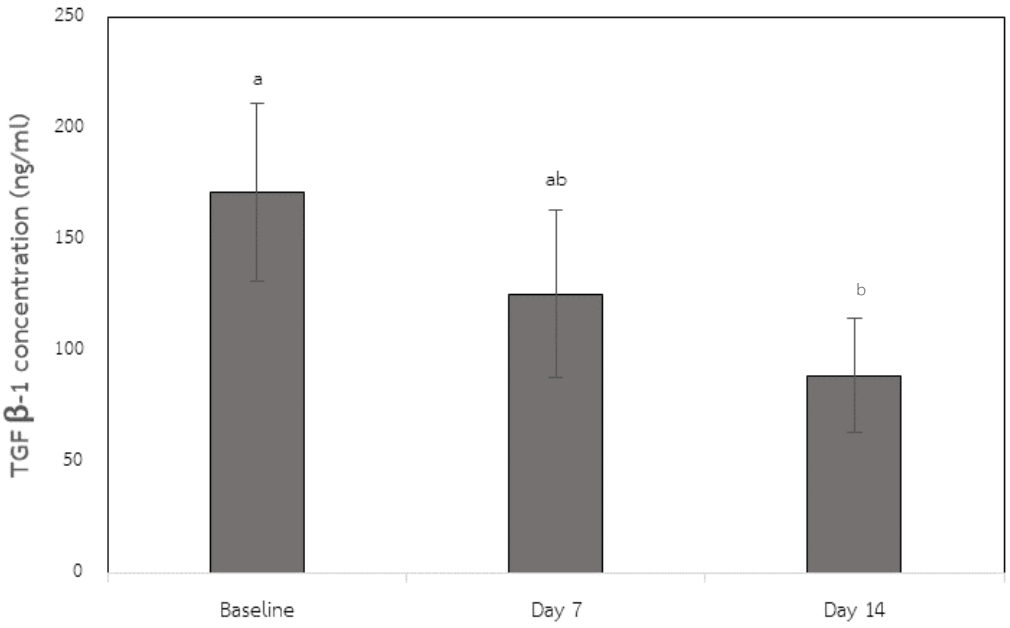

Figure 21 Mean concentration level of TGF β-1 (ng/ml) measured in PRF at baseline (day 0), after kept in -20 °C for 7 days (day 7), and after kept in -20 °C for 14 days (day 14). Different small letters differ significantly at $P < 0.05$ among trial periods.

4.4.2 VEGF-A concentration A

The mean concentrations of VEGF-A in PRF utilized in PD^{+} group were expressed as mean ± S.D. as showed in figure 24. Level of VEGF-A decreased significantly from 88.21 pg/ml (at baseline) to 70.82 pg/ml (1.25-fold) on day 7, and to 63.44 pg/ml (1.39-fold) on day 14. However, no significant difference was found between VEGF-A level measured on day 7 and day 14.

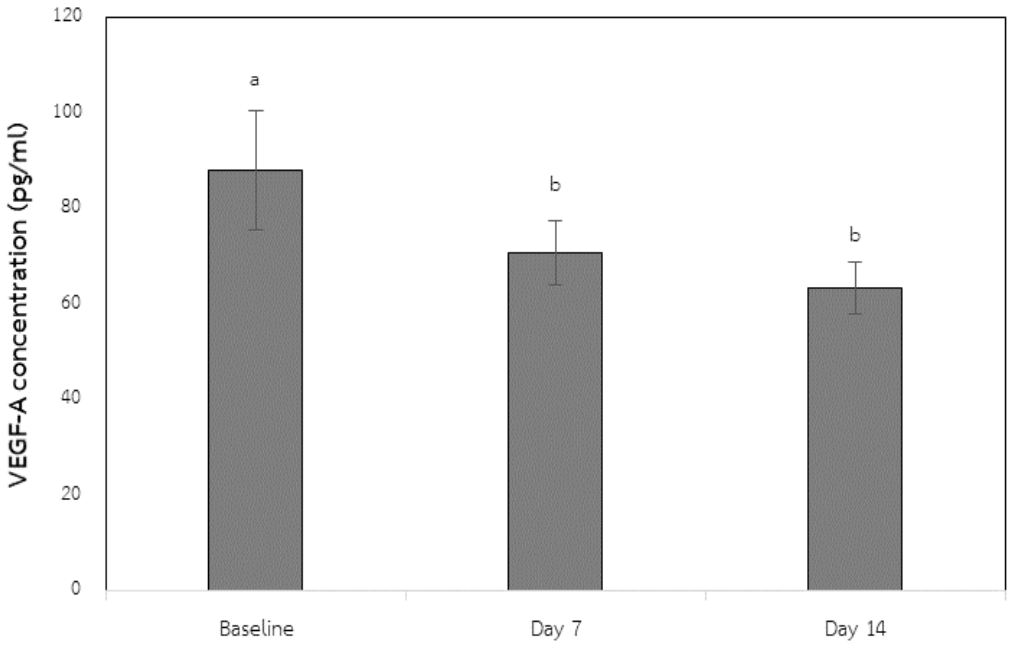

Figure 22 Mean concentration level of VEGF-A (pg/ml) measured in PRF at baseline (day 0), after kept in -20 °C for 7 days (day 7), and after kept in -20 °C for 14 days (day 14). Different capital letters differ significantly at $P < 0.05$ among trial periods.

4.5 Inflammatory cytokine expression analysis

The collected gingival samples were used to evaluate for inflammatory cytokine relative expression level via qRT-PCR technique. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. House-keeping β-actin gene was used as a normalizer and gingival tissues from periodontal healthy dogs (control group) were used as a calibrator for calculation.

4.5.1 Transforming growth factor-beta 1 (TGF-β1)

The TGF-β1 response was expressed as fold expression level as showed in figure 25. C group presented the up-regulation at 7 DPE and then down-regulation at 14 DPE. In both PD and PD^+ group, the up-regulation was observed at both 7 and 14 DPE. Regarding intergroup comparisons, PD^+ group presented the significantly higher TGF-β1 expression level at both 7 and 14 DPE (at 7 DPE; 2.16-fold in C group, 1.98-fold in PD group, and 3.42-fold in PD^+ group, at 14 DPE; 1.32-fold in C group, 3.56-fold in PD group, and 4.12-fold in PD^+ group). The highest level of TGF-β1 observed in the experiment was expressed in PD^+ group at 14 DPE. Regarding intragroup comparisons, expression in C group increased from 1-fold at baseline to 2.16-fold (7 DPE) and then decreased to 1.32-fold (14 DPE). Value at 7 DPE was significantly higher from other trial periods. Both experimental groups showed the same pattern of significant difference among trial periods. In PD group, TGF-β1 expression increased from 1.34-fold at baseline to 1.98-fold (7 DPE) and 3.56-fold (14 DPE). In PD^+ group, TGF-β1 expression increased from 1.38-fold at baseline to 3.42-fold (7 DPE) and 4.12-fold (14 DPE).

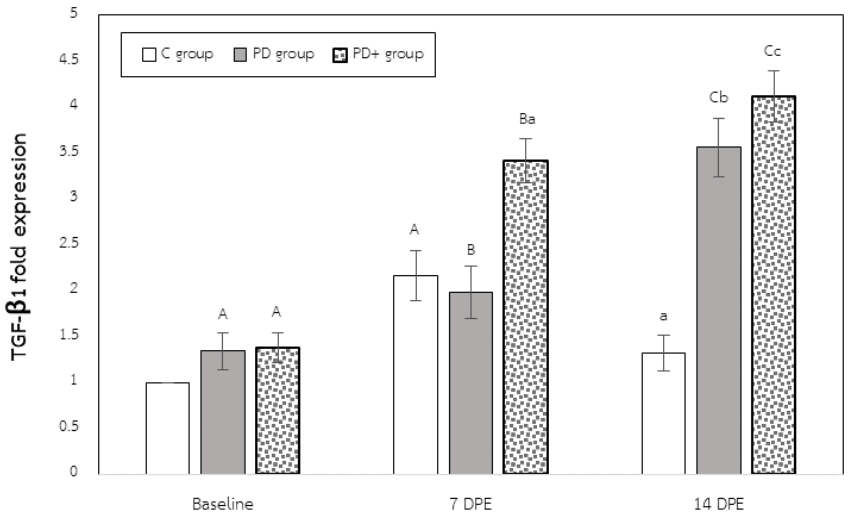

Figure 23 Kinetics of TGF-β1 gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.2 Platelet-derived growth factor-B (PDGF-B)

The PDGF-B response was expressed as fold expression level as showed in figure 26. C and PD^+ group presented similarity in up-regulation at 7 DPE and down-regulation at 14 DPE. PD group presented different pattern of expression, which up-regulation was seen throughout the experimental periods. Regarding intergroup comparisons, C and PD^+ group presented significantly higher expression level compared with PD group at 7DPE. Expression level of PD^+ group was also considered as the highest level of PDGF-B observed in the experiment. However, there was no significant different between C and PD^+ group (3.61-fold in C group, 2.25-fold in PD group and 4.32-fold in PD^+ group). At 14 DPE, PDGF-B expression level of PD^+ group was significantly higher compared with C group, but no significant difference was observed between PD and PD^+ group (1.92-fold in C group, 2.7-fold in PD group and 3.1-fold in PD^+ group). Regarding intragroup comparisons, C group presented an increase expression from 1-fold at baseline to 3.61-fold (at 7 DPE) and then decrease to 1.92-fold (14 DPE). Value at 7 DPE was significantly higher level among trial periods. Both experimental groups showed the same pattern of significant difference among trial periods. In PD group, PDGF-B expression increased from 1.21-fold at baseline to 2.25-fold (7 DPE) and 2.7-fold (14 DPE). In PD^+ group, PDGF-B expression increased from 1.18-fold at baseline to 4.32-fold (7 DPE) and then decreased to 3.1-fold (14 DPE).

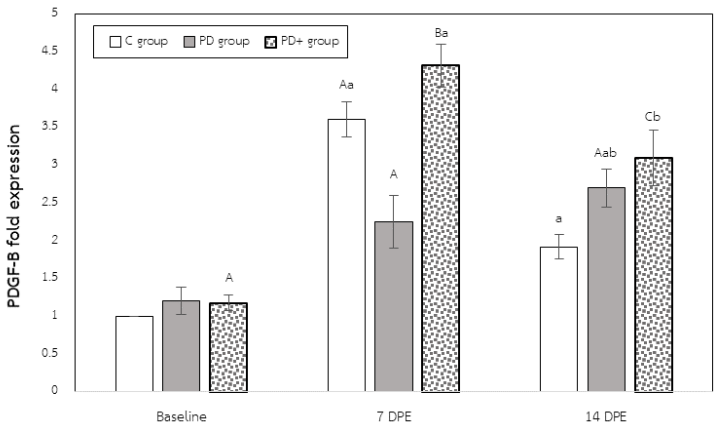

Figure 24 Kinetics of PDGF-B gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.3 Vascular endothelial growth factor-A (VEGF-A)

The VEGF-A response was expressed as fold expression level as showed in figure 27. All groups presented the similar pattern of up-regulation and slight down-regulation at 7 and 14 DPE respectively. Regarding intergroup comparisons, the highest VEGF-A expression level was observed in PD^+ group at both trial periods (at 7 DPE; 2.09-fold in C group, 1.81-fold in PD group, and 2.61-fold in PD^+ group, at 14 DPE; 1.5-fold in C group, 1.71-fold in PD group, and 2.03-fold in PD^+ group). At 7 DPE, there was significant difference only between PD and PD^+ group, which value was considered as the highest level of VEGF-A observed in the experimental periods. At 14 DPE, there was no significant difference among groups. Regarding intragroup comparisons, C group presented an increase in expression level from 1 at baseline to 2.09 (7 DPE) and decreased to 1.5-fold (14 DPE). Value at 7 DPE was considered significant among other trial periods. In PD group, VEGF-A expression increased from 1.20-fold at baseline to 1.81-fold (7 DPE) and then decreased to 1.71-fold (14 DPE). No statistical difference was observe among tiral periods. In PD^+ group, VEGF-A expression increased from 1.16-fold at baseline to 2.61-fold (7 DPE) and then decreased to 2.03-fold (14 DPE). Both value observed at 7 and 14 DPE was statistically different from baseline.

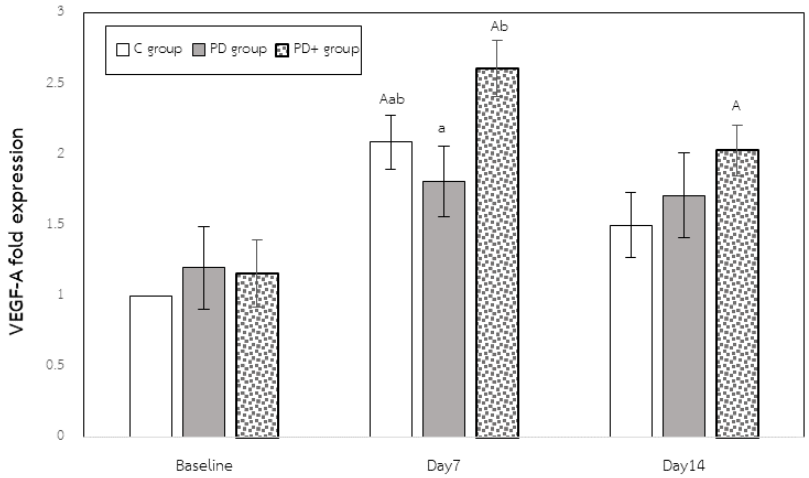

Figure 25 Kinetics of VEGF-A gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.4 Tissue inhibitor of matrix metalloproteinases 1 (TIMP-1)

The TIMP-1 response was expressed as fold expression level as showed in figure 28. C and PD^+ group presented similarity in up-regulation at 7 DPE and down-regulation at 14 DPE. PD group presented gradual up-regulation throughout the experimental periods. Regarding intergroup comparisons, the highest TIMP-1 expression level was observed in PD^+ group at both trial periods (at 7 DPE; 2.84-fold in C group, 1.39-fold in PD group, and 3.26-fold in PD^+ group, at 14 DPE; 1.25-fold in C group, 0.83-fold in PD group, and 2.52-fold in PD^+ group). At 7 DPE, C and PD^+ group presented significant higher level compared with PD group, although there was no significant difference between two groups. However, value of PD^+ group was considered as the highest level TIMP-1 observed in the experiment. At 14 DPE, PD^+ group presented significant higher level among all groups. Regarding intragroup comparisons, C group presented an increase expression from 1-fold at baseline to 2.84-fold (7 DPE) and decreased to 2.01-fold (14 DPE). Value at 7 DPE of C group was significantly higher compared with other trial periods. In PD group, TIMP-1 expression increased from 0.78-fold at baseline to 1.39-fold (7 DPE) and to 1.41-fold (14 DPE). No statistical difference among trial periods was observe. In PD^+ group, TIMP-1 expression increased from 0.75-fold at baseline to 3.26-fold (7 DPE) and then decreased to 2.52-fold (14 DPE). There was significant difference observed among trial periods in this group

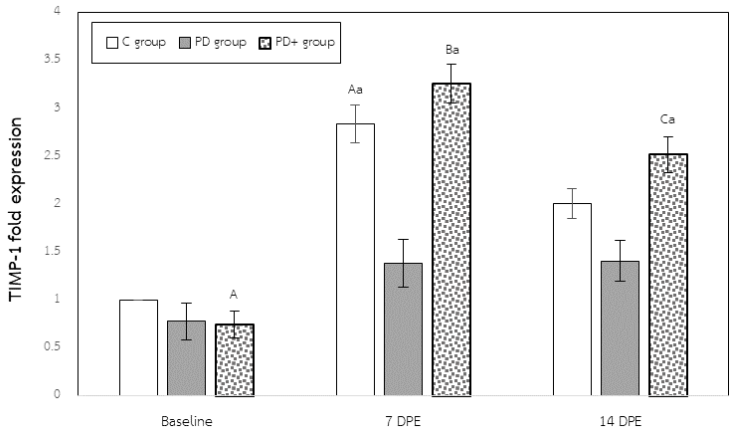

Figure 26 Kinetics of TIMP-1 gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.5 Collagen type I alpha 1 chains (COL1A1)

The COL1A1 response was expressed as fold expression level as showed in figure 29. All groups showed similar pattern of gradual up-regulation throughout the experimental periods. C group presented a slight increase in expression from 1-fold at baseline to 1.12-fold (7 DPE) and to 1.15-fold (14 DPE). PD group presented a relatively slight increase in expression from 0.67-fold at baseline to 0.71-fold (7 DPE) and to 0.95-fold (14 DPE). In PD^{+} group, expression increased from 0.65-fold at baseline to 0.87-fold (7 DPE) and to 1.05-fold (14 DPE). Value of C group at baseline was significant higher compared with others.

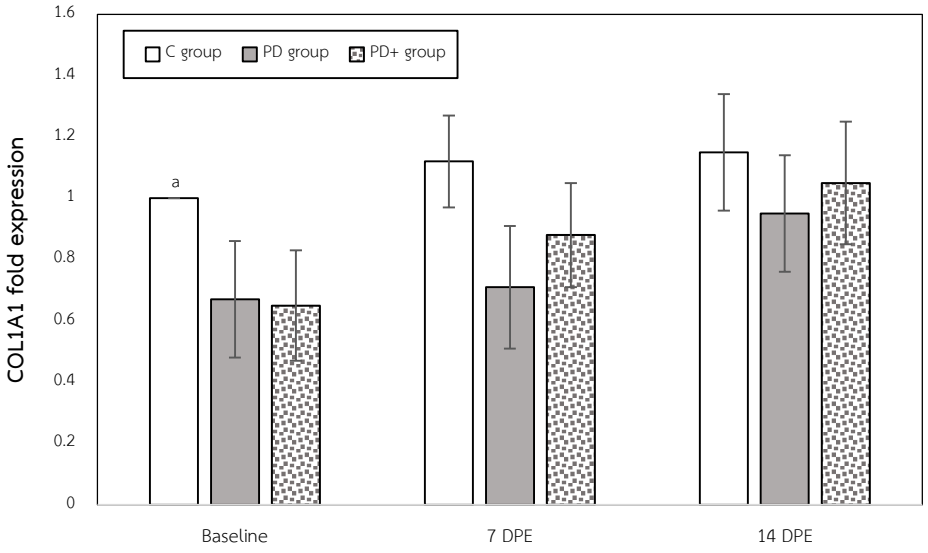

Figure 27 Kinetics of COL1A1 gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.6 Collagen type III alpha 1 chains (COL3A1)

The COL3A1 response was expressed as fold expression level as showed in figure 30. C group presented an up-regulation at 7 DPE and then down-regulation at 14 DPE while PD and PD^{+} groups showed the similar pattern of gradual up-regulation of COL3A1 gene expression throughout the experiment. Regarding intergroup comparisons, PD^{+} and C group presented significant higher expression level compared with PD group at 7 DPE (2.00-fold in C group, 1.58-fold in PD group, and 2.13-fold in PD^{+} group). At 14 DPE, PD+ group presented significant higher level and considered as the highest level of COL3A1 observed in the experiment (1.87-fold in C group, 2.09-fold in PD group, and 3.01-fold in PD^{+} group). Regarding intragroup comparisons, COL3A1 gene expression of C group increased from 1-fold at baseline to 2.00-fold (7 DPE) and then decreased to 1.87-fold (14 DPE). Significant difference was observed between baseline and other trial periods. No significant difference was observed among trial periods. In PD group, expression increased from 0.89-fold at baseline to 1.58-fold (7 DPE) and 2.09-fold (14 DPE). Value at 14 DPE was significantly higher than other trial periods. In PD^{+} group, expression increased from 0.81-fold at baseline to 2.13-fold (7 DPE) and 3.01-fold (14 DPE). Significant difference among trial periods was observed in this group.

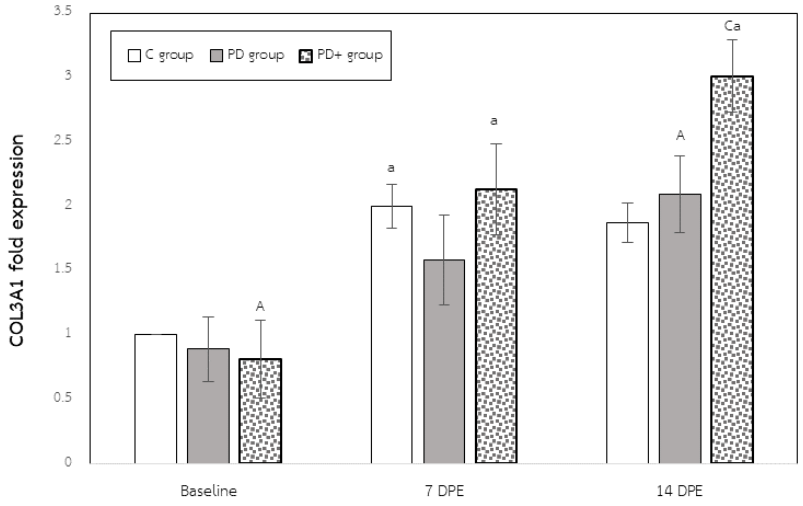

Figure 28 Kinetics of COL3A1 gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.7 Tumor necrosis factor-alpha (TNF-α)

The TNF-α response was expressed as fold expression level as showed in figure 31. C group presented up-regulation at 7 DEP and down-regulation at 14 DPE. PD and PD^+ group showed similarity of highest expression at baseline and down-regulation throughout the trial periods. Regarding intergroup comparisons, C group presented significantly lower expression among groups at baseline. PD group presented higher expression at both 7 and 14 DPE. At 7 DPE, value of PD group was significantly higher compared with C group and considered as the highest expression throughout the experiment. PD^+ group presented lower expression but there was no significant difference neither compared with C group nor compared with PD group (1.81-fold in C group, 3.28-fold in PD group, and 2.45-fold in PD^+ group). At 14 DPE, PD group presented significantly higher expression compared with other groups (1.46-fold in C group, 3.18-fold in PD group, and 1.62-fold in PD^+ group). Regarding intragroup comparisons, expression of C group increased from 1-fold at baseline to 1.81-fold (7 DPE) and decreased to 1.46-fold (14 DPE). PD group presented a slight decreased expression from 3.54-fold at baseline to 3.28-fold (7 DPE) and 3.18-fold (14 DPE). There was no significant difference among trial periods in both groups. PD^+ group presented a gradual decrease expression from 3.6-fold at baseline to 2.45-fold (7 DPE) and 1.62-fold (14 DPE). Significant difference was observed among trial periods in this group.

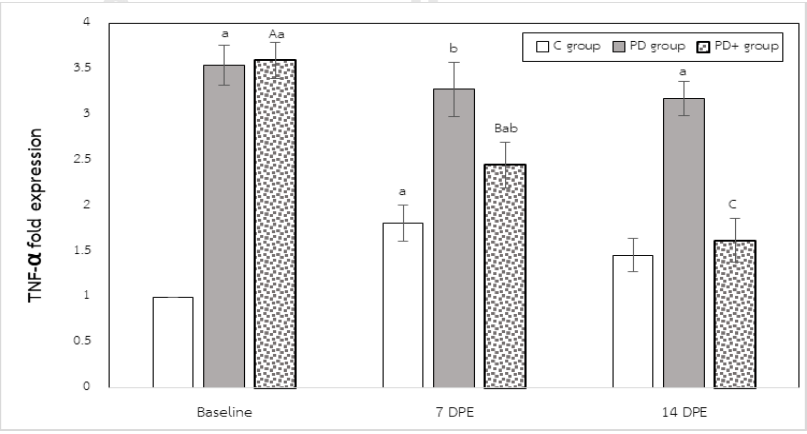

Figure 29 Kinetics of TNF-α gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

4.5.8 Interleukin-1 beta (IL-1β)

The IL-1β response was expressed as fold expression level as showed in figure 32. C group presented up-regulation at 7 DEP and down-regulation at 14 DPE. PD and PD^+ group showed similarity of highest expression at baseline and down-regulation throughout the trial periods. Regarding intergroup comparisons, C group presented significantly lower expression among groups at baseline. PD group presented significantly higher expression among all groups at both 7 and 14 DPE (at 7 DPE; 1.47-fold in C group, 2.27-fold in PD group, and 1.64-fold in PD^+ group, at 14 DPE; 1.12-fold in C group, 1.84-fold in PD group, and 1.37-fold in PD^+ group). There was no significant observed between C and PD^+ group at both trial periods. Regarding intragroup comparisons, expression of C group increased from 1-fold at baseline to 1.47-fold (7 DPE) and decreased to 1.12-fold (14 DPE). There was no significant different observed among trial periods. PD group presented a decreased expression from 2.49-fold at baseline to 2.27-fold (7 DPE) and 1.64-fold (14 DPE). Value at 14 DPE was significantly different from other trial periods. There was no significant difference among trial periods in both groups. PD^+ group presented a significant decrease in expression from 2.45-fold at baseline to 1.64-fold (7 DPE) and 1.37-fold (14 DPE).

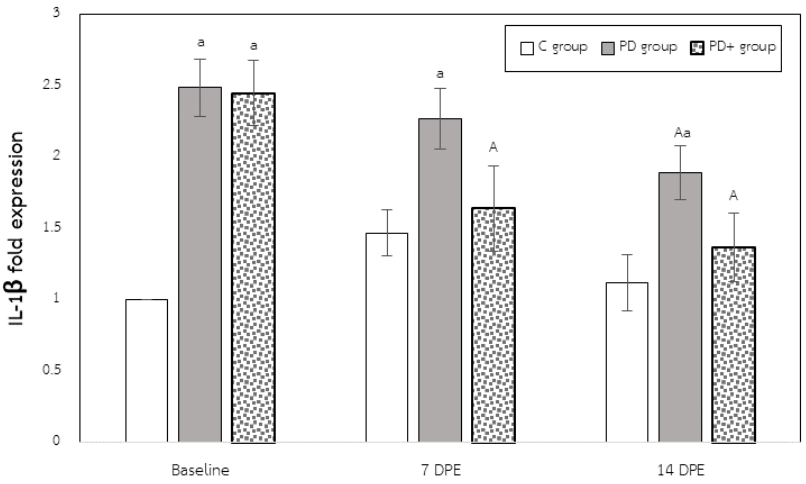

Figure 30 Kinetics of IL-1β gene expression observed at baseline, 7 DPE, and 14 DPE. Different capital letters differ significantly at $P < 0.05$ among trial periods in each group. Different small letters differ significantly among groups in each period.

CHAPTER V

DISCUSSION AND CONCLUSION

PD is one of the most common inflammatory oral diseases in human and companion animals. It is considered a multi-factorial disease, which bacterial accumulation within plaque and calculus is believed to be an initiation and the most important factor for pathogenesis and progression of PD (Riggio et al., 2011). Conventionally, PD management has been performed by non-surgical and surgical method. In recent years, as people has been more aware of surgical disadvantages, probability of dental loss, and prolong usage of antibiotic and anti-inflammatory medication, regenerative periodontology has been extensively researched and emphasized as an alternative approach for PD management in human and also in animals (Niemiec, 2008a; Niemiec, 2008b; Albuquerque et al., 2012). There are various tools concerning regenerative methodology, which one of the most interesting approaches is PRF. PRF, considered from structure and components, is basically a physiological scaffold incorporated with numerous cytokines, which all together play an important roles in favoring tissue engineering, down-regulating of inflammation, and enhancing wound healing process. As regards to fibrin meshwork, physiologic and slow polymerization of fibrin network occurred during PRF preparation allowed the formation of fine and flexible fibrin fibrillae, which results in ability of better cytokine entrapment, cellular migration, and also slow and long-term release of integrated cytokines. During process of preparation, neither anticoagulant nor bovine thrombin is required. The simplified protocol is considered as a chair-sided preparation required only centrifugal machine, blood collection kit, and sterile glass tube, which makes it more economical compared with other platelet derivatives and regenerative methods. Also, membrane form has gained more resistance and can be served as a fibrin bandage to strengthen the wound or post-

extraction socket (Choukroun et al., 2006; Dohan et al., 2006c; Dohan Ehrenfest et al., 2009a; Dohan Ehrenfest et al., 2009b). Advantages of PRF regarding clinical evaluation have been reported in many studies in human PD (Preeja and Arun, 2014). Still, research in animals as well as mechanism of PRF has not yet been fully described. Hence, we conducted this study to determine the synchronization between the mechanism of PRF through expression analysis of cytokine involved in wound healing process and the consequent PD treatment outcome via clinical, radiologic, and histological evaluation. Additionally, we measured the concentration of some key cytokines embedded within PRF to validate the approximate concentration that positively affect the PD treatment.

In the present study, we divided experimental animals into 3 groups. All groups were conducted the OFD procedure in order to validate the same experiment. Control group would demonstrate the normal tissue response to the same experimental approach as the experimental groups, while experimental groups would reflect the response of the periodontal tissue towards the experiment. As regards to clinical outcome, we evaluated the effect of PRF through various clinical parameters. The result demonstrated that the PD^{+} group presented significant decrease in GI (14 DPE) and PPD (21 and 56 DPE) compared with PD group, whereas there was no significant difference in PI and MI between experimental groups. Control group presented all parameters within normal range except for GI at 7 DPE, which increased responding to OFD procedure. However, returning to GI score 0 at 14 DPE implied that healthy gingiva had the capability of rapid healing property. Regarding clinical PD research in human, PRF has been studied in many aspects. For example, intrabony defects, recession defects, furcation defects, and soft tissue healing. In resemblance with human studies, our result was correlated with the studies of Sharma and Pradeep (2011), Ajwani et al. (2015), Joseph V et al. (2014), and Chandradas et al. (2016) who reported that the PRF-treated group had significant GI and PPD reduction compared with OFD alone in an aspect of intrabony defects. Moreover, recent human studies have been focused on clinical application of PRF combined with various types of bone grafts and pharmacologic agents. SEZGIN et al. (2017) designed the split mouth clinical trial to study the effect of PRF by comparing between an-organic

bovine bone mineral (ABBM)-PRF combination and ABBM alone. They found that addition of PRF helped decreasing GI and PPD significantly compared with ABBM alone. However, there was no significant difference in PI between two groups, which was resemble to our study. Same as in other studies, we placed the PRF over the alveolar crest so the crown remained denuded. So we hypothesized that this could be attributed to insignificant difference in PI between experimental groups. Another parameter that we studied was MI. There was no significant difference in MI in our study. Our finding was contradictory with other studies in human i.e. Jadhav et al. (2015) who reported that tooth mobility was decreased after PRF application evaluated at 12 and 18 months post procedure. The possible reason responsible for this might due to our short-term study compared with those studies in human. (Dohan et al., 2006c; Preeja and Arun, 2014)

In the current study, we undergone the intra-oral radiography for alveolar bone assessment. From the result, we found that there was no significant difference in radiological parameter between experimental groups although PD^{+} group presented the lower value in alveolar bone loss compared with PD group at 56 DPE. Our result was differed from other human studies regarding radiologic evaluation. Galav et al. (2016) showed the equal radiographic bone fill between autogenous bone grafting-treated and PRF-treated group at 9 months post-operative, indicating that PRF was capable of osteoinduction and oestogenesis as bone graft. Another human study showed the significant radiographic density and bone filling in PRF-treated group evaluated at 12 months post-operatively (Y-C and J-H, 2011). In this study, they additionally conducted in vitro study to evaluate the PRF effect on human periodontal ligament fibroblasts (PDLFs) and observed the acceleration of factors associated with osteogenesis i.e. phosphorylated extracellular signal-regulated protein kinase (p-ERK), osteoprotegerin (OPG), and alkaline phosphatase (ALP) activity. As regards to study in animals, Samuel et al. (2017) conducted a study in 2 mongrel dogs and revealed that experimental group (PRF mixed with bone substitute material and covered by a PRF membrane and a collagen membrane) showed more quantity of bone filling in mandibular grade II furcation defects compared with control group (bone substitute material and covered with a collagen

membrane) at 2 months post-operatively. Another study in rat also observed the increase expression of other bone markers (bone sialoprotein; BSP, osteocalcin; OC, and runt-related transcription factor 2; RUNX2) after PRF combination with periodontal ligament stem cells (PDLSCs). Also they observed the in vivo significant new bone area percentages in the periodontal fenestration defect created at mandibular bone in the PRF combined with PDLSCs group via histological analysis at 2 months post-operatively (Duan et al., 2018). From aforementioned studies, our result was contradictory with others owning to the fact that bone regeneration requires more time to observe the alteration via radiography. Therefore, in order to achieve early osteogenenis detection, previously mentioned markers associated with bone regeneration or histological analysis would be more appropriate than radiographic evaluation.

In order to investigate the effect of PRF by means of the histopathological examination, the inflammatory reaction and fibrosis score were assessed at baseline and 14 DPE. From the result, we found that PD^{+} group presented significant reduction in inflammatory reaction score whereas there was no significant difference in fibrosis score. Our result was correlated with Kazemi (2013) who reported that PRF application improved and accelerated cutaneous incisional wound healing by increasing epithelialization, neo-angiogenesis, fibroblastic proliferation, and ECM formation in a dog model. In resemblance, human study also found the positive effect of PRF in many aspects. Bansal et al. (2016) reported that PRF provided histologically less inflammatory infiltration and higher healing index compared with commercial oral wound dressing (COE-Pack™) in as aspect of depigmented gingival wound healing. Also, Zhao et al. (2011) found that PRF provided less inflammatory infiltration, dense connective tissue formation, and new bone formation in an aspect of healing of extraction socket. Concerning from property of platelet cytokines, we hypothesized that the reduction of inflammatory reaction score in PD^{+} group resulted from various anti-inflammatory cytokine that embedded in PRF fibrin meshwork as previously mentioned. Our hypothesis was related to Naik et al. (2013), Kimura et al. (2005), and Sunitha Raja and Munirathnam Naidu (2008) who stated that PRF promotes wound healing by releasing platelet growth factors that induced the proliferation of

fibroblasts, endothelial cells, and progenitor cells. As regards to fibrosis score, the insignificance between experimental groups would be attributed to inadequate sensitivity of our evaluation. Other method such as immunohistochemistry, wound healing assay, fibroblast and collagen gene detection might reveal a different result.

In the present study, we measured the concentration of TGF-β1 and VEGF-A in each PRF membrane obtained from individuals by means of ELISA technique. The concentration was measured immediately after PRF procedure, 7 days and 14 days after storage at -20°C. Hatakeyama et al. (2014) reported that the mean concentration of TGF-β1 measured in conventional-centrifugation method PRF collected from 10ml blood from twelve beagle dogs was 63.7±13.7ng/ml. This finding was relatively lower compared with our study. This contrast might be owing to different preparation procedure and variation in experimental animals. Another study of Silva et al. (2012) reported the mean TGF-β1 concentration of plasma and PRP obtained from 4.5ml of fourteen Brazilian Fila dogs blood, which were 22.08ng/ml and 19.55ng/ml respectively. As our TGF-β1 concentration measured in PRF was relatively high compared with value of the previously mentioned research, it has been supported that PRF membrane is theoretically capable of superior cytokine entrapment compared with other platelet concentrates and plasma. In similar terms, Masuki et al. (2016) also reported the significant higher VEGF-A level in PRF compared with PRP. Since recent studies have demonstrated that the PRF membrane has a very significant slow sustained release of key growth factors for at least one week (Su et al., 2009) and up to 28 days with significant decrease in concentration (He et al., 2009). We further stored the PRF membrane at -20°C for 7 and 14 days to determine whether the PRF would be suitable for storage. From our result, it might be concluded that promptly usage would provide more quantity of cytokines.

In order to understand the mechanism of PRF which affected clinical, radiological and histological outcome as previously mentioned, we conducted the various gene expression regarding wound healing and periodontal regeneration by means of qRT-PCR technique. The anti-inflammatory cytokine (TGF-β1, PDGF-B, VEGF-A, TIMP-1, COL1A1, and COL3A1) and pro-inflammatory cytokine (TNF-α and IL-1β) response pattern were

determined in 3 trial periods: day 0 (baseline), 7 DPE, and 14 DPE. Our result revealed that PD^+ group showed the significant up-regulation of all anti-inflammatory cytokines except for COL1A1. Also, significant down-regulation of pro-inflammatory cytokines was observed.

TGF-β1, PDGF, and VEGF are considered as key cytokines found in PRF. TGF-β1 plays important roles in all phase of wound healing by interacting with other cytokines to modulate immune cells, stimulate osteoblastic proliferation, and enhance collagen synthesis (Pakyari et al., 2013). PDGF-B is a member of PDGF family which is considered as a powerful chemoattractant, angiogenesis mediator, and potent activator for migration and proliferation of mesenchymal lineage cells. Its function mainly concerns in inflammatory and proliferative phase of wound healing process (F. et al., 1991). VEGF mainly functions as an endothelial mitogen, chemotactic agent, angiogenesis, and induction of epithelialization and collagen deposit (Bao et al., 2009). Its function mainly cooperate in inflammatory and proliferative process of wound healing (Johnson and Wilgus, 2014). Therefore, high expression of these cytokines would have a positively correlative effect on an entire wound healing process. Our results revealed the up-regulation of mentioned cytokines in PD^+ group, which TGF-β1 and PDGF-B pattern were correlated with study of He et al. (2009), who studied the levels of TGF-β1 and PDGF-B in PRF exudate obtained from human blood at particular time points and evaluate its effect on rat calvaria osteoblast. They reported that the highest amounts of TGF-β1 and PDGF-B released from PRF were found on day 14 and day 7 respectively. Also, osteoblastic cells reached peak mineralization when treated with PRF exudate collected at day 14. In resemblance to this, Wang et al., (2012) reported the same period of time that TGF-β1 and PDGF-B reached their highest release concentration from PRF. Moreover, they also found that adipose tissue-derived stem cells expressed maximum proliferation and alkaline phosphatase activity when cultured with PRF exudate of day 14. The highest gene expression of gingival TGF-β1 and PDGF-B found at 7 and 14 DPE that we found in our study corresponded with the peak concentration levels of TGF-β1 and PDGF-B released from PRF at the same particular day reported in other previously mentioned studies have

been suggested that the anti-inflammatory properties of PRF accelerate the expression of these 2 important cytokines, which in turns promotes the wound healing process and also the PD treatment outcome. Our VEGF expression result was resemble to the study of (Yoon et al. (2014)). They studied the effect of PRF on angiogenesis in guided-bone regeneration of cranial defect in rabbits and found that VEGF expression observed via immunostaining was higher in the group received xenogenic bone combined with PRF compared to the group that received xenogenic bone alone. As mentioned earlier, up-regulation of these anti-inflammatory cytokines is attributed to GI reduction, increase in PPD, and also decrease in inflammatory reaction score as seen in histology of PD^+ group. Concerning expression of control, we hypothesized that up-regulation of these cytokines at 7 DPE of healthy gingiva together with increase in GI at the same period were owing to normal response to inflammation caused by OFD procedure. However, normal tissue has great capacity of healing therefore down-regulation at 14 in an attempt of homeostasis achievement was seen as well as GI returned to score 0.

TIPM-1, COL1A1, and COL3A1 and are mainly concerned in proliferative and remodeling phase. TIMP functions mainly as a Matrix metalloproteinases (MMPs) inhibitor. It plays an important roles in remodeling phase by inhibiting extracellular matrix together with collagen breakdown. Collagen is considered to be a basic element of periodontium. Type III collagen is predominately synthesized at initial phase of wound healing and then gradually replaced with type I collagen 2-3 weeks after initiation of wound healing process. From our results, we found that TIMP-1 and COL3A1 expression was significant higher in experimental group. In resemblance to our result, Wang et al. (2016) studied the effect of PRF when combined with periodontal ligament stem cell (PDLSCs) and jaw bone mesenchymal stem cell (JBMSCs) sheets in an aspect of periodontal tissue engineering. They found that experimental group exhibited higher expression of COL1A1 and COL3A1 than control group. Concerning their function and expression in our study, we concluded that their high expression in PD^+ group would cooperate with previously mentioned anti-inflammatory cytokines to accelerate periodontal ligament production, which resulted in PPD reduction. The expression of these cytokines was correlated with the histological

fibrosis score. In histopath section, C group presented abundant of fibroblastic and fibrocytic proliferation, which was correlated with the higher expression level of these cytokine at baseline compared with experimental groups. Same as mentioned earlier, up-regulation of control group expression at 7 DPE was a normal response to inflammation, which triggered wound healing cascade and leaded to proliferation of connective tissue and extracellular matrix, which could be seen from increased fibrosis score at 14 DPE.

TNF-α and IL-1β are key pro-inflammatory cytokines that involved in the pathogenesis of PD and affected wound healing and regeneration process. High production of these cytokines results in stimulation other inflammatory mediators, tissue destruction by MMPs induction, bone resorption by stimulating osteoclastic activity (Grigoriadou et al., 2010; Ritsu et al., 2017). Therefore, high expression would result in increased inflammatory process and tissue destruction. Our result revealed that PD group presented higher expression of these cytokines, which was related with the higher GI and inflammatory reaction score compared with PD^{+} group. Higher PPD value and also lower fibrosis score were correlated with the cytokine expression result. In control group, expression of pro-inflammatory cytokines increased in response to inflammation as seen in increase in GI score. At 14 DPE, down-regulation was seen together with GI score decrease to 0.

Within the limitation of our present study, we have found that PRF has an ability to potentiate the wound healing and diminish inflammatory response by up-regulating essential anti-inflammatory cytokines and also down-regulating pro-inflammatory cytokines regarding PD. As illustrated in figure 33, up-regulation of anti-inflammatory cytokine (TGF-β1, PDGF-B, VEGF-A, TIMP-1, COL1A1, and COL3A1) results in chemoattractant of other immune cell and fibroblast, inhibiting the degradation of extracellular matrix, promoting angiogenesis, inducing cell proliferation, and stimulating collagen and extracellular matrix synthesis. Likewise, stimulation of inflammatory mediator production, MMP expression, apoptosis of matrix producing cells, and osteoclast activity are also diminished corresponding to down-regulation of pro-inflammatory cytokine (TNF-α and IL1-β). Consequently, increased periodontal attachment gain and decreased

gingivitis and histological inflammatory score. Thus, PRF would be an alternative novel modality for PD managements in dogs. To affirm the observations in our study, however, a long-term and large-scale studies should be carried out. Other essential cytokines concerning pathogenesis of PD and also more parameters regarding PD treatment should be further investigated.

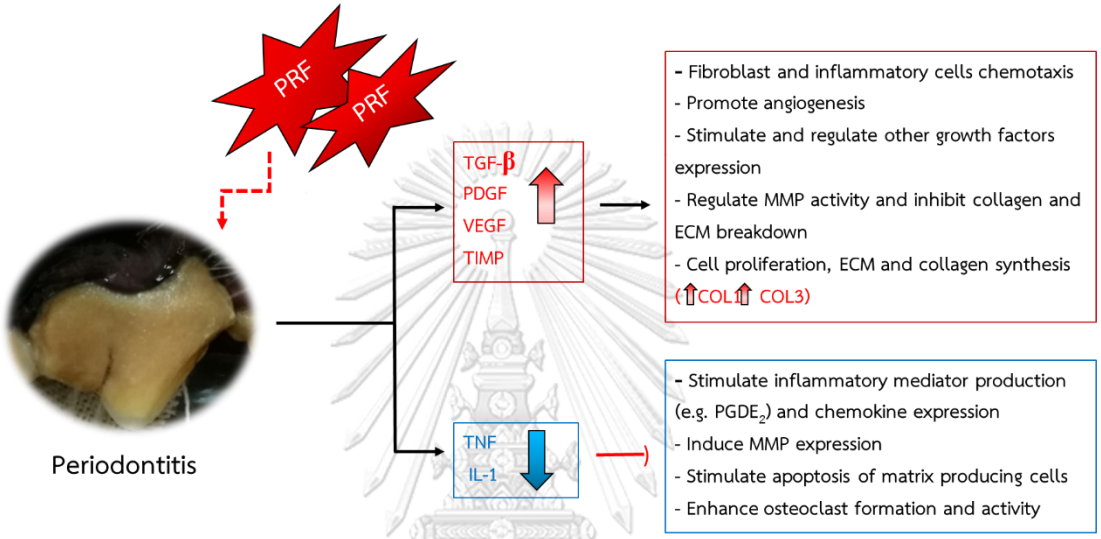

Figure 31 Schematic model of platelet-rich fibrin membrane mechanism. Abbreviations: PRF, platelet-rich fibrin; TGF-β, transforming growth factor-beta; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TIMP, tissue inhibitor matrix metalloproteinase; COL1, type I collagen; COL3, type III; TNF, tumor necrotic factor-alpha; IL-1, interleukin 1; MMP, matrix metalloproteinase; ECM, extracellular matrix; $PGDE_2$, prostaglandin E2.

APPENDIX

APPENDIX

Appendix I Breeds, age, weight of dogs in control and experimental group.

Variables	Control group	Experimental groups
Breeds	mixed	mixed
Age (year)	1.2±0.5	3.5±0.2
Weight (kg)	9.5±0.4	8.2±0.6

Appendix II Complete Blood count of experimental dogs.

	White blood cell ($*10^3$/mm)	Red blood cell ($*10^6$/mm)	Platelet count ($*10^3/mm^3$)
Control group			
1. ภาระ	12.08	5.25	250
2. ไข่	13.60	5.30	290
Experiment group			
1. มณฑา	15.90	5.25	300
2. มู่ทู่	14.50	5.05	290
3. แฝดเล็ก	15.00	4.95	250
4. แฝดใหญ่	16.10	5.00	270
5. จิ๋ว	13.20	5.00	310
6. มีมี่	15.80	5.20	300
7. ตี๊ดตี๋อ	14.90	5.00	280
8. มิเกล	15.10	5.20	290
9. มอมแมม	16.70	5.10	309
10. แบงค์	14.10	5.00	310

LIST OF ABBREVIATIONS

^{o}C	= degree Celcius
β-actin	= Beta-actin gene
µg	= microgram
µl	= microliter
A-PRF	= advanced Platelet-rich fibrin
A-PRF^{+}	= advanced Platelet-rich fibrin plus
ALP	= Alkaline phosphatase
BL	= alveolar bone level
BSP	= bone sialoprotein
cDNA	= Complementary Deoxyribonucleic acid
CEJ	= cemento-enamel junction
COL1A1	= Collagen type 1 alpha 1 chains
COL3A1	= Collagen type 3 alpha 1 chains
cPRP	= concentrated Platelet-rich plasma
CT	= cycle threshold
DPE	= Day post experiment
ECM	= extracellular matrix
EGF	= Epidermal growth factor
GI	= Gingival index
GOI	= Gene of interested
g	= gram
IGF-1	= Insulin growth factor-1
IL-1	= Interleukin-1
IL-4	= Interleukin-1
IL-6	= Interleukin-1
min	= minute
ml	= milliliter

mm = millimeter

mW = millimoles

MMP = matrix metalloproteinase

MWF = Modified Widman Flap

MI = Mobility index

ng = nanogram

nM = nanomoles

OFD = Opened-flap debridement

OC = osteocalcin

OPG = Osteoprotegerin

PBS = Phosphate-buffered saline

PD = periodontitis

PDLFs = Periodontal ligament fibroblasts

PDLSCs = Periodontal ligament stem cells (PDLSCs).

PPD = Periodontal pocket depth

PI = Plaque index

PDGF = Platelet-derived growth factor

p-ERK = Phosphorylated extracellular signal-regulated protein kinase (p-ERK),

pg = picogram

PRF = Platelet-rich fibrin

qPCR = Quantitative real time polymerase chain reaction

RNA = Ribonucleic acid

RUNX2 = Runt-related transcription factor 2; RUNX2

rpm = rounds per minute

s = second

TGF-β1 = Transforming growth factor-β1

TIMP = Tissue inhibitor of matrix metalloproteinase

TNF-α = Tumor necrosis factor α

VEGF = Vascular endothelial growth factor

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