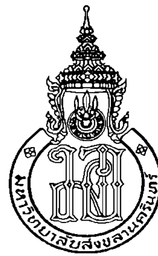

Effects of Leukocyte-Platelet-Rich Plasma on Accelerated Orthodontic Tooth Movement in Rabbits

Theerasak Nakornnoi

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Oral Health Sciences

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ชื่อวิทยานิพนธ์ ผลของลิวโคไซต์เพลทเลทริชพลาสมาที่มีต่ออัตราเร็วในการเคลื่อนฟันทางทันตกรรมจัดฟันในกระต่ายทดลอง

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บทคัดย่อ

บทนำ วิธีในการเพิ่มความเร็วในการเคลื่อนฟันทางทันตกรรมจัดฟันยังคงเป็นประเด็นที่น่าสนใจในหลายการศึกษา เพลทเลทริชพลาสมาเป็นสารชนิดหนึ่งซึ่งประกอบไปด้วยสารชีวโมเลกุลจำพวกโกรทแฟคเตอร์และไซโตไคน์ที่สำคัญหลายชนิดที่มีบทบาทต่อกระบวนการปรับรูปกระดูกและอวัยวะปริทันต์ รวมถึงกระบวนการอักเสบที่เกิดขึ้นในการเคลื่อนฟัน ดังนั้นการนำลิวโคไซต์เพลทเลทริชพลาสมามาประยุกต์ใช้จึงน่าจะส่งผลต่อการเพิ่มความเร็วในการเคลื่อนฟันและลดระยะเวลาในการรักษาทางทันตกรรมจัดฟัน แต่อย่างไรก็ตามยังไม่มีการศึกษาใดที่ศึกษาผลของการฉีดเฉพาะที่ของลิวโคไซต์เพลทเลทริชพลาสมาที่มีต่อความเร็วในการเคลื่อนฟัน

วัตถุประสงค์ เพื่อศึกษาผลของการฉีดเฉพาะที่ของลิวโคไซต์เพลทเลทริชพลาสมาที่มีต่อความเร็วในการเคลื่อนฟัน การตอบสนองของกระดูกเบ้าฟัน และปริมาณของเซลล์สลายกระดูกในกระต่ายทดลอง

วัสดุและวิธีการ กระต่ายทดลองสายพันธุ์ New Zealand white rabbit เพศผู้จำนวน 23 ตัว จะได้รับการทดลองแบบแบ่งส่วนในช่องปาก (split mouth) โดยจะทำการเคลื่อนฟันด้วยการให้แรงจากสปริงในปริมาณ 100 กรัมที่ฟันกรามน้อยบนซี่ที่หนึ่งทั้งสองข้าง จากนั้นขากรรไกรบนด้านซ้ายและด้านขวาจะถูกสุ่มเพื่อใช้ในการทดลองคือ ด้านทดลองจะได้รับการฉีดเฉพาะที่ด้วยลิวโคไซต์เพลทเลทริชพลาสมา ในขณะที่ด้านควบคุมจะได้รับการฉีดเฉพาะที่ด้วยน้ำเกลือ โดยวิธีการฉีดใต้ชั้นเยื่อเมือกที่บริเวณด้านแก้มและเพดานของฟันกรามน้อยซี่ที่หนึ่ง จากนั้นจะทำการเก็บตัวอย่างกระดูกขากรรไกรบนไปวิเคราะห์ปริมาณการเคลื่อนฟันด้วยแบบจำลองฟันดิจิตอลที่ช่วงเวลา 0, 3, 7, 14, 21 และ 28 วัน อีกทั้งทำการวัดอัตราส่วนปริมาตรกระดูก (bone volume fraction) โดยการถ่ายภาพรังสีส่วนตัดขนาดเล็กด้วยคอมพิวเตอร์

(micro-CT) และปริมาณของเซลล์สลายกระดูกด้วยกล้องจุลทรรศน์ที่ช่วงเวลา 0, 7, 14 และ 28 วัน

ผลการวิจัย กลุ่มที่ได้รับลิวโคไซต์เพลทเลทริชพลาสมามีระยะทางการเคลื่อนฟันที่มากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติในทุกช่วงเวลา โดยพบการเพิ่มขึ้นของอัตราการเคลื่อนฟันอย่างมีนัยสำคัญทางสถิติในช่วง 0-7 และ 7-14 วัน อีกทั้งพบการลดลงของอัตราส่วนปริมาตรกระดูกอย่างมีนัยสำคัญทางสถิติที่ช่วงเวลา 7 และ 14 วัน ซึ่งสอดคล้องกับการเพิ่มขึ้นของปริมาณเซลล์สลายกระดูกที่มากกว่ากลุ่มควบคุมที่ช่วงเวลา 7 และ 14 วัน

สรุปผล การฉีดเฉพาะที่ของลิวโคไซต์เพลทเลทริชพลาสมามีผลต่อการเพิ่มขึ้นของความเร็วในการเคลื่อนฟัน ด้วยการลดลงของอัตราส่วนปริมาตรกระดูกและการเพิ่มขึ้นของปริมาณเซลล์สลายกระดูก

Thesis title Effects of Leukocyte-Platelet-Rich Plasma on Accelerated Orthodontic Tooth Movement in Rabbits

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ABSTRACT

Introduction: A need of developing new methods to accelerate tooth movement has been recently investigated. Leukocyte-platelet-rich plasma (L-PRP) has been introduced as a source of autologous growth factors and secretary cytokines that are involved in bone remodeling and inflammatory process during orthodontic tooth movement. Thus, the administration of L-PRP could be used in order to accelerate tooth movement and reduce treatment time. However, no data are available on the use of L-PRP to accelerate orthodontic tooth movement.

Objectives: To determine the effects of a local injection of L-PRP on the rate of orthodontic tooth movement, alveolar bone changes, and osteoclastic activity in rabbits.

Materials and methods: Twenty-three male New Zealand white rabbits were included in a split-mouth design. Tooth movement with a 100-gram nickel-titanium closed-coil spring was performed on the maxillary first premolars. L-PRP was injected submucosally at the buccal and lingual areas of the first premolar in one random side of the maxilla and the other side served as the control and received normal saline. The amount of tooth movement was assessed on three-dimensional digital models at day 0 as the baseline and on days 3, 7, 14, 21, and 28. The bone volume fraction and the osteoclast numbers were assessed at 0, 7, 14, and 28 days by microcomputed tomography (micro-CT) and histomorphometric analysis, respectively.

Results: The L-PRP group showed a significantly increased cumulative amount of tooth movement at all observed periods. However, a significantly higher rate

of tooth movement was observed only at 0-7 days and 7-14 days. The bone volume fraction was significantly decreased in the L-PRP group at 7 and 14 days, which corresponded to a significant increase of the osteoclast numbers in L-PRP group on days 7 and 14.

Conclusion: Local injection of L-PRP resulted in an increased rate of tooth movement by decreased bone volume density and increased the number of osteoclasts.

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CONTENTS

Page

ABSTRACT (THAI LANGUAGE) v
ABSTRACT (ENGLISH LANGUAGE) vii
ACKNOWLEDGEMENT ix
CONTENTS x
LIST OF TABLES xi
LIST OF DIAGRAMS / FIGURES xii
LISTS OF ABBREVIATIONS AND SYMBOLS xiii
CHAPTER
1. INTRODUCTION 1
Background and rationale 1
Review of literatures 2
2. RESEARCH METHODOLOGY 22
3. RESULTS 28
4. DISCUSSION 34
5. CONCLUSION 39
REFERENCES 40
APPENDICES 51
VITAE 54

LIST OF TABLES

Table Page

1. Bioactive molecules in α-granules of platelets……………………………...……...4
2. A comparison of protocols from previous studies used for preparing L-PRP in rabbits……………………………………………………………….………...10
3. The body weights of rabbits during the experimental periods…………...………28

LIST OF DIAGRAMS / FIGURES

Figure Page

1. Conceptual framework in this study..21
2. Diagram of the study design...23
3. Schematic of the preparation process of L-PRP..24
4. (A) Schematic of appliance for orthodontic tooth movement in rabbit………….25
(B) Tooth movement measurement using the 3Shape Ortho Analyzer software.
5. A region of interest……………...26
6. (A) Platelet, (B) Leukocyte concentrations in whole blood and L-PRP…….........30
7. (A) Cumulative distance of tooth movement in L-PRP and control groups.
(B) Rate of tooth movement in L-PRP and control groups................................31
8. Bone volume fraction (BV/TV) in L-PRP and control groups……........................ 32
9. The number of osteoclasts in L-PRP and control groups……............................ 32
10. Histologic sections showed the compression side of the maxillary first premolar in L-PRP group (A, C, E) and the control group (B, D, F) on days 7, 14 and 28 respectively after orthodontic force application…......................... 33

LISTS OF ABBREVIATIONS AND SYMBOLS

Abbreviation		Meaning
L-PRP	=	Leukocyte-platelet-rich plasma
Micro-CT	=	Micro computed tomography
PDL	=	Periodontal ligament
PRP	=	Platelet-rich plasma
IL	=	Interleukin
TNF	=	Tumor necrosis factor
MMP	=	Metalloproteinases
NK-**K**B	=	Nuclear factor kappa B
RANKL	=	Receptor activator of nuclear factor kappa-B ligand
TGF	=	Transforming growth factor
PDGF	=	Platelet derived growth factor
FGF	=	Fibroblast growth factor
IGF	=	Insulin like growth factor
VEGF	=	Vascular endothelial growth factor
EGF	=	Epidermal growth factor
HGF	=	Hepatocyte growth factor
ECGF	=	Endothelial cell growth factor
TIMP-4	=	Tissue inhibitor of metalloprotease-4
PCAM	=	Platelet endothelial cell adhesion molecule
P-PRP	=	Pure platelet-rich plasma
P-PRF	=	Pure platelet-rich fibrin
L-PRF	=	Leukocyte-platelet-rich fibrin
EDTA	=	Ethylenediaminetetraacetic acid
ACD-A	=	Acid-citrate-dextrose solution A
CPD	=	Citrate-phosphate-dextrose
CTAD	=	Citrate-theophylline-adenosine-dipyridamole
MPV	=	Mean platelet volume

LISTS OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

ACD	=	Acid-citrate-dextrose
TEM	=	Transmission electron microscopy
sP-selectin	=	Soluble platelet selectin
MSC	=	Mesenchymal stem cell
AC	=	Anticoagulant
SC	=	Sodium citrate
BT	=	Bovine thrombin
rpm	=	Revolutions per minute
g	=	Gravity
NS	=	Non-specific
BMSC	=	Bone marrow-derived stem cell
BMP	=	Bone morphogenic protein
SDF-1	=	Stromal cell-derived factor-1
OPG	=	Osteoprotegerin
COX	=	Cyclooxygenase
PGE-2	=	Prostaglandin E2
BV/TV	=	Bone volume fraction
BV	=	Bone volume
TV	=	Total volume
kVp	=	Kilovoltage peak
μA	=	Microampere
ROI	=	Region of interest
H&E	=	Hematoxylin and eosin
iNOS	=	Inducible nitric oxide synthase
NO	=	Nitric oxide
TRAP	=	Tartrate-resistant acid phosphatase

CHAPTER 1

INTRODUCTION

Background and rationale

Orthodontic tooth movement occurs by bone remodeling of the alveolar bone and periodontal ligament (PDL) through a biphasic process of bone resorption on the compression side and bone deposition on the tension side. Orthodontic force induces an initial inflammatory response with increased vascular permeability and leukocyte migration. These migrated cells produce inflammatory cytokines that include lymphocyte-derived factors, chemotactic factors, and growth factors. Bone remodeling also occurs with increased bone turnover such as recruitment of osteoclast precursors from the circulation, and enhancement of osteoclast formation and activation. Therefore, a higher cytokine expression on bone remodeling may accelerate the rate of tooth movement.[1,2]

Platelet-rich plasma (PRP) is an autologous concentration of platelets in a small volume of plasma. It has been used extensively as a source of autologous growth factors and secretory cytokines provided by the concentrated platelet suspension.[3] It is known that these factors are involved in the regulation and stimulation of cellular processes such as inflammation, angiogenesis, chemotaxis, proliferation, and differentiation.[4,5] PRP has been suggested as a beneficial therapy to promote tissue repair and regeneration.[6-8] Numerous studies have demonstrated that PRP in bone regeneration has positive effects in stimulating and enhancing the healing process.[9-11] However, there is disagreement on the efficacy of PRP due to controversial results.[12,13] The variety of clinical outcomes possibly resulted from the variations in the formulations and cellular compositions of the PRP.[11,14-18]

Recently, leukocytes have been included in PRP formulations called leukocyte-platelet-rich plasma (L-PRP) and they have received increased attention for their therapeutic effect on tissue regeneration. Previous studies reported that L-PRP

could promote bone regeneration by upregulating cellular proliferation, viability, migration, angiogenesis, and osteogenesis.[11,19] In addition, studies have revealed that the concentration of leukocytes in PRP were correlated with increased levels of catabolic cytokines, including interleukin (IL)-1 , IL-6, and tumor necrosis factor (TNF)-α, and resulted in the production of destructive metalloproteinases (MMP).[17,20-22] Interestingly, with the concentrated leukocytes in L-PRP, it was possible to stimulate the nuclear factor kappa B (NF-κB) pathway via IL-1, TNF-α, and receptor activation of NF-κB ligand (RANKL).[11,20,23] This pathway results in osteoclastogenesis and bone resorptive activity leading to decreased bone density.

Long durations of orthodontic treatment have been associated with adverse effects that include higher risk of caries, root resorption, and gingival recession. A number of attempts have been made to find new methods to accelerate tooth movement with reduced treatment times and side effects. It is known that the rate of tooth movement depends on the turnover rate of bone remodeling. During orthodontic tooth movement, any alterations in alveolar bone density can accelerate the rate of tooth movement. Recent studies[24,25] suggested that the administration of concentrated PRP could effectively accelerate tooth movement. Gulec et al.[25] reported that rats that received PRP had a decrease in the alveolar bone density and increased activity of osteoclasts. However, the limitation of the rat model was autologous PRP was not used because the blood volume of the rats was insufficient for self-produced PRP. Additionally, an immune reaction could result leading to erroneous results. According to a literature review, no data are available on the use of L-PRP to accelerate orthodontic tooth movement. Therefore, the aim of this study was to determine the effect of local administration of L-PRP on accelerating orthodontic tooth movement in rabbits.

Review of literatures

<u>Platelet-Rich Plasma</u>

PRP is defined as a volume of plasma that contains high concentration of platelets above baseline. Normally, the range of platelet in whole blood is 150,000 to

350,000 platelet/μL but the PRP contains a platelet concentration at least 1,000,000 platelets/μL in 5 ml of plasma. It could be defined as a 3-5 fold increase in a platelet concentration over baseline.[26]

Platelets, also called thrombocytes, play a major role in hemostasis and are a natural source of growth factors. Platelets are small anucleate cellular elements that have a characteristic discoid shape and approximately 2-3 μm in diameter.[27] Platelets are derived from the cytoplasm of megakaryocytes in the bone marrow and enter the circulation. Circulating platelets have average life span about 7-10 days before removal from bloodstream and replace with new platelets.[28] At the injured site, the formation of a blood clot occurs through platelet adhesion to the damage tissue. Platelets are activated by collagen-binding and endothelium-derived substance including tissue factor and von Willebrand factor, a major agonist of platelet activation, causing platelet degranulation and aggregation.[27] Tissue factor also activates thrombin to convert fibrinogen to fibrin which as a reservoir of growth factors and cytokines. Moreover, activated platelets release additional their granules contents into surrounding environment.

Platelets have three types of granules including alpha granules, dense granules and lysosomes that store bioactive molecules precisely involved in initiation of coagulation and recruiting other cells during inflammation. Alpha granules are the most abundant platelet granules. These granules contain numerous proteins and growth factors such as transforming growth factor (TGF)-β, platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF)-1, IGF-2, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF).[5,29,30] These factors bind surface receptors on the target cells, causing the regenerative process such as reactivity of blood vessels and blood cell types involved healing mechanism by the regulation and stimulation cellular process such as inflammation, angiogenesis, chemotaxis, differentiation, and metabolism. It could enhance the biological process involved tissue repair and regeneration.[6,8] A list of these factors and their functions in detailed in table 1.

Table 1. Bioactive Molecules in α-Granules of Platelets[8,31]

Categories	Specific Molecules	Biologic Activities
Growth factors	TGF-β	Enhances synthesis of type I collagen, promotes angiogenesis, stimulates chemotaxis of immune cells, inhibits osteoclast formation and bone resorption
	PDGF	Enhances collagen synthesis, proliferation of bone cells, fibroblast chemotaxis and proliferative activity, macrophage activation
	IGF	Promotes cell growth, differentiation, recruitment in bone, blood vessel, skin and other tissues, stimulates collagen synthesis together with PDGF
	FGF	Promotes proliferation of mesenchymal cells, chondrocytes and osteoblasts, stimulates the growth and differentiation of chondrocytes and osteoblasts
	EGF	Stimulates cellular proliferation, differentiation of epithelial cells, promotes cytokine secretion by mesenchymal and epithelial cells
	VEGF	Stimulates angiogenesis, migration and mitosis of endothelial cells, increases permeability of the vessels, stimulates chemotaxis of macrophages and neutrophils
	ECGF	Endothelial cell proliferation, angiogenesis

ECGF; endothelial cell growth factor

Table 1. (Continued)

Categories	Specific Molecules	Biologic Activities
Adhesive proteins	Fibrinogen	Blood clotting cascade (fibrin clot formation)
	Fibronectin	Binds to cell-surface integrins, affecting cell adhesion, cell growth, migration, and differentiation
	Vitronectin	Cell adhesion, chemotaxis
	Thrombospondin-1	Inhibition of angiogenesis
Clotting factors	Factor V, factor XI, protein S, antithrombin	All play a role in thrombin activation and eventual fibrin clot formation
Fibrinolytic factors	Plasminogen	Plasmin production (leads to fibrinolysis)
	Plasminogen activator inhibitor	Regulation of plasmin production
	α-2 antiplasmin	Inactivation of plasmin
Proteases and antiproteases	TIMP-4	Regulation of matrix degradation
	Metalloprotease-4	Matrix degradation
	α1-antitrypsin	Inhibits a wide variety of proteases and enzymes
Basic proteins	Platelet factor 4	Inhibition of angiogenesis
	β-thromboglobulin	Platelet activation, inhibition of angiogenesis
	Endostatins	Inhibitors of endothelial cell migration and angiogenesis

TIMP-4; tissue inhibitor of metalloprotease-4

Table 1. (Continued)

Categories	Specific Molecules	Biologic Activities
Membrane glycoproteins	CD40 ligand	Inflammation, synthesis of interleukins, and integrin production; platelet endothelial cell adhesion, cell signaling, modulation of platelet endothelial cell adhesion molecule (PECAM)-1 on leukocytes

PRP preparation

PRP is prepared by withdrawing autologous blood in the presence of an anticoagulant, which binds calcium and inhibits the clotting cascade by preventing the conversion of prothrombin to thrombin. The anticoagulated whole blood is then achieved to obtain a highly concentration of platelets by the various different methods including selective blood filtration, single- and double-centrifugation method. Centrifugation is one of the most wildly used process in the selective separation of the liquid and solid components of whole blood. From the physical principle described by Stoke's law, the settling velocity of particles in a fluid under condition in gravitational force will be approximately proportional to their diameter. For instance, platelets are approximately 2-3 μm in diameter while red blood cells and white blood cells are approximately 7 and 7-15 μm in diameter respectively. Therefore, under the gravitation force, a platelet will settle proportionally slower than red blood cell or white blood cell.[32]

The double-centrifugation method can be easily to create concentrated platelet. Basically, the first centrifugation separates the blood into three fractions. The red blood cells settle down at the bottom layer while the acellular plasma, called "platelet-poor plasma", is at the top layer. Between two layers, the buffy coat is a whitish color layer which contains the major proportion of the leukocytes and platelets. Plasma and buffy coat are then collected for a second centrifugation to obtain a highly platelet concentrations. The upper part as the platelet poor plasma is discarded while the lower part as PRP that contains the concentrated platelets in suspension is harvested.[33]

Subsequently, PRP may be activated with endogenous or exogenous activators to initiate the clotting mechanism and induce platelet activation. The platelets undergo degranulation to released growth factors, cytokines and adhesion molecules that are involved in tissue healing.

Nowadays, several protocols to creation of PRP have been proposed. Different protocols result in different characters and potential usages of product leading to a confusing application. Dohan Ehrenfest et al.[34] has been purposed the classification of platelet concentrates according to at least two key parameters; the presence of leukocytes and fibrin architecture. Following these criteria, the platelet concentrates is divided in four main families; the pure PRP (P-PRP), L-PRP, pure platelet-rich fibrin (P-PRF), and leukocyte-platelet-rich fibrin (L-PRF). This classification can also allow understanding easily their characteristics and specific clinical potential applications.

Factors determining PRP preparation

Anticoagulant used

During the process in PRP preparation, platelets can adversely affect their structure and function that influence the efficacy of the final platelet product. To minimum aggregation and activation of platelet, anticoagulant plays a major role in preventing the coagulation cascade. Various anticoagulants are available for PRP preparation including heparin, citrate, ethylenediaminetetraacetic acid (EDTA), acid-citrate-dextrose solution A (ACD-A), citrate-phosphate-dextrose (CPD) and citrate-theophylline-adenosine-dipyridamole (CTAD).[35,36]

To clarify the appropriate anticoagulant choice in PRP creation, the effect of anticoagulants on PRP quality and efficacy was investigated. In previous study, the different anticoagulants resulted in the changes in platelet morphology and the extent of spontaneous activation of platelets. They found that the use of EDTA resulted in a higher mean platelet volume (MPV) compared with sodium citrate and acid-citrate-dextrose (ACD). It indicates that EDTA is more spontaneous activation of platelets following the

centrifugation process.[37,38] Furthermore, the use of EDTA is potentially more detrimental to change the platelet morphology.

Heparin is commonly used in collecting blood for PRP production. From study in transmission electron microscopy (TEM) showed that the PRP obtaining heparin had less granules of platelet and earlier lysis than other anticoagulants. It also showed the higher levels of soluble platelet selectin (sP-selectin) after venipuncture.[35] Additionally, heparin treated-blood showed more platelet activation and aggregation after agitation.[38] It may indicate that heparin cannot prevent the spontaneous activation of platelets. Thus, heparin may not an appropriate anticoagulant for PRP preparation.

In clinical or research applications, citrate is the commonly used anticoagulant in PRP preparation. One study suggested that the use of sodium citrate as an anticoagulant in PRP production. They found that the obtaining in sodium citrate had a higher platelet recovery following the first centrifugation step and minimal alteration in mesenchymal stem cell (MSC) gene expression.[36] On the other hand, H. Lei et al.[35] showed that the platelets in citrate had decreased the alpha granules and dense granule, more spontaneous activation and less the amount of growth factor released from PRP.

Although several available anticoagulants are clinically used. ACD-A has been found to support the metabolic needs of platelet and the viable separation of platelets in an undamaged manner.[26] In previous study, it demonstrated that ACD was more efficient in maintaining platelet morphology than heparin and sodium citrate. Additionally, PRP obtained with ACD resulted in higher the amount of growth factor and the efficacy of PRP in stimulating cell proliferation.[35] However, Giraldo et al.[39] found that the type of anticoagulants was not an effect on the amount of platelets and growth factors in equine PRP.

Centrifugation

Several simplified protocols have been developed to facilitate its clinical application. It must be taken into account that the difference in centrifugation methods

may result in varying compositions of PRP according to the different biological responses. These variables in this process include the number of spins, centrifugation force and time period of centrifugation.

In the number of centrifugations, single- and double-centrifugation, the effects of the protocol to create PRP have an influence on the quality and quantity of platelets in PRP samples. Nagata et al.[40] showed that the double-centrifugation had a higher platelet concentration than single-centrifugation. The same finding was reported by Filardo G et al.[41], a platelet concentration of the single-centrifugation could be obtained 1 to 3 times above baseline, while the double-centrifugation achieved 4- to 8-fold baseline levels. Remarkably, a low platelet concentration which achieved by single-centrifugation may possibly suboptimal effects. Accordingly, Marx et al.[42] indicated that a double-centrifugation technique should be utilized to truly concentrated platelets from autologous blood. However, the double centrifugation protocol may cause change in platelet morphology.[40]

The different centrifugation force and time influence the variation of final product. S. Arona et al.[43] found that when the centrifugation time was constant, a higher centrifugation force provided a higher platelet yield and vice versa. However, the higher centrifugation force or time leads to more platelets settling down out of the plasma and may cause an auto-aggregation according to suboptimal effect in clinical outcome.

Numerous studies have been demonstrated the various protocols to optimize the centrifugation procedure using various performance standards and centrifugation variables including volume of whole blood, number of spins, duration of blood centrifugation and range of centrifugal force. Considerably, it should be noted that the blood samples in this study was performed in rabbits. Accordingly, this literature takes into account protocols for making L-PRP from previously studies in rabbit model. A comparison of protocols used for preparing L-PRP is shown in table 2.

Table 2. A comparison of protocols from previous studies used for preparing L-PRP in rabbits.

Study	Blood sample	AC	centrifugation		PRP	Activation	Platelet conc.	Leukocyte conc.
	Ml	type	1st	2nd	ml	type		
Shin KH et al.	10	ACD	2400 rpm/10'	3600 rpm/15'	0.6-0.7	$CaCl_2$ +BT	4x	11x
Giovanini et al.	15	SC	200*g*/10'	400*g*/10'	1	$CaCl_2$	6.5x	5x
Vokurka et al.	8	heparin	400*g*/10'	500*g*/10'	1.3	NS	2.8x	2.4x
Garcia et al.	15	SC	200*g*/20'	400*g*/10'	1	$CaCl_2$	8x	4.6x
Zhang et al.	9	SC	500*g*/10'	3000*g*/5'	NS	$CaCl_2$	3x	4x
Jia et al. ***	15	ACD	250*g*/10'	1000*g*/10'	1	$CaCl_2$ +BT	6.9x	5.9x
Li et al.	15	ACD	250*g*/10'	1000*g*/10'	1	BT	7.2x	5x
Yin W-J et al.	10	ACD	250*g*/10'	1000*g*/10'	1	$CaCl_2$	NS	NS

AC; anticoagulant, SC; sodium citrate, BT; bovine thrombin, rpm; revolutions per minute, *g*; gravity, NS; non-specific

Shin KH et al.[13] used double-spinning approach with two centrifugation techniques for preparation of L-PRP. 10 ml of blood was collected into tubes containing acid citrate dextrose. The sample tube was centrifuged at room temperature at 2,400 rpm for 10 minutes (first spin) and 3,600 rpm for 15 minutes (second spin) that produced a high platelet concentration (approximately 4 times greater than the baseline) and high leukocyte (approximately 11 times greater than base line).

A.F. Giovanini et al.[44] demonstrated that the centrifugation procedure processed 15 ml of autologous blood into a syringe containing 10% sodium citrate for two spins at 200*g* for 20 minutes and 400*g* for 10 minutes at room temperature. This protocol obtained approximately 6.5 times of the platelet concentration and 5 times of the leukocyte concentration above the baseline value.

J. Vokurka et al.[45] obtained a PRP sample that had 2.8 times of the platelet concentration and 2.4 times of the leukocyte concentration compared with initial blood sample. The centrifugation procedure processed 8 ml of blood into a tube with heparin for two spins at 400*g* for 10 minutes and 500*g* for 10 minutes at 21°C.

Garcia et al.[46] collected a 15 ml sample of autologous blood from each animal into a tube containing 10% sodium citrate. The sample was centrifuged at room temperature at 200*g* for 20 minutes and 400*g* for 10 minutes respectively. This protocol achieved an average of 2,500,000 platelets/μl and 43,000 leukocytes/μl while initial blood values were accounted for approximately 300,000 platelets/μl and 9,300 leukocytes/μl.

Zhang et al.[18] found that the concentration of platelets was 3-fold higher than in peripheral blood and the concentration of leukocytes was 4-fold higher than in peripheral blood by applying the double centrifugation. It was processed from the 9 ml of blood mixed with 3.8% sodium citrate and centrifuged at 500*g* for 10 minutes and at 3,000*g* for 5 minutes, respectively.

Jia et al.[47] demonstrated that the L-PRP was prepared by 15 ml of blood together with ACD-A to prevent clotting and then the sample underwent two-step centrifugation at 250*g* for 10 minutes and 1,000*g* for 10 minutes, respectively. This

protocol achieved an average 6.9-fold and 5.9-fold increase in platelets and leukocytes. Meanwhile, Li et al.[48] found that the PRP creation from this protocol represented an approximately of 7.2-fold and 5-fold increase in platelets and leukocytes.

Yin W-J et al.[23] shown that 10 ml of whole blood was collected into centrifuge tube with ACD-A and then it was spun two-step centrifugation according to previously protocol.[47] It had been shown that platelet concentration was 2,184.80 ± 428.19 (10^6/ml) and leukocyte concentration was 51.24 ± 19.53 (10^6/ml).

Activation of PRP

Several methods of platelet activation have been reported such as shear force cause by blood flow, contact with a various factor including collagen and basement membranes of cells, and thrombin. Thrombin is a serine protease derived from prothrombin that convert of fibrinogen to fibrin in order to initiate a clot formation. Simultaneously, platelets are activated to release growth factors of their alpha granules. Thus, the most common activator to trigger platelet activation is bovine or autologous thrombin. Marx[26] reported that within 10 minutes after used thrombin to activate platelet, approximately 70% of stored growth factors were released, and nearly 100% within an hour. Because of a short period of growth factors in minute and hour, activating PRP with exogenous thrombin may actually shorten the exposure of target cells to these growth factors. Furthermore, the bovine thrombin has potential adverse effects including hemorrhage, thrombosis and cause an immune reaction lead to increase antibody levels to human coagulation proteins. In vivo study[49], bovine thrombin can induce antibodies against clotting factors to lead to an autoimmune syndrome with lupus-like reaction.

Resulting from the potential risks with bovine thrombin, calcium chloride has gained popularity in clinical application. It can activate PRP by stimulate the formation of innate thrombin and replenish the binding site previously bound by anticoagulant. Nevertheless, the using an exogenous substance increases a problematic risk of allergic reaction and may also lead to pain and burning sensation due to low pH at the injection sites.

Recently, type I collagen has been reported to be an alternative of platelet activation. Fufa et al.[50] demonstrated that type I collagen was equally potential to stimulate platelet activation as thrombin and to release the growth factors over 10 days. Collagen had also a less retraction of clot form that may be important for wound healing scaffold. The other study that reported by Harrison et al, the use of collagen for activation had a sustained released of cytokines over a week period particularly TGF-β had an 80% greater cumulative released when compared with thrombin activation.[51] Conversely, C. Calvallo et al.[52] found that collagen-activated platelet concentrates released a lower amount of growth factors and exhibited less aggregation.

Interestingly, in vivo and in vitro studies founded that non-activated platelet had a higher cell migration and differentiation, more angiogenesis and faster wound healing when compared with thrombin-activation. Although activated platelets release immediately a higher concentration of growth factor, non-activated platelets likely deliver specifically growth factor to bind receptor on target site.[53] Thus, pre-activation of PRP may not be necessary and could lead to reduction in the final concentration of growth factors at the injury site. Avoidance of exogenous activators may also limit the negative side effects of PRP treatment.

Application of PRP

PRP is becoming a new approach to tissue regeneration. It is widely used in various therapeutic applications including osteoarthritis, tendinitis, muscle injury, bone repair and regeneration, chronic wound healing, and oral surgery. A large variety of growth factors and cytokines that are secreted by α-granules in platelets should be highlighted. These mediators play an important role in tissue repairing mechanism such as promote proliferation, chemotaxis and the differentiation of cells which are essential to enhance soft tissue healing and bone regeneration. There is considerable interest in using these autologous products for therapy in clinical settings.

The efficacy of PRP on healing has extensively investigated in vitro and in vivo studies. Several studies[10,54,55] reported that the PRP could induce mitogenic

effects of various stem cells including bone marrow stem cells (BMSCs), adipose- and muscle-derived stem cells. Zou J. et al.[54] investigated the effect of PRP on the osteogenic potential of BMSCs. They showed that PRP group had positive staining of alkaline phosphatase and mineralized nodules. These results suggested that PRP could promote bone regeneration by enhancing proliferation and differentiation of bone marrow-derived stem cells (BMSCs). In addition, when PRP was used in conjunction with bone morphogenetic protein (BMP) in vitro, their ability to osteoblast differentiation of MSCs was significantly promoted.[9] These finding suggested that sustained release of cytokines from PRP promoted proliferation and differentiation of MSCs, whereas BMP increased the differentiation of MSCs. Furthermore, PRP can also stimulate the migration of BMSCs in vitro.[56] These finding suggested that a high level of growth factors in PRP such as PDGF, FGF, TGF-β could enhance the proliferation of MSCs and chemokines such as stromal cell derived factor 1 (SDF-1) could stimulate chemotaxis of MSCs. Although, numerous in vitro studies have demonstrated a relationship between the chemotactic, mitogenic, and stimuli provided by platelets. The concentration of platelets does not proportionally correlate with the biological effects of PRP.[57]

Interestingly, the different concentration of platelets results in different levels of efficacy in inducing cellular processes.Giusti et al.[55] found that a high concentration of PRP had an inhibitory effect on proliferation, migration and production of collagen whereas MMP production increased. Likewise, in the in vitro study carried out by Choi et al.[58], the viability and proliferation of alveolar bone cells depended on concentrations of PRP manner. They demonstrated that the low concentrations of PRP could stimulate, while the high concentrations suppressed the viability and proliferation of the bone cell. Moreover, in vitro studies[57,59] suggested that a lower concentration of platelet in PRP was suboptimal effect, on the other hand, a higher concentration could be paradoxically inhibitory. Nevertheless, the effect of PRP are not just attributable to concentrated platelets, but the cellular compositions of PRP should be considered.[17]

The different cellular compositions of PRP have an influence on the levels of growth factor and cytokine concentrations. In vivo studies found that the concentration

of platelets correlated positively with anabolic mediators including TGF-β, PDGF.[17] In contrast, the concentration of leukocyte in L-PRP correlated positively with catabolic and inflammatory factors such as MMP-2, MMP-3, MMP-9, TNF-α, IL-1, IL-6, IL-8.[4,11,17,20,60] Interestingly, these finding imply that the inclusion of leukocytes may be associated with increased levels of catabolic signaling, while the platelets were associated with anabolic signaling molecules.

Some PRP preparations include leukocytes in the final product. It is known that leukocyte plays a key role in the initial phase of inflammation. A high concentration of the inflammatory mediators may increase pain and swelling on the target site. In addition, it may have detrimental effects in order to release of reactive oxygen species that can lead to potential tissue damage. Therefore, the inclusion of leukocyte may be associated with an exaggerating local inflammation and impaired remodeling. However, an inflammation response is required to initiate tissue healing and repair. For this reason, the relative presence of leukocytes must be thoroughly investigated.

There are uncertainly results of the inclusion of leukocytes on the clinical efficacy. Some studies recommended that the presence of leukocyte in PRP may be negative for the therapeutic outcome.[11,21,60,61] The in vivo and in vitro studies showed the different therapeutic outcomes between L-PRP and P-PRP that had similar levels of platelets and growth factors. L-PRP was shown to be less effective than P-PRP. These results may be involved the role of leukocytes in L-PRP. It also knows that a higher concentration of leukocytes induces exuberant levels of pro-inflammatory cytokines such as IL-1 and TNF-α. These factors may increase harmful effects on tissue regeneration via the activation of the NF-kB pathway.[11,20,23] Furthermore, leukocyte plays an important role in immune system that influence the detrimental effects through triggering immunological response for a long time.

It seems that leukocyte is not beneficial for therapeutic benefits. On the contrary, several studies have been shown the advantages of L-PRP in the field of tissue healing and regeneration. In vitro study[62] reported that the L-PRP significantly increased

a higher proliferation of bone marrow stromal cell compared with P-PRP. Furthermore, the previous studies reported the potential benefits of including leukocytes in PRP were an antimicrobial activity and immunological resistance. In vitro study[63] showed that the effect of antimicrobial properties of L-PRP could inhibit the growth of Staphylococcus aureus and Escherichia coli. Similarly, Moojen DJ. Et al.[64] found that platelet-leukocyte gel was significant greater microbial activity against bacteria than PRP alone. Moreover, Li GY et al.[48] demonstrated in a rabbit model that L-PRP gel could also have an antimicrobial efficiency in treating chronic osteomyelitis.

PRP in orthodontics

Orthodontic tooth movement occurs by bone remodeling of the alveolar bone and PDL in response to an orthodontic force, resulting in an extensive cellular and molecular changes. The release of various molecules including cytokines, growth factors colony-stimulating factors have an influence a process of bone remodeling to trigger cellular responses such as recruitment of osteoclast precursors from the blood stream, an increase of osteoclast formation and activation. Therefore, the enhancing biological molecules involved in the bone remodeling may affect to accelerate the rate of tooth movement.[1,2]

In orthodontics, the long duration of treatment has been associated with a higher risk of caries, root resorption and periodontal diseases. Accordingly, current knowledge has focused increasingly on the new methods to accelerate tooth movement for reducing treatment times and side effects. Recently, the studies[24,25,65] suggest that the administration of the concentration of PRP can be effective on the accelerated tooth movement. Similarly, in vivo studies[25,65] demonstrated that PRP could increase the rate of tooth movement by decreased alveolar bone density and enhanced osteoclastic activity. There is a considerable interest in understanding how PRP influence bone remodeling process involved in orthodontic tooth movement.

As previously mentioned, PRP has been advocated as a high concentration of growth factors and cytokines that are secreted by activated platelets.

These bioactive molecules play an important role in stimulator of cell proliferation, migration, differentiation and angiogenesis that are involved in the process of bone remodeling. For instance, PDGF is known to be a chemotactic promoter and stimulator of osteoprogenitor cell. In vitro study showed that PDGF-AA could promote osteogenic differentiation and migration.[66] In contrast, PDGF-BB was involved in bone resorption by increasing osteoclasts formation and osteoclast precursor cell chemotaxis.[67]

TGF-β is an essential mediator to enhance the process of bone formation including increasing migration of osteoblastic cells, stimulating osteoblast proliferation and differentiation at early stages. Additionally, this factor inhibits osteoclast formation by decreasing RANKL and increasing osteoprotegerin (OPG) expression.[68] From human study demonstrated that the concentration of TGF-β1 protein in gingival crevicular fluid was significantly increased after 24 hours of orthodontic force application.[69] Therefore, the expression of TGF-β1 may be associated with the more activated bone formation process as part of the stimulated bone remodeling activity during tooth movement.

VEGF is an essential growth factor for stimulating new blood vessel formation and regulating bone repair and regeneration. Nakai et al.[70] reported the expressions of VEGF and its receptor were increased by mechanical force during orthodontic tooth movement. VEGF acts as important mediators during these processes to enhance angiogenesis and osteogenesis by inducing neovascularization into bone tissue by attracting endothelial cell and directly regulating differentiation and function of osteoblasts. From previous study showed that VEGF significantly increased the expression of alkaline phosphatase and osteocalcin; markers of osteogenic differentiation of osteoblasts.[71] Moreover, VEGF increases bone formation by potentially inhibited osteoblast apoptosis.[72] On the other hand, VEGF can enhance osteoclastic bone resorption by induced RANKL expression.[73] Thus, VEGF may have anabolic and catabolic effects that is necessary for maintenance of normal bone remodeling.

Meanwhile, the composition of PRP is not only growth factors, it also contains cytokines, adhesive proteins, proteases, antiproteases and leukocytes.[8]

Because tooth movement is a consequence of an inflammatory process, the presence of leukocytes in PRP can accelerate tooth movement. Cytokines derived from PRP including interleukins or tumor necrosis factors are involved the regulation of immunologic response of bone remodeling during tooth movement. Ren et al.[74] reported that the levels of cytokines such as IL-1, IL-6 and TNF-α were increased after 24 hours of tooth movement. These cytokines have an influence on the induction of osteoclast differentiation and function, contributing to the stimulation of the bone remodeling process and tooth movement.[75,76] Also leukocyte contents in PRP are able to up-regulate cyclooxygenase (COX)-2 expression and prostaglandin E2 (PGE-2) production.[11] These proinflammatory mediators are essential in the bone remodeling process. In vitro study shown that the levels of COX-2 expression and PGE-2 production were higher in compressed bone cells.[77] In addition, PGE-2 is also associated with an increased osteoclast formation and bone remodeling activity by increased RANKL and decreased OPG expression in osteoblasts.[78]

Although research studies indicate that the efficacy of PRP on accelerating tooth movement is attributed to the positive effect of the PRP contents, but the exact mechanism of action of each of the factors on bone remodeling remains unclear. At this point, a major difficulty in understanding PRP biology is that many of the PRP contents have multiple and overlapping biologic effects. There is an obvious need to investigate in more depth the molecular mechanisms of the biological factors in PRP underlying accelerated tooth movement that make the procedure most effective with the fewest side effects to patients.

Rabbit model

Rabbits are small mammals in the family of Leporidae of the order Lagomorpha. When considering a phylogenetic relationship, rabbits are more closely related to primates than rodents. Rabbit strains have a more diverse genetic background than inbred and outbred rodent strains, which makes the model a better overall approximate to humans. However, in some respects have a different physiology

comparable to humans. To illustrate, the characteristic of rabbit bone is diverse to human bone. Rabbit has a faster skeletal change and bone turnover.

The white New Zealand rabbit (Oryctolagus cunuculus) is commonly used as a model for in vivo studies. These strains are less aggressive in nature and have less health problems as compared with other breeds. The using a rabbit as an animal model has an amount of advantages including easy to handle and observe, more economical when compared with the expense of larger animal, short cycle of bleeding and skeletal maturation.[79] Moreover, it has a proper size enough to permit non-lethal monitoring of physiological changes.

Although rabbits are closer to primates than rodents, the rabbit teeth are different to the human teeth. Rabbits have a diphyodont and heterodont dentition. The permanent teeth of rabbit consist of 28 teeth: four incisors, six premolars, and six molars on the upper maxillary, and two incisors, four premolars, and six molars on the mandible. The upper second incisors have typically a peg shape behind the first upper incisors. There is a relatively large diastema between the incisors and premolars in order to the lack of canines. Their teeth have a long anatomical crown, continuously eruption and open-rooted, which defines an erodont dentition.[80]

Research objectives

1. To compare the rates of tooth movement between the L-PRP and control groups at 7, 14, 21 and 28 days.
2. To compare bone volume fraction (BV/TV) between the L-PRP and control groups at 7, 14 and 28 days.
3. To compare osteoclast numbers between the L-PRP and control groups at 7, 14 and 28 days.

Research questions

1. Does the local administration of L-PRP affect the rate of tooth movement?
2. Does the local administration of L-PRP affect the bone volume fraction?
3. Does the local administration of L-PRP affect the osteoclast numbers?

Hypothesis

1. Rate of tooth movement in the L-PRP group at 7, 14, 21 and 28 days is faster than the control group.
2. Bone volume fraction in the L-PRP group at 7, 14 and 28 days is less than the control group.
3. Osteoclast numbers in the L-PRP group at 7, 14 and 28 days are greater than the control group.

Conceptual framework

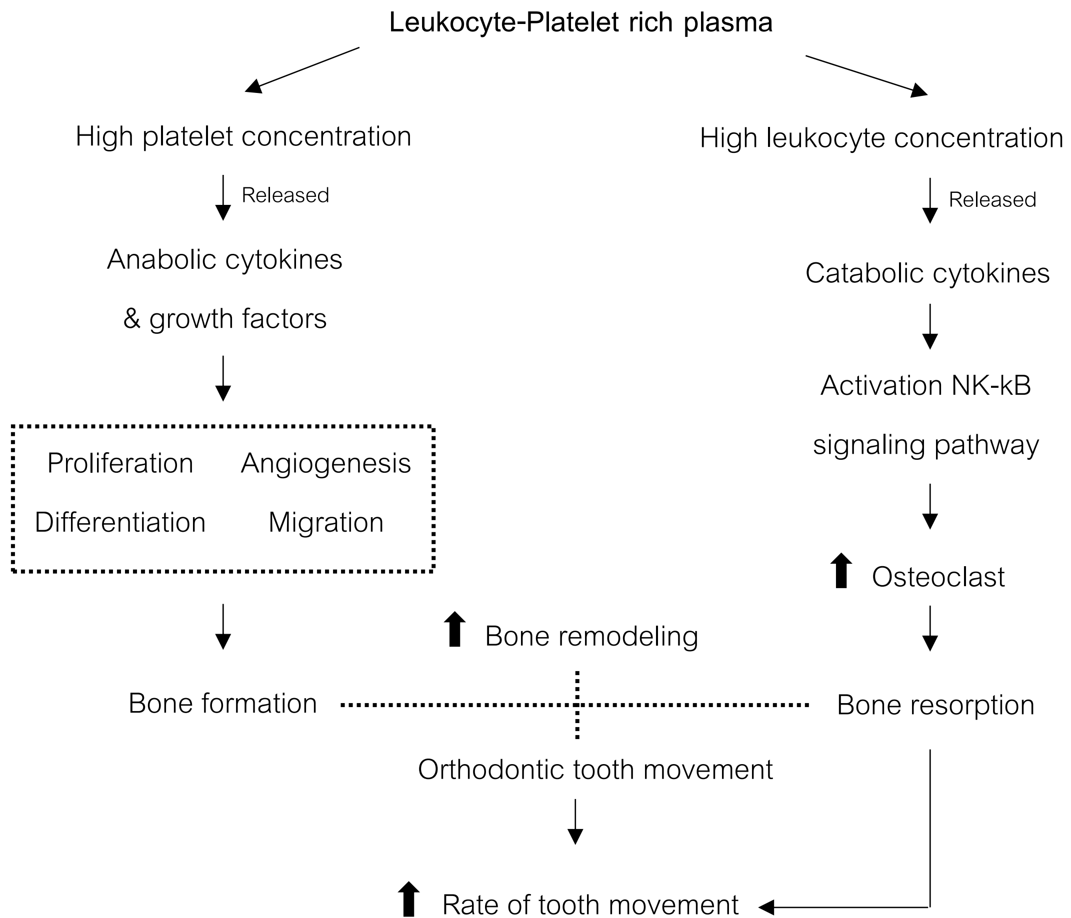

Figure 1. Conceptual framework in this study

CHAPTER 2

RESEARCH METHODOLOGY

Sample

This study was a randomized split mouth experimental design. Twenty-three New Zealand white rabbit males (National Laboratory Animal Center, Mahidol University, Thailand), aged 3 to 4 months weighing 2.5 to 3 kg, were included in this study. The animal experiment was approved by the Animal Ethics Committee, Prince of Songkla University (MOE 0521.11/074). Rabbits were acclimatized for 7 days before the beginning of experiment. Five rabbits were used as control without any intervention. Ten rabbits were randomly divided into 2 groups, the 7-day group (n = 5) and the 14-day group (n = 5), for measuring bone volume fraction and histomorphometry analysis. Eight rabbits were analyzed for the distance of tooth movement at days 0, 3, 7, 14, 21, and 28, and then these animals were included into the 28-day group to evaluate bone volume fraction and histomorphometry analysis. The rabbits were randomly assigned to receive a single injection of L-PRP on one side of the maxilla while the other side served as a control and received an injection of normal saline. All injections were administered in equal volumes with the same technique for the injection and rehabilitation.

The sample size was determined by G*Power software version 3.1.9.2 (Franz Faul, Universität Kiel, Dusseldorf, Germany). According to the results of a previous study[81] (effect size = 1.89), a minimum sample size of 8 animals per group was needed to achieve a significance level of .05 with 90% power. This study was performed as a randomized split-mouth experimental design; therefore, 8 animals were included.

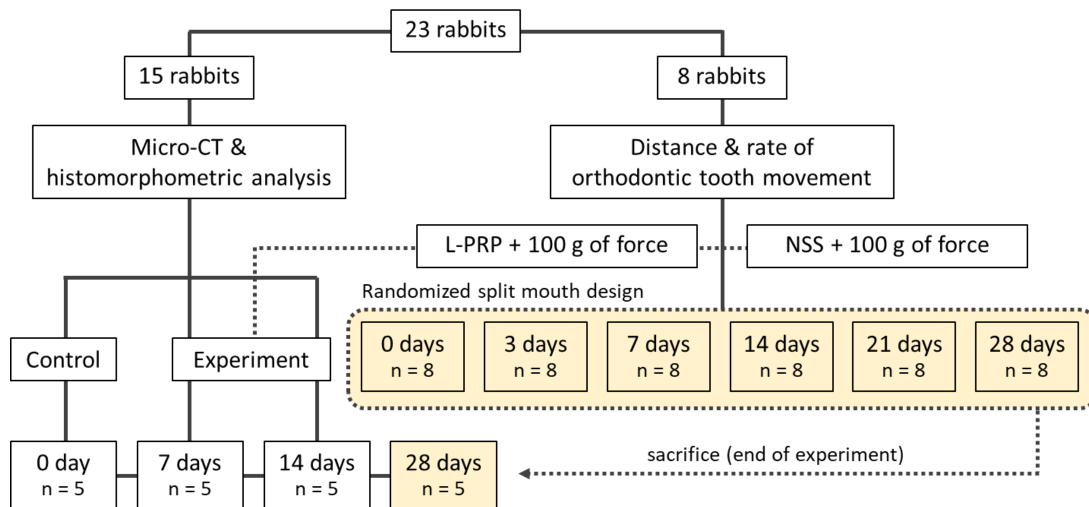

Figure 2. Diagram of the study design

The study consisted of clinical part, which the preparation and application of L-PRP, and orthodontic tooth movement were performed, microcomputed tomography analysis, and histological part.

Clinical procedures

Anesthetic procedure

The rabbits were weighting to calculate accurate the dosage of anesthetic drug. The combination of 25 mg/kg of ketamine hydrochloride (Ketamine-hameln, Hameln Pharmaceuticals Gmbh, Germany) and 5 mg/kg xylazine hydrochloride (X-lazine, LBS Laboratory Ltd, Bangkok, Thailand) was given by intramuscular injection. The rabbits were assessed the depth of anesthesia by the ear-pinch reflex and the jaw muscle tone before the operation. Rabbits were closely monitored of respiration during the experimental procedures.

L-PRP Preparation and Application

A 10 mL sample of autologous blood was collected from each animal via the central auricular artery into a collection tube containing ethylenediaminetetraacetic acid (BD Vacutainer®, Becton Drive, Franklin Lakes, NJ, USA). The blood samples were

spun in a two-step centrifugation procedure according to a previously reported protocol.[47] Briefly, the tubes were centrifuged at 250*g* for 10 minutes to separate the sample into three fractions: the upper fraction of platelet-containing plasma, the middle fraction of buffy coat, and the lower fraction of red blood cells. The top two fractions were collected and centrifuged again at 1,000*g* for 10 minutes. The platelet poor plasma in the upper layer was discarded. Precipitated platelets and leukocytes were homogenized in the residual plasma to achieve 1 mL of L-PRP (Figure 3). A quantity of 0.5 mL of L-PRP was analyzed for the concentration of platelets and leukocytes with a hematology analyzer. The remaining 0.5 mL of L-PRP was injected submucosally at the buccal and lingual areas of the first maxillary premolar using a 27-gauge dental needle.

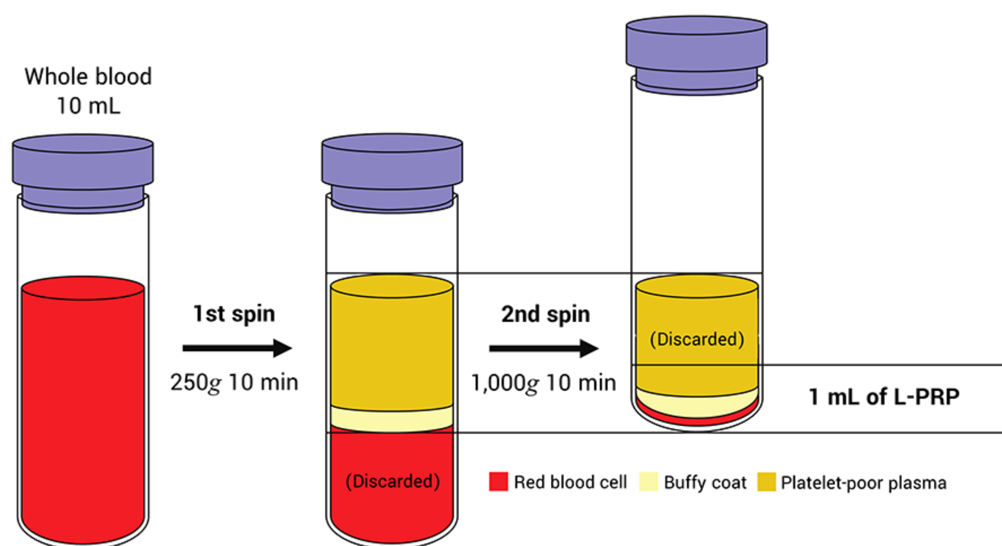

Figure 3. Schematic of the preparation process of L-PRP.

<u>Orthodontic Procedures</u>

The force was applied using a light-type nickel-titanium closed-coil spring, (Dentos, Daegu, Korea) with a length of 13 mm and a diameter of 1.5 mm, between the maxillary first premolar and incisor on each side. The spring was fixed in place via 0.008-inch stainless steel ligature wire and secured with composite resin (Flow Tain, Reliance Orthodontic products inc, USA) on the incisors to prevent sliding. (Figure

4A) The force was measured using a force gauge (Correx, Haagstreit, Bern, Switzerland) to generate 100 g of activation. The appliances were monitored at each time point to ensure they were intact.

Measuring the Distance of Tooth Movement

Tooth movement distance was measured using silicone impression material (Silagum DMG; Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany). The impressions were taken of the rabbits' maxillary dentition at each of the observed periods. These impressions were scanned directly and used to construct three-dimensional models with a 3D scanner (Trios3, 3Shape, Copenhagen, Denmark). The amounts of tooth movement in the digital models were determined using 3Shape Ortho Analyzer™ software (version 1.7.1.0; Copenhagen, Denmark). Briefly, the occlusal plane was chosen as the reference plane. This plane was created by the cusp tips of the maxillary second premolars and molars. Then the distance of tooth movement was determined by measuring the linear distance on the 2-dimentional schematic image of the cross-section. The nearest distance between the distal surface of the crown of the first premolar and the mesial surface of the crown of the second premolar was assessed. Measurements were performed with the digital caliper function within 3Shape Ortho Analyzer software (Figure 4B).

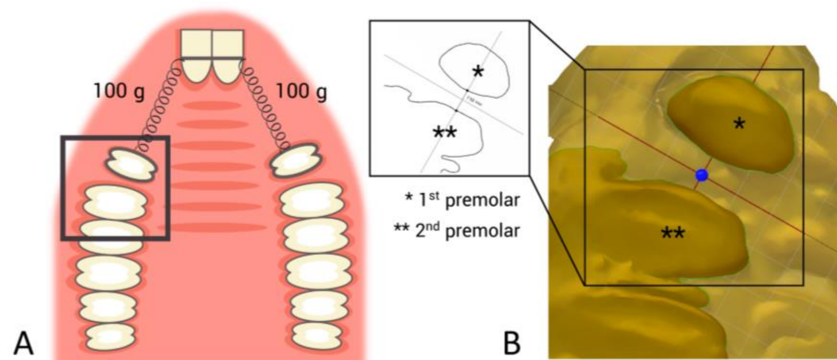

Figure 4. (A) Schematic of appliance for orthodontic tooth movement in rabbit.
(B) Tooth movement measurement using the 3Shape Ortho Analyzer software.

Microcomputed tomography analysis

The rabbits were euthanized with an overdose of anesthetic drug at each time point (0, 7 ,14 and 28 days). Then the maxillae were harvested and fixed in 10% neutral buffered formalin within 48 hours. The dissected maxilla was scanned using a Scanco μCT 35 system (Scanco Medical, Bassersdorf, Switzerland) with a voxel size of 18.5 μm at the condition of 70 kVp, 114 μA. After scanning, the image reconstruction was performed with the manufacturer's software. To analyze images, the region of interest (ROI) was defined as the area of alveolar bone at the mesial side near the coronal thirds of the root of maxillary first premolar (Figure 5). Measurements of bone volume fraction were determined using the image analysis software (μCT 35 Version 4.1, Scanco Medical, Bassersdorf, Switzerland).

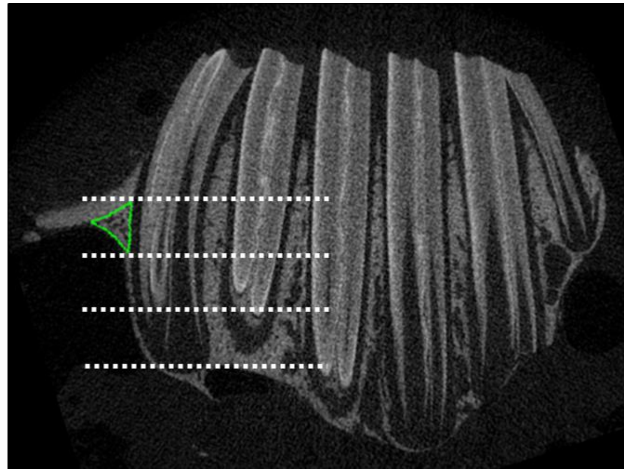

Figure 5. A region of interest

Histological procedure

<u>Tissue preparation</u>

After the micro-CT scanning, the maxillae were decalcified with 10% EDTA (Fluka, Sigma-Aldrich Inc., Singapore) in pH 7 at room temperature for 28 days. Then the samples were dehydrated with graded alcohols and embedded in paraffin.

The specimens were sagitally sectioned parallel to the long axis of maxillary first premolar at a thickness of 5 micrometers and stained with hematoxylin and eosin (H&E).

Histomorphometric analysis

Osteoclasts were counted at the compression side of the maxillary first premolar. The coronal half of the root and the adjacent alveolar bone were determined for histologic analysis because of the tipping movement of the first premolar. In each animal, three sections from the alveolar crest to the middle level were examined to quantify the osteoclast numbers under a light microscope (Carl Zeiss Microscopy GmbH, Germany) with 20-times magnification. Osteoclast was defined as large multinucleated cells that were located on or near bone surfaces.

Statistical analysis

All sample measurements were reassessed by the same investigator at an interval of 2 weeks. Intraclass correlation coefficients were calculated to assess intraobserver reliability. All data are reported as means and standard deviations of each group. The statistical analyses were performed using SPSS version 17 (SPSS, Chicago, IL, USA). The Shapiro-Wilk test for the distances of tooth movement, bone volume fraction, and osteoclast numbers showed that the data were normally distributed. A comparison of the results between the groups was carried out by independent-sample *t*-test. A probability value less than 0.05 indicated statistical significance.

CHAPTER 3

RESULTS

All animals were weighed weekly to determine the animal health during the experiment. The body weights of rabbits increased throughout the observation period. Weight loss of rabbits were not detected when compared with initial (Day 0).

Table 3. The body weights of rabbits during the experimental periods.

Sample Number	Weight (kilograms)				
	Day 0	Day 7	Day 14	Day 21	Day 28
1	2.73	-	-	-	-
2	2.53	-	-	-	-
3	2.57	-	-	-	-
4	2.61	-	-	-	-
5	2.56	-	-	-	-
6	2.53	2.61	-	-	-
7	2.74	2.79	-	-	-
8	2.51	2.54	-	-	-
9	2.62	2.75	-	-	-
10	2.81	2.90	-	-	-
11	2.55	2.58	2.68	-	-
12	2.86	3.02	3.18	-	-
13	2.91	2.99	3.03	-	-
14	2.88	2.93	3.01	-	-
15	2.51	2.56	2.71	-	-
16	2.56	2.68	2.72	2.78	2.85

Table 3. (Continued)

Sample Number	Weight (kilograms)				
	Day 0	Day 7	Day 14	Day 21	Day 28
17	2.88	2.94	3.07	3.11	3.15
18	2.97	3.08	3.20	3.25	3.28
19	2.64	2.76	2.84	2.93	3.04
20	2.52	2.59	2.65	2.75	2.78
21	2.60	2.75	2.81	2.88	2.90
22	2.53	2.62	2.69	2.73	2.75
23	2.74	2.79	2.83	2.86	2.88

All the appliances remained intact during the experiment. The proper direction of maxillary first premolars was performed. The intraclass correlation coefficients were 0.97 for the distances of tooth movement, 0.91 for bone volumetric analysis, and 0.93 for histomorphometric analysis. The reliability results indicated excellent correlation of measurements.

Components of Whole Blood and L-PRP

The mean concentrations of platelets and leukocytes in the whole blood were 351.14 ± 78.40 × 10^3 cells/μL and 3.69 ± 0.98 × 10^3 cells/μL, respectively. After L-PRP preparation, these concentrations increased to 2,314.44 ± 570.82 × 10^3 cells/μL and 6.87 ± 2.29 × 10^3 cells/μL (Figure 6). The results demonstrated that L-PRP had a 6.6-fold and a 1.9-fold increase in the concentrations of platelets and leukocytes, respectively.

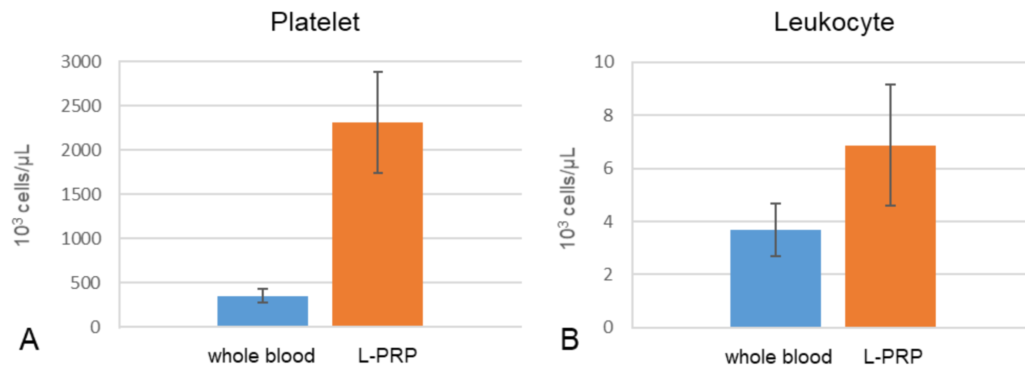

Figure 6. (A) Platelet, (B) Leukocyte concentrations in whole blood and L-PRP.

Distance and rate of tooth movement

The distance of tooth movement increased from the beginning up to 28 days in both groups but the L-PRP group had greater cumulative tooth movement than the control group at all observation times. The cumulative amount of tooth movement in the L-PRP group was significantly greater than the control group at day 3 (0.84 ± 0.05 mm vs 0.76 ± 0.07 mm), day 7 (1.04 ± 0.05 mm vs 0.94 ± 0.09 mm), day 14 (1.62 ± 0.09 mm vs 1.39 ± 0.12 mm), day 21 (1.95 ± 0.2 mm vs 1.64 ± 0.17 mm), and day 28 (2.3 ± 0.32 mm vs 2.01 ± 0.12 mm) (Figure 7A).

The rates of tooth movement in the L-PRP and control groups increased steeply for the first 7 days. After that there was a continuous decrease from 14 days to 21 days and then the tooth movement rate remained constant until 28 days. The L-PRP group showed a significantly greater rate of tooth movement than the control group from 0 to 7 days (1.04 ± 0.05 mm vs 0.94 ± 0.09 mm) and from 7 to 14 days (0.58 ± 0.09 mm vs 0.45 ± 0.12 mm). However, the rate of tooth movement between the two groups was not significantly different at the intervals of 14-21 days and 21-28 days (Figure 7B).

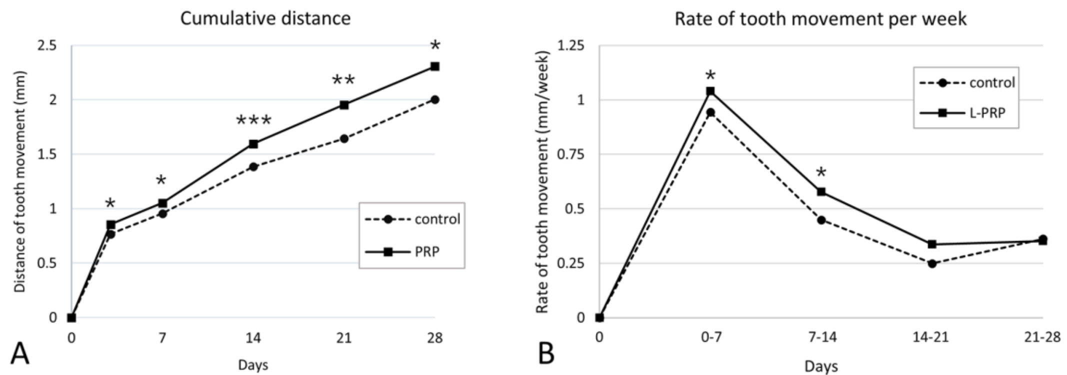

Figure 7. (A) Cumulative distance of tooth movement in L-PRP and control groups, (B) Rate of tooth movement in L-PRP and control groups. * P < .05; ** P < .01; *** P = .001

Alveolar bone change

The initial BV/TV values between the L-PRP and control groups were not significantly different (75.85 ± 5.34% vs 77.45 ± 7.09%, respectively). The BV/TV values in L-PRP and control groups showed similar trends at all time points. The results demonstrated that the BV/TV in the L-PRP and control groups was a continuous decrease from day 0 to day 14 and then the BV/TV maintained a steady level until 28 days. The BV/TV values on days 7, 14 and 28 were less than the baseline (day 0) in both groups. The L-PRP group showed a significantly lower the BV/TV values than the control group at day 7 (61.98 ± 3.60% vs 71.45 ± 2.66%) and day 14 (58.23 ± 3.53% vs 63.22 ± 2.83%). However, there was no a significant difference at day 28 (57.96 ± 1.74% vs 60.68 ± 3.40%) (Figure 8).

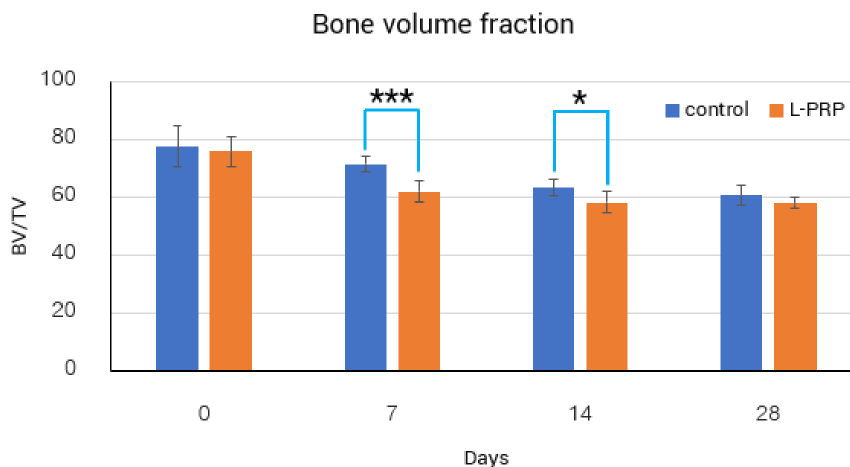

Figure 8. Bone volume fraction (BV/TV) in L-PRP and control groups.

Osteoclast numbers

The osteoclast numbers increased from 7 days to 14 days and subsequent declined on 28 days. The data exhibited a peak of the number of osteoclasts on day 14. The L-PRP group showed significantly greater osteoclast numbers than the control group at day 7 (10.6 ± 2.07 vs 7.4 ± 2.30) and day 14 (16.2 ± 3.03 vs 11.6 ± 3.04), but there was no a significant difference at day 28 (4.2 ± 1.78 vs 3.8 ± 1.48) (Figure 9).

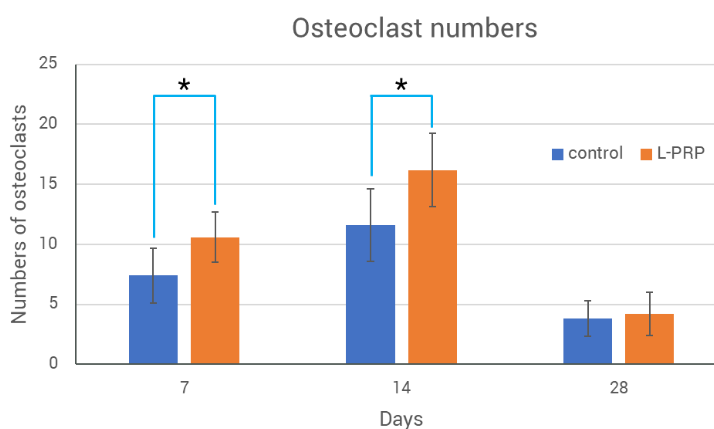

Figure 9. The number of osteoclasts in L-PRP and control groups.

Histological findings

Histologic sections along the compression side of the root of the maxillary first premolar on days 7 and 14 showed hyperemia of the periodontal ligament and the adjacent alveolar bone surface. The hyperemia was more noticed in the L-PRP group, who presented the dilated blood vessels congested with numerous blood cells, compared with the control group. Moreover, rich cellularity in which a discrete inflammatory infiltrate adjacent to the bone surface was more obvious in the L-PRP on day 7.

The scalloped surface of alveolar bone with few osteoclasts in resorption lacunae was showed on day 7, while a discrete bone resorption with an irregular surface was more observed on day 14. Furthermore, a number of osteoclasts in the L-PRP group were more detected on the bone surfaces than the control group on days 7 and 14. However, the differences in both groups were not noticed on day 28. (Figures 10)

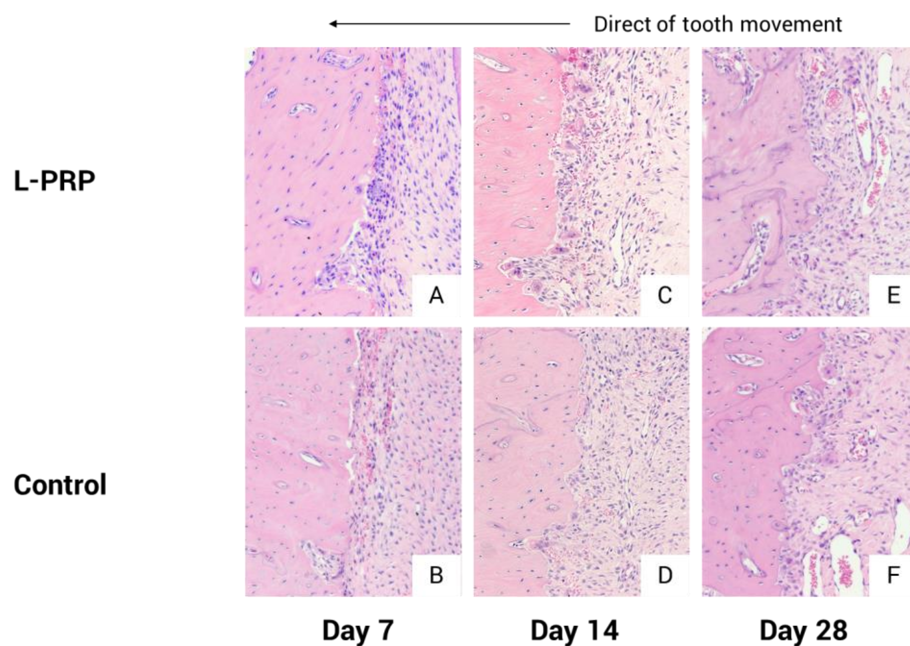

Figure 10. Histologic sections showed the compression side of the maxillary first premolar in L-PRP group (A, C, E) and the control group (B, D, F) on day 7, 14 and 28 respectively after orthodontic force application.

CHAPTER 4

DISCUSSION

Local injection of L-PRP produced accelerated tooth movement. There was significantly more tooth movement in the L-PRP group than in the control group at all time periods. These findings were consistent with previous results[25,65] which reported the positive effects of PRP that promoted rapid tooth movement. The reason for accelerated tooth movement could be attributed to a high concentration of growth factors and cytokines secreted by activated platelets in PRP. The release of these molecules, including PDGF, TGF-β, and VEGF, influenced the process of bone remodeling to trigger cellular responses in the various cell types which provided a favorable environment for tooth movement.[2] However, our results differed to some extent from those of Rashid et al.[65] who concluded that the rate of tooth movement in the PRP group did not significantly increase in the first week. This may be explained by the presence of leukocytes in the PRP in this study. The leukocytes contributed mainly to the initial burst release of pro-inflammatory cytokines (ie IL-1, IL-6, and TNF-α) which could be considered the initiating factors for cellular and molecular events in the early phase of orthodontic tooth movement.[20,22] It was assumed that the presence of leukocytes in PRP contributed to increase orthodontic tooth movement.

This study found that tooth movement in the L-PRP group was approximately 1.2 times higher than the control group at day 21. According to previous studies, the magnitudes of tooth movement were increases of 1.7 times in a rat model[25] and 2.13 times in a dog model.[65] The differences between the animal models used to investigate tooth movement can reasonably explain the different results. It is known that tooth movement is a process of bone remodeling. Therefore, the rate of bone remodeling can be used to quantify the rate of tooth movement. In the rabbit model, the rate of bone turnover in the jaw bones is approximately 2% per year, while the bone remodeling rate in the alveolar bone of canines is estimated to be 20-36% per year.[82] It was assumed

that a slower tooth movement rate in this study was possibly due to lower bone turnover. Interestingly, a previous study using corticomy to accelerate tooth movement in a rabbit model,[81] showed tooth movement increases of 1.2 times, similar to the current. Administration of L-PRP may be an alternative procedure that offers benefits for the acceleration of orthodontic tooth movement.

This study demonstrated a significant increase in tooth movement in the L-PRP group during the first and second weeks, while the differences were not significant in the last two weeks. This may have occurred because of a reduced level of pro-inflammatory cytokines and growth factors overtime. According to Schar et al.,[19] L-PRP released most of the growth factors and catabolic cytokines at the beginning. They found that the release pattern of IL-1β peaked at day 3 followed by a dramatic decrease at day 7, and then almost no further release at day 14. In addition, a previous study found that alveolar bone changes occurred early at 3 days and were more significant at day 7 after force application and the injection of PRP.[25] Corresponding to the histological findings, osteoclasts were seen on day 3 and peaked on day 7. It was assumed that the effects of a single injection of L-PRP occurred in the early stages of orthodontic tooth movement.

Alveolar bone change at the maxillary first premolar demonstrated that the bone volume fraction in the L-PRP group was significantly less than the control group on days 7 and 14 after force application, which corresponded to high levels of the osteoclasts in the results. It was assumed that a rapid reduction of bone density resulted from a large amount of bone resorption that occurred with high osteoclast activities.

Interestingly, our results found that the bone volume fraction in the L-PRP group was significantly decreased during the first week, while the control group showed a sharp decrease of bone volume fraction in the second week. This finding agreed with the previous study[25] who reported that the decrease of alveolar bone density in rats received PRP injection was begun on day 3 and more significantly on day 7. Meanwhile, the results in the control group demonstrated that the reduction of alveolar bone density was remained steady from day 3 to 7 and then dropped considerably after the first week.

In accordance with the phases of orthodontic tooth movement, the initial phase is a period of rapid tooth movement that occurred between 1 to 3 days and then the lag phase which known as the minimal or no tooth movement was begun until day 7. The post lag phase in which the gradually or suddenly increased tooth movement occurred thereafter[83]. Therefore, our findings could be implied that L-PRP may accelerate tooth movement due to a decrease an interval of lag phase after the initial phase of orthodontic tooth movement.

Correspondingly, a significant higher number of osteoclasts were found in the L-PRP group during the first and second weeks when compared with the control group. The possible reason for this finding may be the increased pro-inflammatory cytokines released from the leukocytes in L-PRP including IL-1, IL-6, and TNF-α. The roles of these cytokines are involved in the process of bone resorption initiated by cytokine-induced inflammation during orthodontic tooth movement[84]. The in vitro studies[11,20] demonstrated that the pro-inflammatory cytokines in L-PRP induce the activation of NF-κB pathway via the canonical pathway. They found that L-PRP treatment on cells in vitro upregulated the expression of downstream inflammation-related genes, including COX-2 and inducible nitric oxide synthase (iNOS), and increased the levels of PGE2 and nitric oxide (NO). These factors are known as important inflammatory mediators to induce osteoclastic bone resorption. The previous studies[85-87] demonstrated that iNOS-mediated NO production is essential for inflammation-induced bone resorption by stimulating osteoclast differentiation and activation. Also, in vitro study[88] found that PGE2 production is an influence on TNF-α-induced RANKL expression. Moreover, the role of pro-inflammatory cytokines was related to the process of bone resorption through recruitment of osteoclast precursors from the circulation[89] in accordance with this histologic finding that showed a higher inflammatory infiltrate in the L-PRP group on day 7.

Moreover, histologic findings in this study demonstrated that L-PRP group increased vascularity compared with the control group in the first and second weeks. This could be explained by the released angiogenic growth factors in L-PRP,

particularly VEGF which is known as an important mediator of angiogenesis and increased vascular permeability[90]. In vitro studies[11,91] demonstrated that the presence of angiogenic growth factors in PRP promoted vascular growth and stimulated endothelial progenitor cells to form vessel-like structure. In vivo studies[91,92] also revealed that the animal treated with PRP showed an increase newly vessel formation. Interestingly, the VEGF mediated angiogenesis is related to an indirectly increase of bone resorption. The new capillaries were formed to enhance the recruitment of osteoclast precursors to the bone surface adjacent to the resorption site[93]. In accordance with our study, the numerous differentiated osteoclasts in close to the blood vessel were presented along the bone surface adjacent to the compressed PDL. Moreover, VEGF is essential for the recruitment, differentiation, and activation of osteoclast precursors, and enhancing osteoclastic bone resorption[94,95]. Thus, due to the aforementioned reasons, this study could be explained to the increased osteoclast numbers in L-PRP group.

The peak of the osteoclast numbers in this study was showed on day 14. These histologic findings similar with a rabbit study of Chen et al.[81], found that the higher osteoclast numbers were seen in the second week. However, their results also found an initial peak of osteoclast numbers on day 3. They suggested that the first peak of osteoclasts derived from the local PDL or the bone marrow of the alveolar bone, while the second peak of osteoclasts originated from the differentiation of mononuclear cells from peripheral blood[96]. However, this study did not investigate histologic finding on day 3.

Although our findings are consistent with Rashid et al.[65], in that the received PRP group exhibited an increased number of osteoclasts at week 9, they did not examine the early stages of histologic findings. However, these results were in contrast with previous findings[25,97], in which the effects of PRP did not increase the osteoclastic activity. Gulec et al.[25] reported that the osteoclast cell counts in PRP group were less than the control group at first, second and third weeks despite a higher rate of tooth movement in PRP group. Akbulut et al.[97] also demonstrated that the tartrate-resistant acid phosphatase (TRAP) expressions between the groups were not

differences at all observation times. One reason for this is that the possible immune reaction from usage of allogenic PRP injection led to different results. The other reason is possible due to the inclusion of leukocytes in PRP.

The inability to characterize the release characteristics of growth factors and cytokines in this study was a limitation because measurements of these molecules were not performed. Knowing the expression of various biological molecules may be needed to understand how long these factors maintain biologic activity. In addition, the specific biomarkers for examining bone remodeling are needed to better understand the exact molecular mechanism of L-PRP injection on tooth movement. Moreover, the results from this animal experiment cannot be directly extrapolated to a clinical situation. Thus, further studies are needed to investigate the efficacy of L-PRP administration for accelerated orthodontic tooth movement in clinical trials as well as determining the frequency of application for optimal effectiveness to enhance orthodontic tooth movement.

From these results, a single submucosal injection of L-PRP may have a positive influence on orthodontically induced tooth movement and may increase the rate of tooth movement in the initial stages through increased osteoclast activity resulted in decreased bone volume fraction.

CHAPTER 5

CONCLUSION

1. Cumulative orthodontic tooth movement in the group that received L-PRP was significantly greater in all time periods
2. The injection of L-PRP significantly increased the rate of tooth movement during the first 14 days, but there was no significant difference in rate of tooth movement after day 14.
3. The injection of L-PRP significantly decreased the bone volume fraction on days 7 and 14.
4. The number of osteoclasts increased significantly at 7 and 14 days after L-PRP injection.

REFERENCES

1. Teixeira CC, Khoo E, Tran J, et al. Cytokine expression and accelerated tooth movement. ***J Dent Res*** 2010; 89(10): 1135-1141.
2. Andrade Jr I, Taddei SRA, Souza PEA. Inflammation and Tooth Movement: The Role of Cytokines, Chemokines, and Growth Factors. ***Semin Orthod*** 2012; 18(4): 257-269.
3. Sanchez AR, Sheridan PJ, Kupp LI. Is platelet-rich plasma the perfect enhancement factor? A current review. ***Int J Oral Maxillofac Implants*** 2003; 18(1): 93-103.
4. Dohan DM, Choukroun J, Diss A, et al. Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part III: Leucocyte activation: A new feature for platelet concentrates? ***Oral Surg Oral Med Oral Pathol Oral Radiol. Endod*** 2006; 101(3): e51-e55.
5. Martinez CE, Smith PC, Palma Alvarado VA. The influence of platelet-derived products on angiogenesis and tissue repair: a concise update. ***Front Physiol*** 2015; 6: 290.
6. Alsousou J, Ali A, Willett K, Harrison P. The role of platelet-rich plasma in tissue regeneration. ***Platelets*** 2013; 24(3): 173-182.
7. Fernandez-Moure JS, Van Eps JL, Cabrera FJ, et al. Platelet-rich plasma: a biomimetic approach to enhancement of surgical wound healing. ***J Surg Res*** 2017; 207: 33-44.
8. Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. ***Am J Sports Med*** 2009; 37(11): 2259-2272.
9. Fernandes G, Wang C, Yuan X, Liu Z, Dziak R, Yang S. Combination of Controlled Release Platelet-Rich Plasma Alginate Beads and Bone Morphogenetic Protein-2 Genetically Modified Mesenchymal Stem Cells for Bone Regeneration. ***J Periodontol*** 2016; 87(4): 470-480.

10. Tajima S, Tobita M, Orbay H, Hyakusoku H, Mizuno H. Direct and indirect effects of a combination of adipose-derived stem cells and platelet-rich plasma on bone regeneration. ***Tissue Eng Part A*** 2015; 21(5-6): 895-905.

11. Yin W, Qi X, Zhang Y, et al. Advantages of pure platelet-rich plasma compared with leukocyte- and platelet-rich plasma in promoting repair of bone defects. ***J Transl Med*** 2016; 14: 73.

12. Knapen M, Gheldof D, Drion P, Layrolle P, Rompen E, Lambert F. Effect of leukocyte- and platelet-rich fibrin (L-PRF) on bone regeneration: a study in rabbits. ***Clin Implant Dent Relat Res*** 2015; 17 Suppl 1: e143-152.

13. Shin KH, Lee H, Kang S, et al. Effect of Leukocyte-Rich and Platelet-Rich Plasma on Healing of a Horizontal Medial Meniscus Tear in a Rabbit Model. ***Biomed Res Int*** 2015; 2015: 179756.

14. Russell RP, Apostolakos J, Hirose T, Cote MP, Mazzocca AD. Variability of platelet-rich plasma preparations. ***Sports Med Arthrosc*** 2013; 21(4): 186-190.

15. Wasterlain AS, Braun HJ, Dragoo JL. Contents and Formulations of Platelet-Rich Plasma. ***Oper Tech Orthop*** 2012; 22(1): 33-42.

16. Zhou Y, Zhang J, Wu H, Hogan MV, Wang JH-C. The differential effects of leukocyte-containing and pure platelet-rich plasma (PRP) on tendon stem/progenitor cells - implications of PRP application for the clinical treatment of tendon injuries. ***Stem Cell Res Ther*** 2015; 6(1): 173.

17. Sundman EA, Cole BJ, Fortier LA. Growth factor and catabolic cytokine concentrations are influenced by the cellular composition of platelet-rich plasma. ***Am J Sports Med*** 2011; 39(10): 2135-2140.

18. Zhang L, Chen S, Chang P, et al. Harmful Effects of Leukocyte-Rich Platelet-Rich Plasma on Rabbit Tendon Stem Cells In Vitro. ***Am J Sports Med*** 2016; 44(8): 1941-1951.

19. Schär MO, Diaz-Romero J, Kohl S, Zumstein MA, Nesic D. Platelet-rich Concentrates Differentially Release Growth Factors and Induce Cell Migration In Vitro. ***Clin Orthop Relat Res*** 2015; 473(5): 1635-1643.

20. Anitua E, Zalduendo M, Troya M, Padilla S, Orive G. Leukocyte Inclusion within a Platelet Rich Plasma-Derived Fibrin Scaffold Stimulates a More Pro-Inflammatory Environment and Alters Fibrin Properties. ***PLoS One*** 2015; 10(3): e0121713.

21. Pifer MA, Maerz T, Baker KC, Anderson K. Matrix metalloproteinase content and activity in low-platelet, low-leukocyte and high-platelet, high-leukocyte platelet rich plasma (PRP) and the biologic response to PRP by human ligament fibroblasts. ***Am J Sports Med*** 2014; 42(5): 1211-1218.

22. Anitua E, Zalduendo MM, Prado R, Alkhraisat MH, Orive G. Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: evaluation of the effect of leukocyte inclusion. ***J Biomed Mater Res A*** 2015; 103(3): 1011-1020.

23. Yin W-J, Xu H-T, Sheng J-G, et al. Advantages of Pure Platelet-Rich Plasma Compared with Leukocyte- and Platelet-Rich Plasma in Treating Rabbit Knee Osteoarthritis. ***Med Sci Monit*** 2016; 22: 1280-1290.

24. Liou E. The development of submucosal injection of platelet rich plasma for accelerating orthodontic tooth movement and preserving pressure side alveolar bone. ***APOS Trends Orthod*** 2016; 6(1): 5-11.

25. Güleç A, BakkalbaŞı BÇ, Cumbul A, Uslu Ü, Alev B, Yarat A. Effects of local platelet-rich plasma injection on the rate of orthodontic tooth movement in a rat model: A histomorphometric study. ***Am J Orthod Dentofacial Orthop*** 2017; 151(1): 92-104.

26. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? ***Implant Dent*** 2001; 10(4): 225-228.

27. Ghoshal K, Bhattacharyya M. Overview of Platelet Physiology: Its Hemostatic and Nonhemostatic Role in Disease Pathogenesis. ***Sci World J*** 2014; 2014: 16.

28. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. ***J Cell Biol*** 2013; 201(6): 785-796.

29. Everts PA, Knape JT, Weibrich G, et al. Platelet-rich plasma and platelet gel: a review. ***J Extra Corpor Technol*** 2006; 38(2): 174-187.

30. Boswell SG, Cole BJ, Sundman EA, Karas V, Fortier LA. Platelet-rich plasma: a milieu of bioactive factors. ***Arthroscopy*** 2012; 28(3): 429-439.

31. Pavlovic V, Ciric M, Jovanovic V, Stojanovic P. Platelet Rich Plasma: a short overview of certain bioactive components. ***Open Med*** 2016; 11(1): 242-247.

32. Arnoczky SP, Delos D, Rodeo SA. What Is Platelet-Rich Plasma? ***Oper Tech Sports Med*** 2011; 19(3): 142-148.

33. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). ***Trends Biotechnol*** 2009; 27(3): 158-167.

34. Dohan Ehrenfest DM, Andia I, Zumstein MA, Zhang C-Q, Pinto NR, Bielecki T. Classification of platelet concentrates (Platelet-Rich Plasma-PRP, Platelet-Rich Fibrin-PRF) for topical and infiltrative use in orthopedic and sports medicine: current consensus, clinical implications and perspectives. ***Muscles Ligaments Tendons J*** 2014; 4(1): 3-9.

35. Lei H, Gui L, Xiao R. The effect of anticoagulants on the quality and biological efficacy of platelet-rich plasma. ***Clin Biochem*** 2009; 42(13-14): 1452-1460.

36. do Amaral RJ, #xe9, Corr F, et al. Platelet-Rich Plasma Obtained with Different Anticoagulants and Their Effect on Platelet Numbers and Mesenchymal Stromal Cells Behavior In Vitro. ***Stem Cells Int*** 2016; 2016: 11.

37. Golański J, Pietrucha T, Baj Z, Greger J, Watala C. Molecular insights into the anticoagulant-induced spontaneous activation of platelets in whole blood - various anticoagulants are not equal. ***Thromb Res*** 1996; 83(3): 199-216.

38. Nishioka T, Yokota M, Tsuda I, Tatsumi N. Flow cytometric analysis of platelet activation under calcium ion-chelating conditions. ***Clin Lab Haematol*** 2002; 24(2): 115-119.

39. Giraldo CE, Álvarez ME, Carmona JU. Effects of sodium citrate and acid citrate dextrose solutions on cell counts and growth factor release from equine pure-platelet rich plasma and pure-platelet rich gel. ***BMC Vet Res*** 2015; 11: 60.

40. Nagata MJH, Messora MR, Furlaneto FAC, et al. Effectiveness of Two Methods for Preparation of Autologous Platelet-Rich Plasma: An Experimental Study in Rabbits. ***Eur J Dent*** 2010; 4(4): 395-402.

41. Filardo G, Kon E, Pereira Ruiz MT, et al. Platelet-rich plasma intra-articular injections for cartilage degeneration and osteoarthritis: single- versus double-spinning approach. ***Knee Surg Sports Traumatol Arthrosc*** 2012; 20(10): 2082-2091.

42. Marx RE. Platelet-rich plasma: evidence to support its use. ***J Oral Maxillofac Surg*** 2004; 62(4): 489-496.

43. Arora S, Doda V, Kotwal U, Dogra M. Quantification of platelets and platelet derived growth factors from platelet-rich-plasma (PRP) prepared at different centrifugal force (g) and time. ***Transfus Apher Sci*** 2016; 54(1): 103-110.

44. Giovanini AF, Deliberador TM, Tannuri Nemeth JE, et al. Leukocyte-platelet-rich plasma (L-PRP) impairs the osteoconductive capacity of the autograft associated to changes in the immunolocalization of TGF-β1 and its co-expression with Wnt10b and CD34 cells. ***J Craniomaxillofac Surg*** 2013; 41(7): e180-e186.

45. Vokurka J, Gopfert E, Blahutkova M, Buchalova E, Faldyna M. Concentrations of growth factors in platelet-rich plasma and platelet-rich fibrin in a rabbit model. ***Vet Med*** 2016; 61(10): 567-570.

46. Garcia AGW, Scariot R, Araujo MRd, et al. Platelet-leukocyte-rich plasma (L-PRP) prevents the collagen III degradation and impairs the bone matrix development in artificial defect of rabbit calvaria, associated with suppression of the immunohistochemical expression of MMP-2 and MMP-9. ***RSBO (Online)*** 2014; 11: 328-335.

47. Jia WT, Zhang CQ, Wang JQ, Feng Y, Ai ZS. The prophylactic effects of platelet-leucocyte gel in osteomyelitis: an experimental study in a rabbit model. ***J Bone Joint Surg Br*** 2010; 92(2): 304-310.

48. Li GY, Yin JM, Ding H, Jia WT, Zhang CQ. Efficacy of leukocyte- and platelet-rich plasma gel (L-PRP gel) in treating osteomyelitis in a rabbit model. ***J Orthop Res*** 2013; 31(6): 949-956.

49. Schoenecker JG, Johnson RK, Lesher AP, et al. Exposure of mice to topical bovine thrombin induces systemic autoimmunity. ***Am J Pathol*** 2001; 159(5): 1957-1969.

50. Fufa D, Shealy B, Jacobson M, Kevy S, Murray MM. Activation of Platelet-Rich Plasma Using Soluble Type I Collagen. ***J Oral Maxillofac Surg*** 2008; 66(4): 684-690.

51. Harrison S, Vavken P, Kevy S, Jacobson M, Zurakowski D, Murray MM. Platelet activation by collagen provides sustained release of anabolic cytokines. ***Am J Sports Med*** 2011; 39(4): 729-734.

52. Cavallo C, Roffi A, Grigolo B, et al. Platelet-Rich Plasma: The Choice of Activation Method Affects the Release of Bioactive Molecules. ***Biomed Res Int*** 2016; 2016: 7.

53. Scherer SS, Tobalem M, Vigato E, et al. Nonactivated versus thrombin-activated platelets on wound healing and fibroblast-to-myofibroblast differentiation in vivo and in vitro. ***Plast Reconstr Surg*** 2012; 129(1): 46e-54e.

54. Zou J, Yuan C, Wu C, Cao C, Yang H. The effects of platelet-rich plasma on the osteogenic induction of bone marrow mesenchymal stem cells. ***Connect Tissue Res*** 2014; 55(4): 304-309.

55. Giusti I, Ascenzo S, Manc, et al. Platelet Concentration in Platelet-Rich Plasma Affects Tenocyte Behavior In Vitro. ***Biomed Res Int*** 2014; 2014: 12.

56. Murphy MB, Blashki D, Buchanan RM, et al. Adult and umbilical cord blood-derived platelet-rich plasma for mesenchymal stem cell proliferation, chemotaxis, and cryo-preservation. ***Biomaterials*** 2012; 33(21): 5308-5316.

57. Weibrich G, Hansen T, Kleis W, Buch R, Hitzler WE. Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration. ***Bone*** 2004; 34(4): 665-671.

58. Choi BH, Zhu SJ, Kim BY, Huh JY, Lee SH, Jung JH. Effect of platelet-rich plasma (PRP) concentration on the viability and proliferation of alveolar bone cells: an in vitro study. ***Int J Oral Maxillofac Surg*** 2005; 34(4): 420-424.

59. Ranly DM, McMillan J, Keller T, et al. Platelet-Derived Growth Factor Inhibits Demineralized Bone Matrix-Induced Intramuscular Cartilage and Bone Formation A Study of Immunocompromised Mice. ***J Bone Joint Surg Am*** *.* 2005; 87(9): 2052-2064.

60. Boswell SG, Schnabel LV, Mohammed HO, Sundman EA, Minas T, Fortier LA. Increasing platelet concentrations in leukocyte-reduced platelet-rich plasma decrease collagen gene synthesis in tendons. ***Am J Sports Med*** 2014; 42(1): 42-49.

61. McCarrel TM, Minas T, Fortier LA. Optimization of leukocyte concentration in platelet-rich plasma for the treatment of tendinopathy. ***J Bone Joint Surg Am*** 2012; 94(19): e143(141-148).

62. Perut F, Filardo G, Mariani E, et al. Preparation method and growth factor content of platelet concentrate influence the osteogenic differentiation of bone marrow stromal cells. ***Cytotherapy*** 2013; 15(7): 830-839.

63. Cieslik-Bielecka A, Dohan Ehrenfest DM, Lubkowska A, Bielecki T. Microbicidal properties of Leukocyte- and Platelet-Rich Plasma/Fibrin (L-PRP/L-PRF): new perspectives. ***J Biol Regul Homeost Agents*** 2012; 26(2 Suppl 1): 43s-52s.

64. Moojen DJ, Everts PA, Schure RM, et al. Antimicrobial activity of platelet-leukocyte gel against Staphylococcus aureus. ***J Orthop Res*** 2008; 26(3): 404-410.

65. Rashid A, ElSharaby FA, Nassef EM, Mehanni S, Mostafa YA. Effect of platelet-rich plasma on orthodontic tooth movement in dogs. ***Orthod Craniofac Res*** 2017; 20(2): 102-110.

66. Li A, Xia X, Yeh J, et al. PDGF-AA Promotes Osteogenic Differentiation and Migration of Mesenchymal Stem Cell by Down-Regulating PDGFRα and Derepressing BMP-Smad1/5/8 Signaling. ***PLoS One*** 2014; 9(12): e113785.

67. Li D-q, Wan Q-l, Pathak JL, Li Z-b. Platelet-derived growth factor BB enhances osteoclast formation and osteoclast precursor cell chemotaxis. ***J Bone Miner Metab*** 2017; 35(4): 355-365.

68. Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming Growth Factor-β1 to the Bone. ***Endocr Rev*** 2005; 26(6): 743-774.

69. Uematsu S, Mogi M, Deguchi T. Increase of transforming growth factor-beta 1 in gingival crevicular fluid during human orthodontic tooth movement. ***Arch Oral Biol*** 1996; 41(11): 1091-1095.

70. Nakai T, Yoshimura Y, Deyama Y, Suzuki K, Iida J. Mechanical stress up-regulates RANKL expression via the VEGF autocrine pathway in osteoblastic MC3T3-E1 cells. ***Mol Med Rep*** 2009; 2(2): 229-234.

71. Tan YY, Yang YQ, Chai L, Wong RW, Rabie AB. Effects of vascular endothelial growth factor (VEGF) on MC3T3-E1. ***Orthod Craniofac Res*** 2010; 13(4): 223-228.

72. Street J, Lenehan B. Vascular endothelial growth factor regulates osteoblast survival - evidence for an autocrine feedback mechanism. ***J Orthop Surg Res*** 2009; 4: 19.

73. Zhang Q, Guo R, Lu Y, et al. VEGF-C, a lymphatic growth factor, is a RANKL target gene in osteoclasts that enhances osteoclastic bone resorption through an autocrine mechanism. ***J Biol Chem*** 2008; 283(19): 13491-13499.

74. Ren Y, Hazemeijer H, de Haan B, Qu N, de Vos P. Cytokine profiles in crevicular fluid during orthodontic tooth movement of short and long durations. ***J Periodontol*** 2007; 78(3): 453-458.

75. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. ***J Clin Invest*** 2005; 115(2): 282-290.

76. Wu Q, Zhou X, Huang D, Ji Y, Kang F. IL-6 Enhances Osteocyte-Mediated Osteoclastogenesis by Promoting JAK2 and RANKL Activity In Vitro. ***Cell Physiol Biochem*** 2017; 41(4): 1360-1369.

77. Sanuki R, Shionome C, Kuwabara A, et al. Compressive Force Induces Osteoclast Differentiation via Prostaglandin E2 Production in MC3T3-E1 Cells. ***Connect Tissue Res*** 2010; 51(2): 150-158.

78. Kanematsu M, Sato T, Takai H, Watanabe K, Ikeda K, Yamada Y. Prostaglandin E2 Induces Expression of Receptor Activator of Nuclear Factor–**K**B Ligand/Osteoprotegrin Ligand on Pre-B Cells: Implications for Accelerated Osteoclastogenesis in Estrogen Deficiency. ***J Bone Miner Res*** 2000; 15(7): 1321-1329.

79. Mapara M, Thomas BS, Bhat KM. Rabbit as an animal model for experimental research. ***Dent Res J (Isfahan)*** 2012; 9(1): 111-118.

80. Campillo VE, Langonnet S, Pierrefeu A, Chaux-Bodard AG. Anatomic and histological study of the rabbit mandible as an experimental model for wound healing and surgical therapies. ***Lab Anim*** 2014; 48(4): 273-277.

81. Chen YW, Wang HC, Gao LH, et al. Osteoclastogenesis in Local Alveolar Bone in Early Decortication-Facilitated Orthodontic Tooth Movement. ***PLoS One*** 2016; 11(4): e0153937.

82. Ibrahim AY, Gudhimella S, Pandruvada SN, Huja SS. Resolving differences between animal models for expedited orthodontic tooth movement. ***Orthod Craniofac Res*** 2017; 20 Suppl 1: 72-76.

83. An J, Li Y, Liu Z, Wang R, Zhang B. A micro-CT study of microstructure change of alveolar bone during orthodontic tooth movement under different force magnitudes in rats. ***Exp Ther Med*** 2017; 13(5): 1793-1798.

84. Alhashimi N, Frithiof L, Brudvik P, Bakhiet M. Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines. ***Am J Orthod Dentofacial Orthop*** 2001; 119(3): 307-312.

85. Tan SD, Xie R, Klein-Nulend J, et al. Orthodontic force stimulates eNOS and iNOS in rat osteocytes. ***J Dent Res*** 2009; 88(3): 255-260.

86. van't Hof RJ, Armour KJ, Smith LM, et al. Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption. ***Proc Natl Acad Sci U S A*** 2000; 97(14): 7993-7998.

87. Herrera BS, Martins-Porto R, Maia-Dantas A, et al. iNOS-derived nitric oxide stimulates osteoclast activity and alveolar bone loss in ligature-induced periodontitis in rats. ***J Periodontol*** 2011; 82(11): 1608-1615.

88. Park H-J, Baek K, Baek J-H, Kim H-R. TNFα Increases RANKL Expression via PGE_2-Induced Activation of NFATc1. ***Int J Mol Sci*** 2017; 18(3): 495.

89. Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. ***Trends Immunol*** 2003; 24(1): 25-29.

90. Dai J, Rabie AB. VEGF: an essential mediator of both angiogenesis and endochondral ossification. ***J Dent Res*** 2007; 86(10): 937-950.

91. Mammoto T, Jiang A, Jiang E, Mammoto A. Platelet rich plasma extract promotes angiogenesis through the angiopoietin1-Tie2 pathway. ***Microvasc Res*** 2013; 89: 15-24.

92. Lyras DN, Kazakos K, Verettas D, et al. The influence of platelet-rich plasma on angiogenesis during the early phase of tendon healing. ***Foot Ankle Int*** 2009; 30(11): 1101-1106.

93. Cheung W-Y, Liu C, Tonelli-Zasarsky RML, Simmons CA, You L. Osteocyte apoptosis is mechanically regulated and induces angiogenesis in vitro. ***J Orthop Res*** 2011; 29(4): 523-530.

94. Nakagawa M, Kaneda T, Arakawa T, et al. Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. ***FEBS Lett*** 2000; 473(2): 161-164.

95. Kohno S, Kaku M, Tsutsui K, et al. Expression of Vascular Endothelial Growth Factor and the Effects on Bone Remodeling during Experimental Tooth Movement. ***J Dent Res*** 2003; 82(3): 177-182.

96. Rody WJ, Jr., King GJ, Gu G. Osteoclast recruitment to sites of compression in orthodontic tooth movement. ***Am J Orthod Dentofacial Orthop*** 2001; 120(5): 477-489.

97. Akbulut S, Yagci A, Yay AH, Yalcin B. Experimental investigation of effects of platelet-rich plasma on early phases of orthodontic tooth movement. ***Am J Orthod Dentofacial Orthop*** 2019; 155(1): 71-79.

APPENDICES

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January 22 , 2018

This is to certify that the research project entitled " Effects of leukocyte-platelet rich plasma on accelerated orthodontic tooth movement by local administration in rabbits" conducted by Mr. Theerasak Nakornnoi, Faculty of Dentistry, Prince of Songkla University, has been approved by Institutional Animal Care and Use Committee, Prince of Songkla University.

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Ref.2/2018

หนังสือรับรอง

รหัสโครงการ 2560-03-65

โครงการวิจัย ผลของลิวโคไซต์เพลทเลทริชพลาสมาที่มีต่ออัตราความเร็วในการเคลื่อนฟันทางทันตกรรมจัดฟันในกระต่ายทดลอง

นักวิจัย นายธีรศักดิ์ นครน้อย
สังกัด คณะทันตแพทยศาสตร์

ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์ของสถาบัน มหาวิทยาลัยสงขลานครินทร์ โดยขอให้รายงานความก้าวหน้าของโครงการวิจัยทุก 6 เดือน

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