Growth Factor Releasing of Platelet-Rich Fibrin and It's Application in Sinus Augmentation

Narumol Pattaraporncharoen

A Thesis Submitted in Partial Fulfillment of Requirements for the Degree of Master of Science in Oral and Maxillofacial Surgery

Prince of Songkla University

2011

Thesis Title	Growth Factor Releasing of Platelet-Rich Fibrin and It's Application in Sinus Augmentation
Author	Miss Narumol Pattaraporncharoen
Major Program	Oral and Maxillofacial Surgery

Major Advisor :

……..……

(Assoc. Prof. Prisana Pripatnanont)

Co-advisors :

……..……

(Asst. Prof. Dr. Srisurang Suttapreyasri)

……..……

(Asst. Prof. Dr. Sompid Kintarak)

……..……

(Dr. Narit Leepong)

Examining Committee :

……Chairperson

(Assoc. Prof. Dr. Theeralaksna Suddhasthira)

……..……

(Assoc. Prof. Prisana Pripatnanont)

……..……

(Asst. Prof. Dr. Srisurang Suttapreyasri)

……..……

(Asst. Prof. Dr. Suwanna Jitpukdeebodintra)

……..……

(Prof. Nabil Samman)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Oral and Maxillofacial Surgery

……

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์ การปลดปล่อยโกรธแฟคเตอร์ของเพลตเลทริชไฟบริน และการนำไปใช้ในการปลูกกระดูกของพื้นโพรงอากาศแมกซิลา

ผู้เขียน นางสาว นฤมล ภัทรพรเจริญ

สาขาวิชา ศัลยศาสตร์ช่องปากและแมกซิโลเฟเชียล

ปีการศึกษา 2554

บทคัดย่อ

เพลทเลท ริช ไฟบรินเป็นแหล่งของโกรธแฟกเตอร์ที่ผลิตจากตัวผู้ป่วยเอง ซึ่งมีผลส่งเสริมการเจริญของเซลล์กระดูก โดยที่สารโกรธแฟคเตอร์คาดว่าจะถูกปลดปล่อยออกมาอย่างต่อเนื่องจากร่างแหไฟบริน และน่าจะส่งผลเร่งการเจริญเติบโตของเซลล์กระดูกใหม่ในการผ่าตัดยกพื้นโพรงแมกซิลา

วัตถุประสงค์: เพื่อตรวจหาปริมาณโกรธแฟคเตอร์ชนิดบีบี ในเพลทเลท-ริช-ไฟบริน และประเมินความคงตัวของเพลทเลท-ริช-ไฟบริน รวมถึงประสิทธิภาพในส่งเสริมการสร้างกระดูกใหม่ในการยกพื้นโพรงอากาศแมกซิลาในหมูมินิพิค

วิธีการทดลอง:การศึกษาแบ่งเป็นสองขั้นตอนคือ ขั้นตอนศึกษาในห้องปฏิบัติการเพื่อตรวจหาปริมาณของ โกรธแฟคเตอร์ในเพลทเลท-ริช-ไฟบริน และขั้นตอนการทดลองในสัตว์ทดลองเพื่อตรวจสอบประสิทธิภาพการเร่งการเจริญเติบโตของเซลล์กระดูกใหม่ในโพรงอากาศแมกซิลาในหมูมินิพิค

ปริมาณของส่วนประกอบต่างๆของเลือดในเพลทเลท-ริช-ไฟบรินถูกประเมินโดยการคำนวณโดยอ้อมจากตัวอย่างเลือดก่อนการเตรียมเพลทเลท-ริช-ไฟบริน เทียบกับตัวอย่างสารเลือดที่เหลือหลังจากการนำเพลทเลท-ริช-ไฟบริน ออกจากหลอดซึ่งใช้ปั่นเลือด ปริมาณของโกรธแฟคเตอร์ที่ถูกปลดปล่อยออกมาจากเพลทเลท-ริช-ไฟบริน ถูกวัดโดยวิธีอิไลซ่าที่เวลา 10, 20, 30 และ 60 นาที เปรียบเทียบกับปริมาณของโกรธแฟคเตอร์ที่ถูกปลดปล่อยออกมาทันทีหลังจากทำให้เพลทเลท-ริช-ไฟบรินสลายตัวด้วยยาสเตรปโตไคเนส และ การทำให้เพลทเลท-ริช-ไฟบรินแตกตัวด้วยการบดเพลทเลท-ริช-ไฟบริน ที่ทำให้แข็งตัวแล้ว

ขั้นตอนการศึกษาในสัตว์ทดลอง สัตว์ทดลองถูกแบ่งออกเป็นสองกลุ่ม กลุ่มทดลองทำการผ่าตัดยกพื้นโพรงอากาศแมกซิลาโดยใช้ เพลทเลท-ริช-ไฟบริน และกลุ่มควบคุมทำการผ่าตัดด้วยวิธีเดียวกันแต่ใช้ก้อนเลือด ความคงตัวของช่องว่างที่ได้รับการยกพื้นโพรงอากาศถูก

วัดโดยวิธีการทางรังสีคอมพิวเตอร์ และปริมาณของการสร้างกระดูจะประเมินทางจุลพยาธิวิทยา ที 2 และ 6 สัปดาห์หลังการผ่าตัด

ผลการศึกษา: ปริมาณของเกล็ดเลือด ลิมโฟไซท์ และ เม็ดเลือดขาว ในเพลทเลท-ริช-ไฟบริน มีปริมาณมากกว่าในเลือด 2.4, 2.24 และ 1.87 เท่า ตามลำดับ โกรธแฟคเตอร์ถูกปลดปล่อยออกมามากที่สุดในช่วง 10 นาทีแรก และค่อยๆลดลงใน นาทีที่ 30 ปริมาณของโกรธแฟคเตอร์ที่ถูกปลดปล่อยออกมาทั้งหมดใน 60 นาที มีปริมาณไม่แตกต่างจากปริมาณของ โกรธแฟคเตอร์ทั้งหมดที่ถูกสกัดออกมาทั้งวิธีที่สลายเพลทเลท-ริช-ไฟบรินด้วยยาสเตรปโตไคเนส และ การทำให้เพลทเลท-ริช-ไฟบรินแตกตัวด้วยการบดเพลทเลท-ริช-ไฟบริน ที่ทำให้แข็งตัวแล้ว

เพลทเลท-ริช-ไฟบรินและก้อนเลือด สามารถคงอยู่ได้ในโพรงอากาศแมกซิลาได้นาน 2 สัปดาห์ และยุบตัวลงใน 6สัปดาห์ ปริมาณของกระดูกใหม่ถูกสร้างขึ้นในกลุ่มทดลองมากกว่ากลุ่มควบคุมทั้งสัปดาห์ที่ 2 และ 6อย่างมีนัยสำคัญ

สรุป:โกรธแฟคเตอร์, PDGF-BB ถูกปลดปล่อยจากเพลทเลท-ริช-ไฟบรินตลอดระเวลาการทดลองในช่วง 60 นาที และมีปริมาณไม่แตกต่างกับปริมาณของโกรธแฟคเตอร์ที่ถูกสกัดออกมาจากการแตกตัวของเพลทเลท-ริช-ไฟบริน และ เพลทเลท-ริช-ไฟบริน สามารถคงอยู่ได้ในสิ่งมีชีวิตอย่างน้อย 2 สัปดาห์ซึ่งอยู่ในช่วงการหายของแผลและการสร้างกระดูกในระยะแรก และสามารถส่งเสริมให้มีการสร้างกระดูกได้มากกว่าก้อนเลือดโดยไม่ใช้สารทดแทนกระดูกตัวอื่น

Thesis Title	Growth Factor Releasing of Platelet-Rich Fibrin and It's Application in Sinus Augmentation
Auther	Miss Narumola Pattaraporncharoen
Major Program	Oral and Maxillofacial Surgery
Academic year	2011

Abstract

Background: Platelet rich fibrin (PRF), a new generation of platelet concentration and a source of autologous growth factors, is expected to slowly release growth factors embedded in fibrin matrix over time. It may be beneficial for enhancing new bone regeneration in sinus grafting space.

Aim of the study: To verify the growth factor (PDGF-BB) entrapped in the PRF and determine the stability of PRF and new bone formation efficacy in sinus space in minipigs.

Materials and methods: The study was divided into two parts, in vitro part for quantifying growth factors and in vivo part for evaluating early effect of PRF in bone regeneration. In vitro, the amount of blood component in platelet rich fibrin were evaluated by indirect calculation from whole blood and the remnant after retrieving of PRF. To quantify growth factors released from PRF in the exudate, PDGF-BB was measured by ELISA at each time point:10, 20, 30 and 60 min. Total immediate releasing of PDGF-BB was measured after dispersing fibrin with streptokinase and milling the frozen fibrin. These levels of PDGF-BB were compared.

In vivo animal model, PRF (test group) and blood clot (control group) were used to fill the lifted space in the sinus of minipigs. The stability of lifted space were evaluated by Cone beam CT and amount of new bone regeneration were measured from histomorphometric study at 2 and 6 weeks after sacrificing.

Result The platelet concentration in PRF increased approximately 2.4 folds to the amount in whole blood. The concentration of lymphocytes and white blood cells increase 2.24 and 1.87 folds respectively. PRF progressively released PDGF-BB in 60 minutes especially in the first 10 minutes and decreased in 30 minutes. The total amount of PDGF-BB in 60 minutes was not different from total extraction of dispersed PRF by streptokinase or freezer mill.

In minipig sinus, both PRF and blood clot were remained and maintained space in 2 weeks but collapsed in 6 weeks. New bone formation was higher in PRF group in both 2 weeks and 6 weeks period.

Conclusion PRF sustained slow releasing of growth factor, PDGF-BB, during ex-vivo period for 60 minutes and the amount was comparable to the total amount of immediate growth factors extraction. PRF was retained in the body at least 2 weeks period of initial wound healing and able to promote more bone regeneration than blood clot without any grafting material.

Acknowledgement

This thesis is the end of my journey in obtaining my degree in Msc. in oral and maxillofacial surgery. I would like to express my gratitude to my supervisors, Associate Professor Prisana Pripatnanont, Head of the Department of Oral and Maxiilofacial Surgery, Faculty of Dentistry, Prince of Songkla University and Assistance Professor Srisurang Suttapreyasri for their understanding, encouraging and personal guidance those have provided a good basis for this thesis.

I am deeply grateful to my supervisor, Doctor Narit Leepong for his teaching and kindly help in sinus augmentation procedure in minipig and Associate Professor Sompid Kintarak for histology knowledge in this study.

Next, thanks to the Animal Science Unit, Faculty of Natural Resources, Prince of Songkla University HatYai, Songkhla for animal surgical facilities, The Pathology Department, Faculty of Medicine, Naresuan University, Phitsanulok for histomorphometric softwere and equipment in this study.

Finally, I wish to express my love and gratitude to all my friends and co-workers. I would like to express my thankfulness to my parent for their support and constant inspiration in my life.

Narumol Pattaraporncharoen

Content

List of Tables

List of Figures

List of Figures (Continued)

List of Abbreviations and Symbols

ANOVA	=	One-way analysis of variance
cm	=	Centimeter
conc.	=	Concentration
°C	=	Degree celcius
DMEM	=	Dulbecco's modified eagle's medium
ELISA	=	Enzyme-linked immunosorbent assay
FDBA	=	Freeze-dried bone allograft
g	=	Gram
IGF-I	=	Insulin growth factor 1
IL-1ß	=	Interleukin-1 beta
IL-6	=	Interleukin-6
I.U	=	International unit
kd	=	Kilo Daltons
kg	=	Kilogram
ml	=	Milliliter
min	=	Minute
N_cm	=	Newton centimeter
ng	=	Nanogram
nm	=	Nanometer
PDGF	=	Platelet derived growth factor
pg	=	Picogram
plt	=	Platelet
PRF	=	Platelet rich fibrin
SEM	=	Scanning electron microscope
TGF-ß	=	Transforming growth
TNF-α	=	Tumor necrosis factor alpha
µm.	=	Micrometer

VEGF	=	Vascular endothelial growth factor
WBC	=	White blood cell
wk	=	Week

Chapter 1

Introduction

Platelets are the end product of megakaryocytes that formed in bone marrow. They are round or oval in shape, approximately 2 μm. in diameter. They have no nuclei and cannot replicate so the life span of platelet is 5-9 days [1]. They have trilaminar cell membrane with glycoprotein receptor surface overlying and partially penetrating a bilayer of phospholipid and cholesterol. Platelet contain organelles and granules, alpha,delta and lamda respectively.[2] Functions of platelet are involved with both hemostasis and initiation of wound healing. Whenever tissue has been injured and blood vessel is damaged, platelets become exposed to damaged vessel, and direct contact with collagen, the basement membrane of capillaries, which causes the platelets aggregate at the site and change from a round shape to a large , sticky pseudopodia shape [3]. This process is called platelet activation. During activation, the alpha granules within platelets fuse with the platelet plasma membrane and release some of their protein content to surroundings (degranulation).

The alpha granules are 200-500 nm in diameter and contain more than 30 bioactive proteins that have important role in hemostasis and/or tissue healing (Table 1). These proteins include growth factor cytokines and inflammatory cytokines such as PDGF (including AA BB, AB isomers), TGF-ß (including ß1 and ß2 isomers), platelet factor 4, interleukin-1, platelet-derived angiogenesis factor, VEGF, epidermal growth factor, platelet derived endothelial growth factor, epithelial growth factor, insulin-like growth factor, osteocalcin, osteonectin, fibrinogen, vitronectin, fibronectin, and thrombospondin-1[4]. Platelets begin actively secreting these proteins within 10 minutes after clotting, and more than 95% of the presynthesized growth factors are secreted within 1 hour. After the initial burst, the platelets still synthesize and secrete additional growth factors for the remaining several days of their life span[5].These growth factor play role in bone and soft tissue healing.

Table 1: Growth factors released from platelets and their biologic action (Harrison and Cramer, 1993)

Growth factor	Source cells	Target	Biologic action
Platelet-derived growth factor	Platelets, macrophages, monocytes, endothelial cells, smooth muscle cells	Fibroblasts, smooth muscle cells, glial cells, macrophages, neutrophils	Stimulates DNA and protein synthesis in osseous tissues; mitogenic effects on mesenchymal cells; angiogenic effect on endothelial cells
Transforming growth factor β	Platelets, T-lymphocytes, macrophages/monocytes, neutrophils	Fibroblasts, marrow stem cells, endothelial cells, epithelial cells, preosteoblasts	Stimulates angiogenesis; enhanced woven bone formation; stimulate matrix synthesis in most culture systems; chemotactic effect on osteoblastic cells; stimulates endothelial chemotaxis; stimulates bone formation by inhibitory effect on osteoclasts
Platelet-derived angiogenesis factor	Platelets, endothelial cells	Endothelial cells	Mitogenic effect on endothelial cells; increased angiogenesis and vessel permeability
Insulin-like growth factor 1	Osteoblasts, macrophages, monocytes, chondrocytes	Fibroblasts, osteoblasts, chondroblasts	Stimulates proliferation of osteoblasts and matrix synthesis; increases expression of bone matrix proteins, such as osteocalcin; in combination with PDGF it enhances the rate and quality of wound healing
Platelet factor 4	Platelets	Fibroblasts, neutrophils	Chemoattractant for neutrophils and fibroblasts

PDGF - platelet-derived growth factor, TGF-β - transforming growth factor β, PDAF - platelet-derived angiogenesis factor, IGF-1 - insulin-like growth factor -1, PF-4 - platelet factor - 4

Platelet-derived growth factor is a glycoprotein with molecular weight approximately 30 kd. It seems to be the first growth factor found in a wound and initiated connective tissue ,including bone healing[6]. In human it exists a dimeric protein consisting of 2 subunits: A and B exists in 3 combinations: PDGF-AA, PDGF-BB, and PDGF-AB. The role of each isomer in bone and soft tissue healing is not well explored. There are approximately 0.06 ng of PDGF per one million platelets. When activated, the growth factor attaches to transmembrane receptors on target cells such as osteoblasts and fibroblasts. The main function of PDGF is stimulating cellular replication (mitogenesis). This growth factor increases cell populations of healing cells, including mesenchymal stem cells and osteoprogenitor cells, which are part of the connective tissue-bone healing; cellular composite and endothelial cells, causing budding of new capillaries into the wound (angiogenesis). PDGF also stimulates bone resorption by increasing the number of osteoclasts, which can lead to faster bone remodeling. Furthermore, PDGF activates macrophages, resulting in debridement of the surgical or traumatic site. The macrophage activation then triggers a second source of growth factors released from the host tissues under the influence of the macrophage action. This secondary release of endogenous factors continues the process of repair and bone regeneration. In vivo, 20-100 ng of PDGF in demineralized bone matrix carrier has been

shown to increase ectopic bone formation and alkaline phosphatase activity in rats. PDGF, when locally applied, also stimulated bone healing in rat osteotomies, as well as in rabbit calvaria defects in combination with barrier membranes. This protein has also been found to be expressed by many cell types during different stages of normal fracture healing [7].

In the past two decades, an increased understanding of the physiological role of platelet during wound healing and after tissue injury has led to the idea of using platelets as therapeutic tools. After fibrin glue was introduced in the early 1990 as a biomaterial with hemostasis and adhesive properties, the strategic modification of the fibrin including platelets was reported [8] In vitro, there is a dose-response relationship between platelet concentration and the proliferation of human adult mesenchymal stem cells, the proliferation of fibroblasts, and the production of type I collagen[9]. This suggests that the application of platelet concentration can enhance wound healing.

The preparation obtained from a patient known as platelet-rich plasma (PRP) is defined as a portion of the plasma fraction of autologous blood having high platelet concentration above baseline. PRP has been first introduced for oral and maxillofacial reconstruction by Marx et al in early 1990s. Once the platelet concentrate is activated by thrombin and calcium, a three-dimensional and biocompatible fibrin scaffold is formed, and a myriad of growth factor and protein are released contributing to the accelerate postoperative wound healing and tissue repair [10] (Figure 1). PRP contains not only a high level of platelets but also the full complement of clotting factors and secretory proteins includes TGF-b, vascular endothelial growth factor (VEGF), and PDGF. Due to the increased concentration and released of these factors, PRP potentially enhances the recruitment and proliferation of stem cells, and endothelial cells[11].

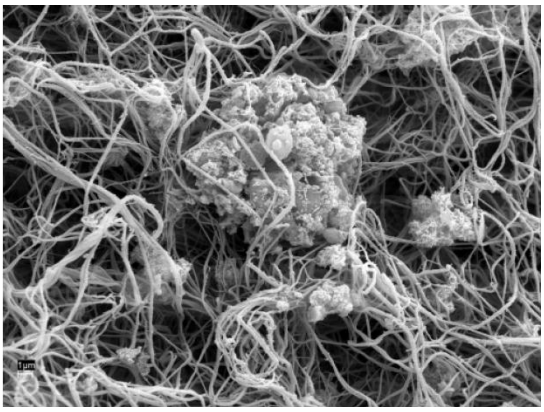


Figure1: Scanning electron microscopy of human platelet-rich plasma. Multiple platelet cell elements can be observed, forming a cell conglomerate trapped among fibrillar elements (fibrin)

There are many studies of application of PRP in several animal and clinical studies. In sinus augmentation, PRP accelerated vascularization of the graft, improve soft tissue healing, less postoperative morbidity and enhance bone regeneration (Table 2). However there are controversies in literature regarding the potential benefits of the autogeneous PRP. The positive effects of using PRP to enhance bone regeneration in sinus graft are summarized in Table 2. Table 3 summarized human studies that PRP had no effect on bone regeneration ([12])

Table 2: The positive effect of PRP on sinus augmentation in human studies([12])

Investigators	Study Model	Outcomes	Major Critique
Rosenberg and Torosian	1 pt SA with PRP and composite graft	Reduced treatment time by half (placed implant at 3 mo)	1 case report
Rodriguez *et al*	15 pts, 24 SAs with anorganic bovine bone and PRP Simultaneous placement of 70 implants	Bone density: grafted = native bone	Faster bone consolidation not supported by study design
Philippart *et al*	25 SAs with a "bone paste" made of: PRP, rhTF, calvarial bone chips, and tetracycline	Vital bone formation with 91.3% implant success rate	No controls
Maiorana *et al*	11 SAs in 10 pts grafted with PRP gel and anorganic bovine bone	Bovine graft particles in all samples with variable amounts of new bone	No controls
Wiltfang *et al*	45 SAs in 39 pts Randomized, prospective design Test: β-TCP granules and PRP; Control: β-TCP granules alone	Bone formation: 32% to 43% (test), compared to 25% to 37% (control)	Wide range of results
Monov *et al*	105 pts with <5 mm crestal height SA with composite graft of: autogenous bone (30% to 40%), xenograft (60% to 70%), and PRP	Exposure time reduced by 3 mo Subjective claims of less analgesic use and edema	No controls
Velich *et al*	Retrospective study of failure 5 y period (1996–2001) and 810 SAs Various materials used	Graft resorption: 2.7% overall compared to 0% in β-TCP and PRP group	Small number of cases used PRP
Philippart *et al*	4 SAs in 3 patients with autogenous calvarial bone powder, PepGen P-15 (DENTSPLY Friadent CeraMed, Lakewood, CO), PRP, and rhTF	Bone paste had a high bone regeneration capacity	Small sample size No controls
Graziani *et al*	6 pts had unilateral SA using autogenous bone, PRP and autologous fibrinogen	Average bone augmentation of 6.27 mm (range 3.5-10)	No controls
Kassolis and Reynolds	10 pts Randomized, single-blinded Bilateral SA Test: FDBA and PRP; Control: FDBA and resorbable membrane	Percentage of vital tissue in sinuses: 78.8 ± 8.3 (test) compared to 63.0 ± 15.7 (control)	Platelet yield not given
Steigmann and Garg	20 pts with bilateral SA Test: Autologous PRP alone Control: β-TCP alone	Higher percent of new bone formation in the test group	No negative control

pt indicates patient; SA, sinus augmentation; TCP, tricalcium phosphate.

Table 3: Non beneficial effect of PRP on sinus augmentation in human studies ([12])

Investigators	Study Description	Outcomes	Critiques
Raghoebar *et al*	5 pts Bilateral SA Test: Autogenous graft from iliac crest and PRP Control: Autogenous iliac crest graft alone	Bone density: No significant difference between groups	Resorption of iliac crest graft may have masked regenerative capacity of PRP
Froum *et al*	Bilateral SA in 3 pts. Test: Anorganic bovine bone and PRP Control: Anorganic bovine bone alone	BIC for test implants: 37.6% (test) compared to 33.8% (control)	Case report

pts indicates patients; SA, sinus augmentation.

Although PRP had advantages of using an autologous blood product that carries no risk of cross-reactivity, immune reaction, or disease transmission, the preparation of PRP still uses calcium and bovine thrombin to activate gel formation. It has been reported that there was antibody to the factor V and XI and thrombin, resulting in the risk of life-threatening coagulopathies .Bovine thrombin preparation has been shown to contain factor V, which could result in the stimulation of immune system when challenged with foreign protein ([13])

The recent form of platelet concentration, platelet rich fibrin, was first developed by Choukroun et al ([14]). A procedure is simple that blood sample is taken without anticoagulant to allow natural clot when contact with glass tube in which are immediately centrifuged at 3000 rpm (approximately 400g) for 10 minutes. Fibrinogen is initially concentrated in the top part of the tube, before the circulating thrombin transforms it into fibrin. A fibrin clot is then obtained in the middle of the tube, just between the red corpuscles at the bottom and acellular plasma at the top (Figure 2). This protocol attempted to accumulate platelet and release cytokine in fibrin clot. Preliminary hematologic studies revealed that platelets did not remain in the acellular supernatant (platelet-poor plasma (PPP)) or in the red blood corpuscles base ([15])

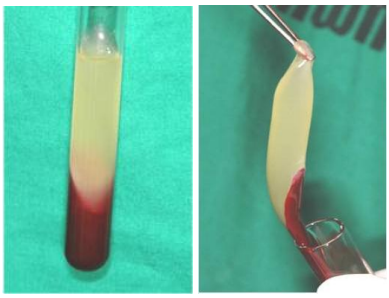


Figure 2: A fibrin clot (platelet rich fibrin) in the middle of the tube, between the red corpuscles at the bottom and acellular plasma at the top.

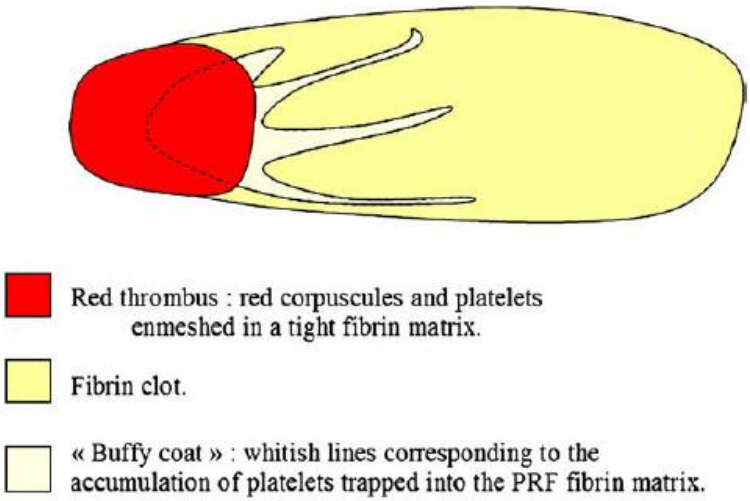


Figure 3: The three part of PRF fibrin clot: a red thrombus in contact with the red blood corpuscle base, an acellular fibrin gel, and a network of buffy columns ([15]).

The slow polymerization with physiologic thrombin concentrations implies very elastic matricial architecture. The junctions between fibrin particularly favorable to cell migration and soluble molecule retention ([14]). The overall architecture of the fibrin clot was examined with a scanning electron microscope (Figure 4-6) and showed that RBCs were widely predominant in the red part of the PRF clot, and the leukocytes were distributed at the junction between the red and yellow parts of the clot. Only a few RBCs were identified in the rest of the clot, which were probably artifacts due to clot handling. Platelet morphology was totally modified by aggregation and clotting processes ([16]).

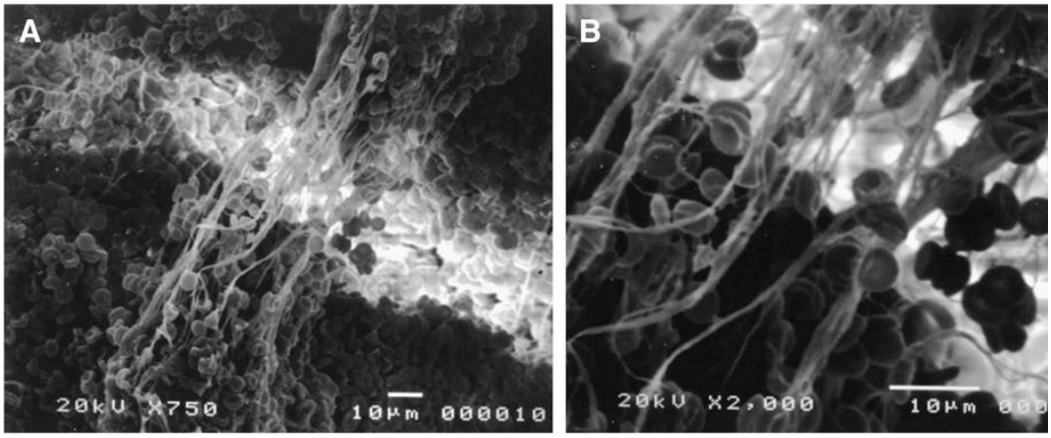


Figure 4: The red area of the PRF clot contain many RBCs trapped within an immature and very loose fibrin matrix ([16])

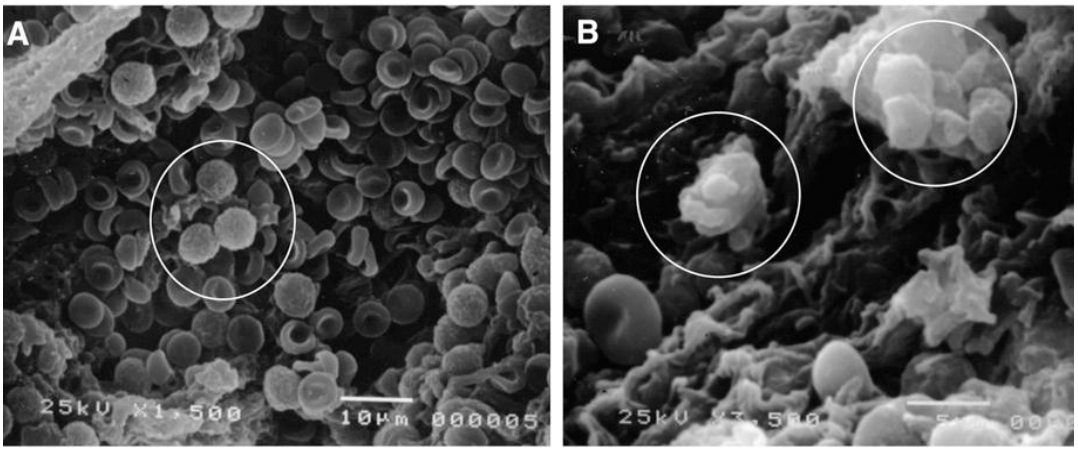


Figure 5: A) At the borderline between the red area and yellow clot, clusters of RBCs and leukocytes found. Leukocytes appear spherical structures with an irregular surface (white circles). B) Platelets are often enmeshed in the fibrin network but sometimes appear as aggregates (white circles) ([16]).

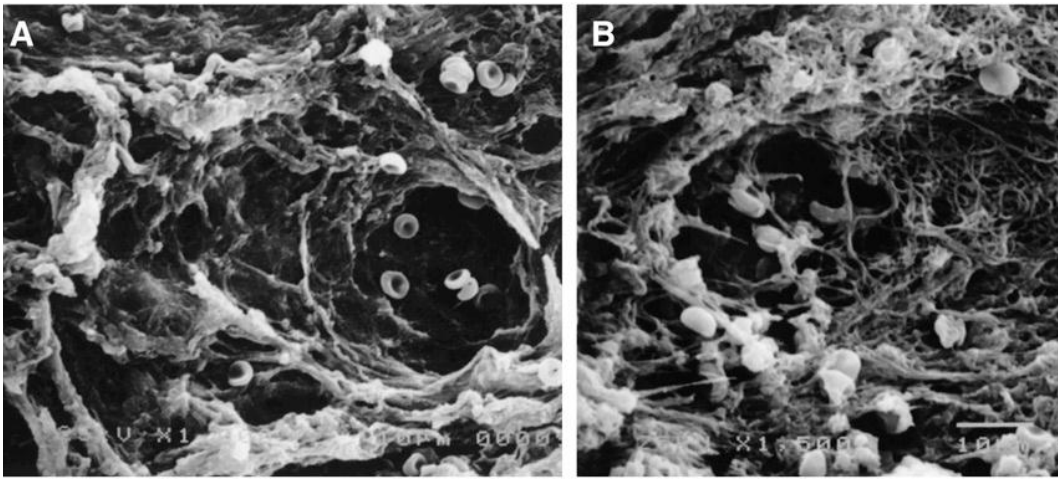


Figure 6: A and B) The yellow clot, the platelet aggregates are closely merged into a dense and mature fibrin matrix ([16]).

The previous study reported that during autogenous PRF processing, the absence of anticoagulant in the collection tube induced massive platelet activation, bolstered by the presence of a mineral phase on the glass tube walls (residual glass particles). These growth factor cytokines, PDGF-BB and TGF-ß1, are small soluble molecules which centrifugation could naturally concentrate in the upper part of the tube (the supernatant). However the majority of PDGF-BB and TGF-ß1 were found neither in the supernatant nor in the exudates. They were hypothesized to be remained and trapped in the PRF fibrin matrix ([15, 17])

Since 2006, the used of platelet-rich fibrin has been widely and some studies reported that mixing PRF with graft material could shorten of healing time of bone graft healing ([14, 15]) and the PRF can be used as an adjunct to osteotome sinus floor elevation ([18]).

The platelet cytokine quantification in the autogenous PRF constitutes a significant step in the understanding of this biomaterial, because these soluble molecules are key inflammatory and healing mediators ([19]). A previous study had investigated the quantity of growth factor cytokine (PDGF-BB and TGF ß-1) and inflammatory cytokine (IL-1ß, IL-6 and TNF-α, anti-inflammatory cytokines such as IL-4) in the components of blood products such as platelet poor plasma, exudates from PRF,

serum or plasma and platelet rich plasma. It was found that the exudate from PRF clot leaving in a sterile metal cup for 10 minutes contained a low level of PDGF-BB and TGF ß-1 which was significantly lower than those obtained from the several cPRP protocols. On the other hand, IGF-I was found in the supernatant and the exudates in a significant higher level than in the cPRP of various protocol ([15]) (Figure 7-9).

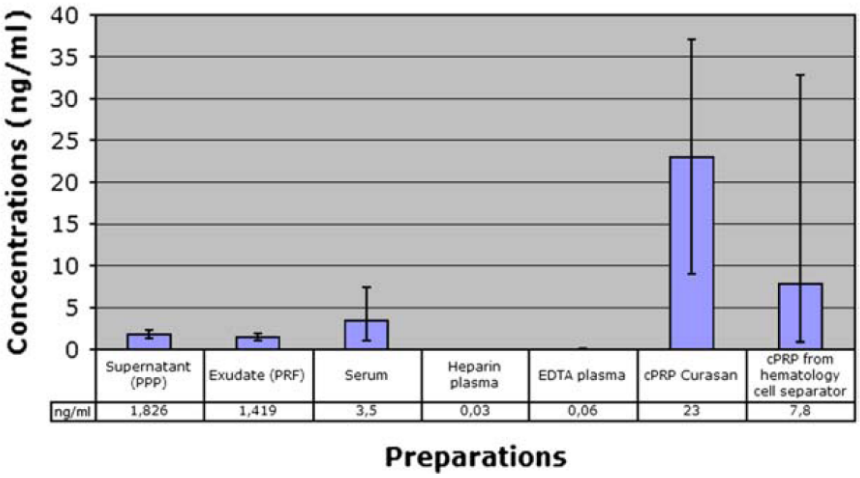


Figure 7: PDGF-BB ELISA quantifications ([15])

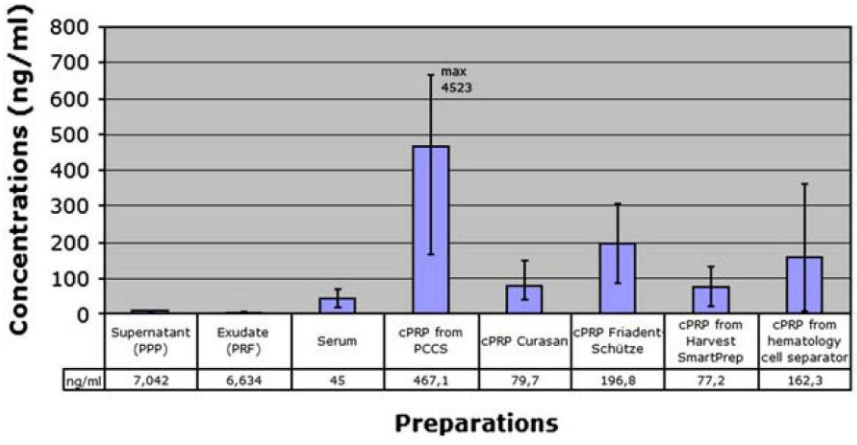


Figure 8: TGFß-1 ELISA quantifications ([15])

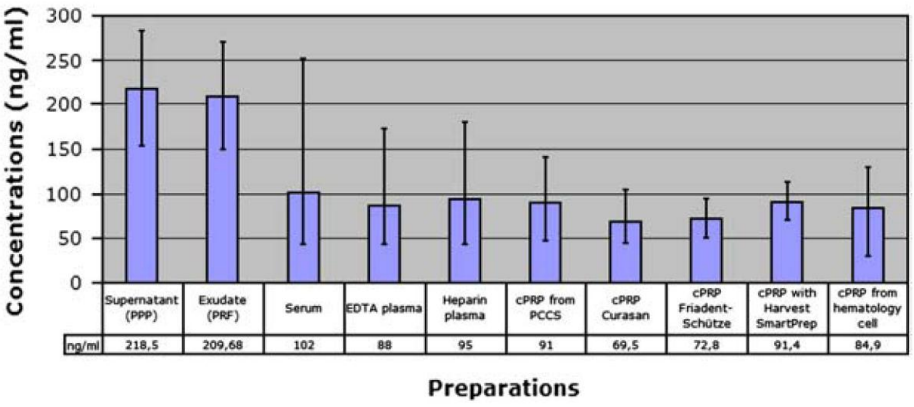


Figure 9: IGF-1 ELISA quantifications ([15])

From this study it was proposed that the whole PRF platelet cytokine possibly to be trapped in the fibrin meshes. In 2009 Dohan presented that when the platelet-rich fibrin has been pressed into a membrane, there was a slow release of growth factors in the exudates such as PDGF, TGF- β and VEGF for at least 7 days in vitro([20]) (Figure 10 and 11).

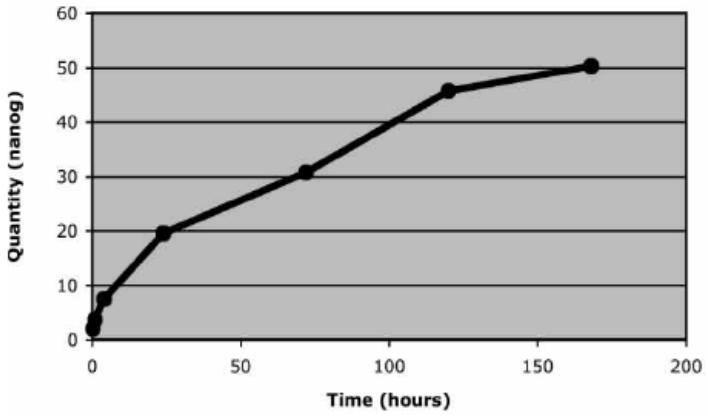


Figure 10: Slow release of PDGF AB from PRF membrane ([20])

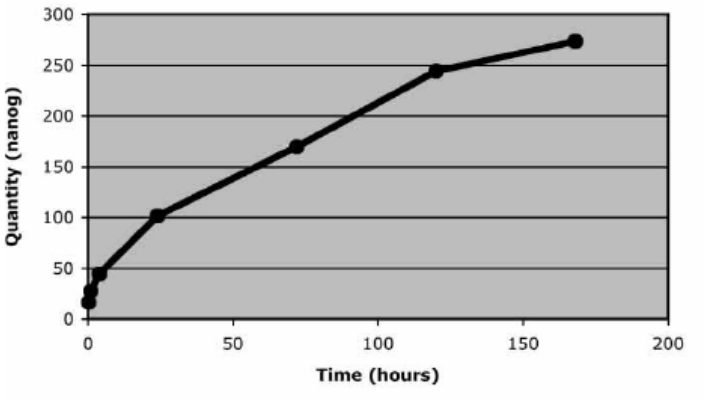


Figure 11: Slow release of TGF β from PRF membrane ([20])

Platelet rich fibrin was used clinically in sinus augmentation in 2006. The histologic examination of the specimen after sinus augmented at 8 months of FDBA grafting alone and 4 months of FDBA with PRF grafting were similarly observed. It is possible that PRF facilitated shorter healing period then grafting without PRF (4 months instead of 8 months) ([21]).

Diss et al. ([18]) used platelet rich fibrin (PRF) as a grafting material for osteotome sinus floor elevation with microthread implant and found endosinus bone gained. Despite a limited residual bone height, a healing period of 2-3 months was found to be sufficient to resist a torque of 25 N_cm applied during abutment tightening. At 1 year, the formation of a new recognizable bone structure delimiting the sinus floor was identified radiologically and led to a predictable implant function.

Pripatnanont P et al. (Pripatnanont et al 2009) investigated the effect of platelet-rich fibrin on autogenous bone and deproteinized bovine bone in bone formation in rabbit calvarial defects. The adjuvant of PRF to either the autogenous bone, the empty defect or deproteinized bovine bone showed better result than the condition of non-PRF particularly in the autogeneous bone. It was concluded that PRF might be useful for enhancing bone regeneration in situation that contained osteogenic cells.

Based on an experimental animal model by Lindhe et al ([22]) , bone regenerated was found in an isolated space created between the periosteum and the calvarial cortex. The isolated space was filled initially with clotted blood and then was

occupied later by newly formed bone. This observation is consistent with the possibility that no filler is necessary in the created space for sinus lifting if the space is maintained for adequate time to allow regeneration of new bone. Some previous studies found the positive effect of the clotted blood alone in the space maintained by implant in sinus augmentation. It is conceivable that formation of new bone in the maxillary sinus does not require the presence of any graft or scaffold but derived of bone cell from the sinus periosteum or peripheral cancellous marrow in the maxilla ([23-25]).

In bone healing and regeneration process, several components within blood constituents eg. fibrin, fibronectin, vitronectin, platelet derived growth factor, transforming growth factor are parts of natural healing process which can be altered or accelerated by concentrating these factors. These proteins set the stage for tissue healing which include cellular chemotaxis, proliferation and differentiation removal of tissue debris, angiogenesis, and the laying down of extracellular metrix ([10, 26]). The blood clot formation after surgical intervention or trauma initiates the healing cascade. The composition of clot is 95% red blood cell, 4% platelet, and 1% white blood cell. However an analysis of platelet enrich clot reveals different composition compared to natural clot with 95% platelets (as opposed to 4%), 4% red blood cell (as opposed to 95% red blood cell), and a similar amount of white blood cell ([11]) . According to high concentration of platelet, de novo bone formation may be accelerated at the surgical site when platelet concentration is applied.

Platelet-rich fibrin presents a complex tridimensional architecture ([17])and does not dissolve quickly after application([14, 15]) therefore it could be used as a growth factor carrier and a degradation scaffold. From SEM study of PRF demonstrated that PRF composed of two components: fibrin filaments and cellular component containing platelet cells that are essential for tissue regeneration (Figure 12 and 13). When compared to SEM study of natural blood clot, PRF showed more organized dense fibrin filaments with more platelets entrapped in the matrix (Pripatnanont et al 2009).

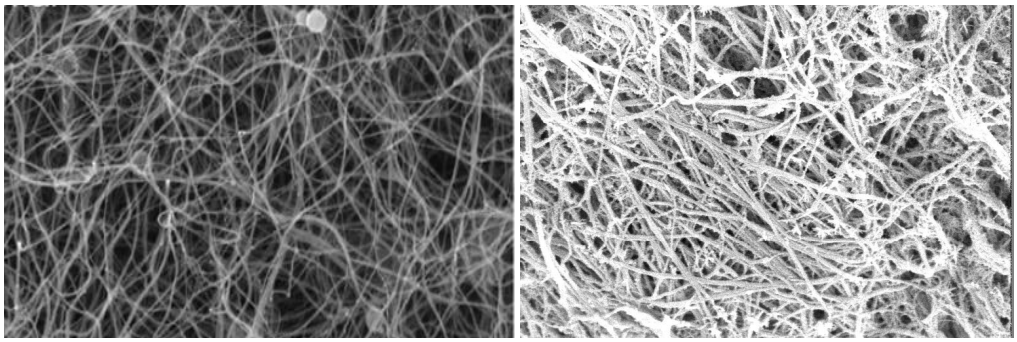


Figure 12: (left) Scanning electron microscope image of natural blood clot (Pripatnanont et al 2009).

Figure 13: (right) Scanning electron microscope image of platelet rich fibrin (Pripatnanont et al 2009).

The previous report (20) showed that the amount of growth factors released from pressed PRF membrane were presented through 7 days. In clinical application, after PRF is processed, it might not be used instantly, then some exudates might be leaked even it is not pressed. The amount of leaked growth factors in the exudates at each time point and the growth factors those were still entrapped in the PRF clot have never been studied before.

Moreover the previous work in our institute showed that PRF still remained in the socket of extracted tooth after 14 days (unpublished data), (Suttapreyasri et al 2009). Therefore the stability of fibrin and its ability to release growth factors during initial period of wound healing should facilitate bone formation in the early stage. Therefore it is hypothesized that platelet-rich fibrin can maintain the created space in sinus augmentation and slowly release growth factors to promote new bone formation in the early stage.

The aim of this study were to determine the growth factor (PDGF-BB) entrapped in the PRF in vitro and to evaluate the effect of platelet rich fibrin on the early stage of bone regeneration in maxillary sinus of minipigs The study was divided into two part, in vitro and in vivo study.

Objective of the study

General objective :

To determine the enmeshed growth factor, PDGF-BB, in the autogenous platelet-rich fibrin and to evaluate the effect of platelet rich fibrin in bone regeneration.

Specific objective

In vitro :

1. To determine the concentration of released growth factor, PDGF, in the exudates of the autogenous platelet-rich fibrin at various time point.
2. To determine the concentration of the growth factor, PDGF, enmeshed in autogenous platelet-rich fibrin

In vivo :

1. To evaluate early bone formation potential of platelet-rich fibrin compare to blood clot in sinus augmentation procedure
2. To evaluate stability of platelet rich fibrin in the created space in sinus augmentation

Benefit of the study

In vitro:

1. To provide the scientific knowledge of growth factor contained in platelet-rich fibrin.
2. To develop the protocol of clinical use of platelet-rich fibrin.

In vivo:

To utilize platelet rich fibrin in clinical application.

Hypothesis:

In vitro:

1. There is less growth factor, PDGF, in the exudates released from platelet rich fibrin at any time point.
2. There is more growth factor, PDGF, in degraded platelet-rich fibrin than in the exudates.

In vivo:

The platelet rich fibrin is stable in the sinus during 2 weeks period and promotes bone formation in the created space in the sinus.

Chapter 2

Part I

In vitro : Materials and methods

Scope of study

This study was divided into in vitro and in vivo studies. The *in vitro* study was performed to investigate the quantity of growth factor, PDGF-BB, released from the fibrin into the exudates at each time point when fibrin was left unused, and the amount of PDGF-BB that entrapped in the PRF clot. The *in vivo* study was to evaluate the stability of PRF in the created space in the maxillary sinus of the minipigs and determine the effect of PRF on bone formation in the sinus at 2 and 6 weeks.

In vitro study

Sample size

Four healthy volunteers, two male and two female without any systemic disease, 26 to 30 years of age were participated in the study.

Groups of study

Sixtyfive mililitres of blood from each volunteers was divided in 5 tubes as follows:

5 ml for determining whole blood component count

10 ml for preparing PRF for testing of PDGF-BB released in the exudates

10 ml for preparing PRF for testing PDGF-BB extracted by force with bone mill

30 ml for preparing PRF for testing PDGF-BB released after PRF clot lysis with streptokinase

10 ml for preparing serum PDGF in normal blood as a control of normal group

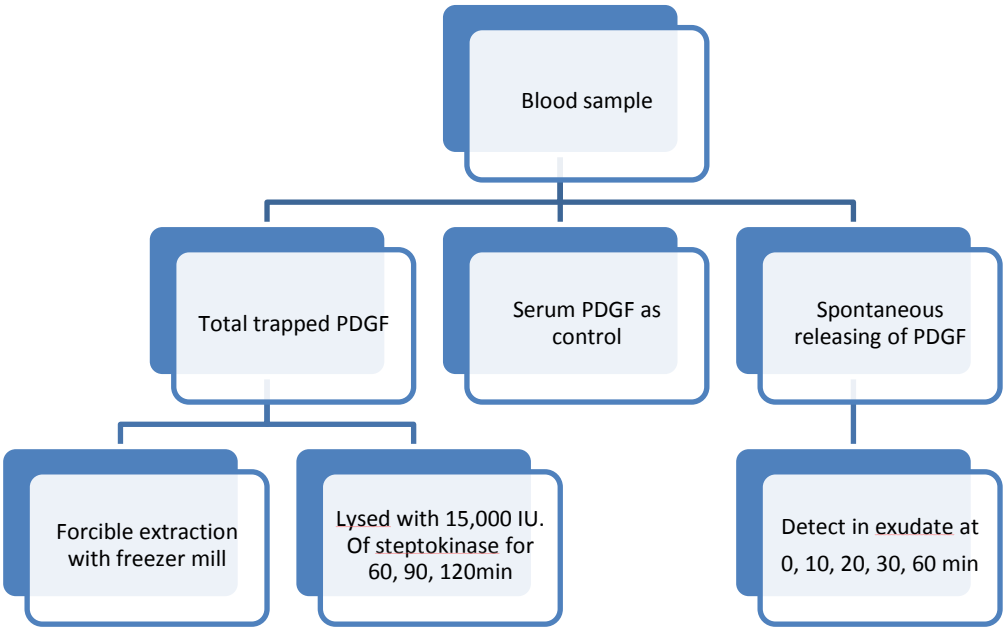
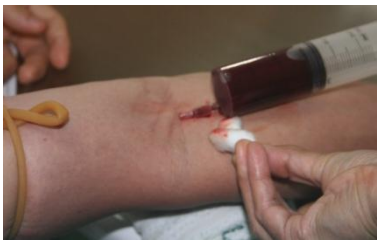


Figure 14: In vitro groups of the study

Material

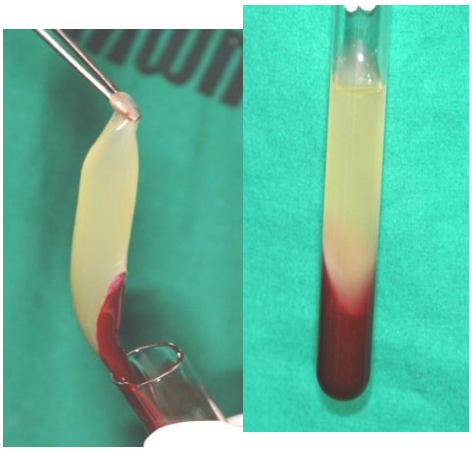
Platelet-rich fibrin (PRF) preparation

Blood samples were treated according to the PRF protocol ([14]): 50 ml of whole blood was drawn and divided into 5 glass tubes, without anticoagulant. Each tube of 10ml blood was immediately centrifuged at 3,000 rpm. for 10 minutes. The result was a fibrin clot located in the middle of the tube, between the red corpuscles at the bottom and acellular plasma at the top, with a maximum number of platelets caught in the mesh of fibrin ([15]).The fibrin clot was elevated by tissue forceps up from the tube and left for 10 seconds to get rid of the supernatant. The red cell part was cut which preserve the platelet that was high concentration at the buffy coat between fibrin clot and red corpuscles.



Blood was drawn without anticoagulant

Each 10 ml was centrifuged



Platelet-rich fibrin

Figure 15: Platelet rich fibrin preparation

Method

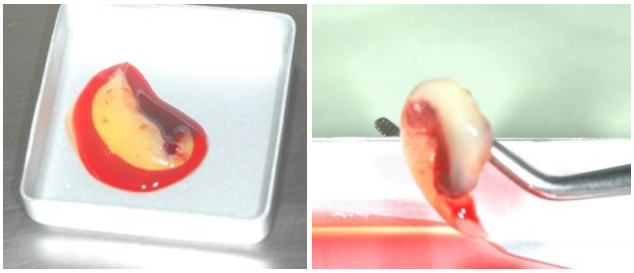
Blood component count examination

The platelet, lymphocyte, white blood cell and red blood cell concentration were examined in the whole blood and in the PRF remnant part. After PRF processing, PRF clot was retrieved from the 10 ml syringe for the experiment, the remaining substance of serum part and red blood cell part were well agitated. Two ml of the mixing fluid was used to determine blood components apart from PRF that left in the remnant. Examination of blood components in the PRF was done by using the automated hematology analyzer. The blood component count in the PRF was calculated from subtraction the actual amount from whole blood with the amount counted from the

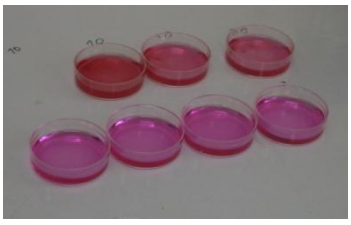
remnant which were converted the proportion to 10 ml of the blood and then normalized to 1 ml.

Releasing of PDGF in the PRF exudates

Immediately after processing of PRF, PRF clot was transferred into a sterile metal cup with 4 ml of Dulbecco's modified eagle's medium (DMEM) and retransferred to a new cup of 4ml sterile DMEM after each time point 0, 10, 20, 30, 60 min, and the previous 4ml of DMEM with the exudate leaked from PRF at each time point was stored at -80°C before ELISA quantification. The quantity of growth factor cytokine, PDGF-BB in the exudates leaked from PRF clot was measured by ELISA kit (Quantikine, R&D Systems,Minneapolis, MN, USA).The tests were repeated twice to reduce a measurement error.



Transferred PRF from glass tube after centrifuged in the sterile metal cup



Dulbecco's modified eagle's medium (DMEM)

Figure 16: Preparation of Platelet rich fibrin exudates at each time point

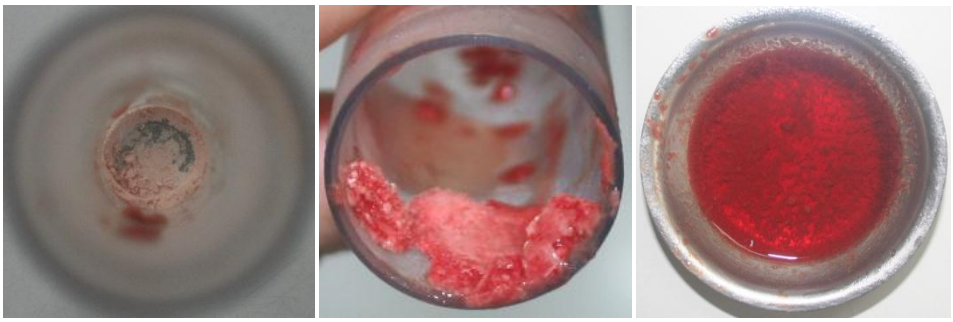
Releasing of PDGF after PRF clot dispersed and lysis

PDGF-BB extracted from physical treatment with frozen mill

Fresh PRF clot was immediately frozen in liquid nitrogen and milled by a frozen mill. The supernatant was collected and kept at -80°C. PDGF-BB concentration was measured using ELISA kit (Quantikine, R&D Systems,Minneapolis, MN, USA) according to the manufacturer's. The tests were repeated twice to reduce a measurement error.

Chopped PRF in plastic tube for freezer mill

Frozen PRF with liquid nitrogen and milled



PRF after milling and left in room temperature

Figure 17: Dispersion of platelet rich fibrin with freezer mill

PDGF-BB extracted from chemical treat with streptokinase

Another three fresh PRF clots were transferred to the petri dishes and treated with 15,000 I.U of streptokinase and incubated in a humidified incubator at 37°C at various time point. After 60, 90 and 120 minutes period, the lysis clots were stored at -80°C. PDGF-BB was measured by using ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. The tests were repeated twice to reduce a measurement error.

Figure 18: Lysed PRF with 15,000 IU of streptokinase

Serum PDGF in whole blood

The 10 ml of blood sample was kept in a dry glass tube for 30 minutes, to allow adequate time to coagulate thoroughly. Then it was centrifuged at 1000g for 15

minutes to recover the representative serum for cytokine released from completely activated blood. Then 2 ml of serum was used to measure PDGF-BB twice.

Data analysis

The differences between the mean of PDGF-BB of the exudates at 0, 10, 20, 30, 60 minute and in the whole blood were analyzed by using repeated ANOVA at p-value 0.05. The difference between each pair of time was assessed by pair t-test.

Chapter 3

Part I

Invitro : Result

Four healthy volunteers, 2 males and 2 females, aged 27.5±1.91 ([26-30]) years were participated in the present study. Sixty ml of blood was drawn from each volunteer, 50 ml was used for preparing PRF, and 10 ml was used as normal blood and its serum was used for PDGF examination. Average volume of PRF prepared from each 10 ml of whole blood was 4.1±0.54 ml as shown in Table4.

Table 4: The PRF volume from each sample

Sample	A	B	C	D	Mean±SD (ml)
Whole blood (ml)	10	10	10	10	10
PRF (ml)	4	3.5	4	4.8	4.1±0.54

Whole blood count examination

Two mililitres of whole blood from each volunteer was used to determine platelet count, white blood cell, red blood cell count and lymphocyte count. Table 5 presents the amount of each item that was determined from the whole blood.

Table 5: Platelet count, lymphocyte, white blood cell count and red blood cell count concentration from of whole blood.

Sample	A	B	C	D	Mean±SD	Normal value
Platelet ($x10^3$/μm)	221	195	316	309	260.25±61.32	150-450
Lymphocyte	41.40%	41.20%	42.50%	43.90%	42.25±1.2	20-50 %
WBC ($x10^3$/μm)	3.84	3.59	5.88	6.27	4.89±1.38	4.5-10.0
RBC ($x10^6$/μm)	4.2	3.86	4.59	5.07	4.43±0.52	4.2-5.5

PRF examination

The amount of each item was shown in Table 6. These amounts were used for indirect calculation of the amount of the components in the PRF because the PRF is in a gelling stage that is not possible for direct counting by automatic counting machine.

Table 6 : Platelet count, lymphocyte, white blood cell count and red blood cell count concentration of the remaining substance.

Sample	A	B	C	D	Mean±SD
Platelet ($x10^3$/μm)	11	5	19	7	10.5±6.19
Lymphocyte	2%	2%	4%	3%	2.75±0.96
WBC ($x10^3$/μm)	1.24	1.24	2.92	3.46	2.22±1.15
RBC ($x10^6$/μm)	4.97	4.16	5.05	6.96	5.28±1.19

Blood component concentration of PRF

The platele, lymphocyte, white blood cell and red blood cell concentration of PRF were calculated. The platelet concentration in PRF was increase about 2.4 fold to the whole blood (Table 7). The concentration of lymphocyte and white blood cell were increase 2.24 and 1.87 fold respectively (Table 8 and 9). Unlike red

blood cell, the concentration in the remnant was increase from the whole blood. Therefore no any red blood cells were trapped in the PRF clot.

Table 7: Calculated concentration of platelet in PRF

Sample	Plt. Conc. of whole blood ($x10^3$/μm)	Plt. Conc. of remnant ($x10^3$/μm)	PRF volume (ml)	Plt. Conc. Of PRF ($x10^3$/μm)	Increasing fold
A	221	11	4	533.5	2.41
B	195	5	3.5	547.88	2.82
C	316	19	4	761.50	2.42
D	306	7	4.8	629.92	2.06
Mean	260.25±61.32	10.5±6.19	4.1±0.54	618.2±104.52	2.43±0.31

Table 8 : Calculated concentration of lymphocyte in PRF

Sample	lymphocyte of whole blood (%)	lymphocyte of remnant (%)	PRF volume (ml)	lymphocyte Of PRF (%)	Increasing fold
A	41.40	2	4	99.5	2.40
B	41.20	2	3.5	114	2.77
C	42.50	4	4	75.25	1.77
D	43.90	3	4.8	88.16	2.01
Mean	42.25±1.2	2.75±0.96	4.1±0.54	94.21±16.49	2.24±0.44

Table 9 : Calculated concentration of white blood cell in PRF

Sample	WBC Conc. of whole blood ($x10^3$/μm)	WBC Conc. of remnant ($x10^3$/μm)	PRF volume (ml)	WBC Conc. Of PRF ($x10^3$/μm)	Increasing fold
A	3.84	1.24	4	7.74	2.02
B	3.59	1.24	3.5	7.95	2.21
C	5.88	2.92	4	10.32	1.76
D	6.27	3.46	4.8	9.31	1.49
Mean	4.89±1.38	2.22±1.15	4.1±0.54	8.83±1.21	1.87±0.31

Table 10 : Concentration of red blood cell of whole blood and the remaining substance.

Sample	RBC Conc. of whole blood ($x10^3$/μm)	RBC Conc. of remnant ($x10^3$/μm)
A	4.20	4.97
B	3.86	4.16
C	4.59	5.05
D	5.07	6.96
Mean	4.43±0.52	5.28±1.19

Releasing of PDGF in the PRF exudates

The concentration of PDGF-BB level in exudates leaked from PRF while it was left in the metal cup at each time point and the accumulative concentration are presented in Table 11. A large amount of PDGF-BB was released in the first 10 minutes and then slowly released when time passed at the steady rate (Figure 27). The releasing rate at 30 minutes was lower than the first and second 10 minutes. The cumulative quantity of PDGF-BB levels leaked from PRF is presented in Figure 26.

Table 11: PDGF-BB concentration and rate of release in exudates leaked from PRF in each time point

Time point (min)	Mean conc. (pg/ml) N=4	Mean cumulative conc (pg/ml)	Releasing rate/min
First 10	585.16 ± 228.66	585.16 ± 228.65	58.52±22.87
Second 10	379.59 ± 133.88	964.75 ± 347.21	37.96±13.39
* Third 10	214.25 ± 120.61	1179.00 ± 446.09	21.43±12.06*
At 30 – 60	784.93 ± 343.92	1963.93 ± 380.17	21.66±11.46

*Significant difference from first and second 10 min., tested by paired t-test

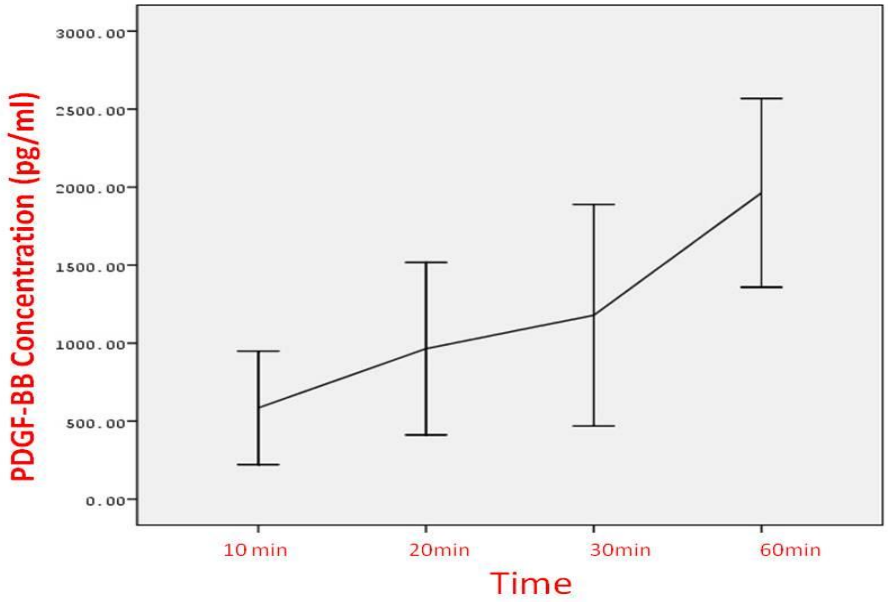


Figure 19: Cumulative mean concentration of leaked PDGF-BB from PRF

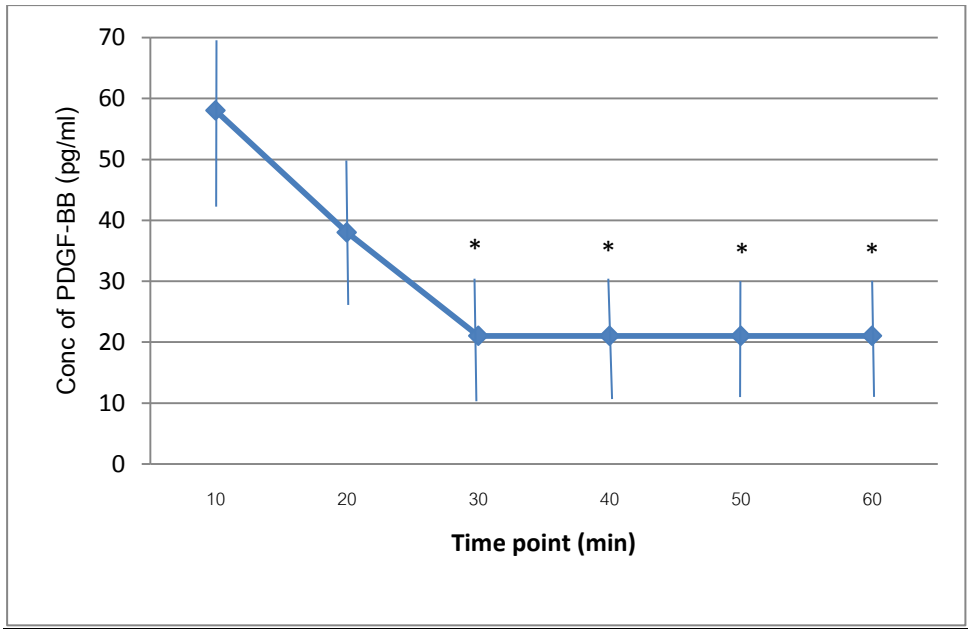


Figure 20: Rate of PDGF-BB releasing at each time point

*Significant difference from the first and the second 10 min., tested by paired t-test

Total PDGF-BB extracted

PDGF-BB extracted from chemical treated with streptokinase

From each volunteer, 30 ml of whole blood was used to prepare 3 pieces of platelet-rich fibrin clot for detection of total PDGF extracted from clot lysis with streptokinase 3 times interval. PRF was treated with 2 ml of 15,000 IU streptokinase and incubated in humidified incubator for 60, 90 and 120 minutes. It was found that the lysis was not complete even at after 120 minutes of streptokinase treated, some PRF fibrin still remained. There was no significant difference of PDGF-BB level at any time point treated with streptokinase. The concentrations of PDGF-BB are shown in Table 12

Table 12 : Concentration of PDGF-BB after lysis of PRF with streptokinase

Time (min)	Mean conc.(pg/ml) N=4	Lysis rate (pg/ml/min)
60	1872.20 ± 960.22	31.20
90	2131.64 ± 564.80	23.68
120	2227.32 ± 566.59	18.56

PDGF-BB extracted from physical treatment with frozen mill

Whole PRF clot was frozen in liquid nitrogen and milled in the plastic tube in a frozen condition. Then PRF was thawed and 2 ml of exudates was used for measuring PDGF-BB level.(Table 13)

Table 13: Concentration PDGF-BB from dispersed PRF with frozen mill

Sample	A	B	C	D	Mean±SD
PDGF-BB level from dispersed PRF (pg/ml)	2507.54	2562.54	2684.81	2213.90	2492.20±199.78

Whole blood serum PDGF-BB

Two ml of whole blood was collected without anticoagulant for measuring level of PDGF-BB in each volunteer (Table 14). The amount of serum PDGF-BB was not difference when compared to PDGF-BB from extracted PRF. Therefore PDGF-BB was presented in both serum and activated platelet

Table 14: Concentration of serum PDGF-BB

Sample	A	B	C	D	Mean±SD
Serum PDGF-BB (pg/ml)	1628.45	2513.00	2318.90	2527.54	2246.97±423.18

The level of PDGF-BB leaked from exudates, from extracted PRF both mechanical and chemical and in the serum were compared (Table15). There was no significant difference of PDGF-BB amount from all methods and serum.

Table 15: The amount of PDGF-BB released from all method

Experiment	Amount of PDGF-BB (Mean±SD)
Leaked from exudates at 60 min	1963.93 ± 380.17
Treated with streptokinase at 120 min	2227.32 ± 566.59
Dispersed with frozen mill	2492.20±199.78
Serum	2246.97±423.18

Correlation of platelet count and PDGF-BB level

There was strong correlation of whole blood platelet count and PRF platelet count (r = 0.86). But correlation between PDGF-BB from four experiments (exudates at 60 min, PDGF-BB from chemical and physical extract and serum PDGF-BB) and platelet count of whole blood and PRF were weak correlated (Table 16)

Table 16: Correlation examination of all experiment and platelet count

Experiment	PDGF-BB	Plt. count whole blood	Plt count PRF	Correlation Whole blood	Correlation PRF
Leaked from exudates at 60 min	1963.93 ± 380.17 pg/ml			-0.27	-0.5
Treated with streptokinase at 120 min	2227.32 ± 566.59 pg/ml	260.25±61.32 X 10^6µm	618.2±104.52 X 10^6µm	0.39	0.52
Dispersed with frozen mill	2492.20±199.78 pg/ml			-0.22	0.29
Serum	2246.97±423.18 pg/ml			0.32	0.36

Chapter 4

Part I

In vitro : Discussion

The blood components of whole blood in our study composed of 94% red blood cell, 5.5% platelet and 0.1% white blood cell. This result was agree with previous study that reported the composition of clot ; 95% red blood cell, 4% platelet, and 1% white blood cell ([11]).The blood composition count of the remaining substance from PRF showed reduced amount of platelets, lymphocytes and white blood cells from whole blood. There were few platelets, lymphocytes and white blood cells left in the remnant after PRF clot was retrieved PRF clot from the tube. In the PRF clot,the concentrations of blood component were increased 2.43, 2.24 and 1.87 folds of platelets, lymphocytes and white blood cells respectively. This result showed that most of platelets, lymphocytes and white blood cells were trapped in the PRF clot.The compositions of PRF clot in this study were 98.55% of platelet, 1.4% of white blood cell and absent of red blood cell. This proportion was comparable to previous platelet rich plasma concentration; 95% of platelet, 4% of white blood cell and 1% of red blood cell ([11]).This result confirms the previous studies([20]) that the concentration of platelets and white blood cells were increased in PRF clot and its proportion seemed to be better than PRP in terms of higher platelets and lower white blood cell concentration. According to the increasing fold, PRP has more increasing folds([2, 11]) , from 3 to 8 folds depending on processing techniques than those get from PRF. It can be explained that 350-450 ml of whole blood has been centrifugeed to get 50-100 ml of PRP whereas PRF use only 10 ml of whole blood to get 4-5ml of PRF. Thus the increasing folds of platelets in PRP are supposed to be 3-8 folds with PRF is 2 folds depending on the initial whole blood used for centrifugation.

This study showed that PRF constituted of the platelet and lymphocyte concentration that released growth factor cytokine and inflammatory cytokine when it

was applied in the surgical site. From previous reports demonstrated the high level of growth factors, PDGF, IGF, VEGF and inflammatory cytokine IL-1, I-6 or TNF-β([15, 20]).

The releasing of PDGF-BB in the exudates leaked from PRF in the first 10 minutes was only 29.89% of the cumulative volume and 23.48% of the total volume of extracted PDGF-BB from PRF with streptokinase and freezer mill. This result agreed with Dohan et al that the initial leak of growth factors during first 10 minutes was minimal and the remained growth factors still entrapped in the fibrin gel (Dohan *et al*., 2006b;Dohan *et al*., 2006c). Our study showed continuous leakage of PDGF-BB but in a slow rate in every 10 minutes to 30 minutes. After 30 minutes the releasing rate is significant lower than first and second 10 minutes. Other studies present more PDGF releasing in the same time point (Table 17). However materials and methods of the study were different. All of other studies determined PDGF in the exudate after PRF were pressed and squeezed into a membrane. Therefore growth factors were forced by a positive pressure to be leaked from the fibrin membrane into the exudates. The quantity of the growth factors were more than spontaneous leakage.Su et al. reported an increasing content of PDGF-AB, TGF-β1, VEGF, and EGF in the PRF exudates over the time course of the study. The content in PRF exudates increased at a constant rate until 60 minutes and then increased in a significant higher level of all growth factors within 120 to 300 minutes ([27]). It inferred that the non-pressed fibrin stored a larger amount of growth factor than the pressed fibrin. In case of using PRF as a membrane, it should be pressed instantly before using and its exudates should be used with grafting material as a mixture

Table 17: Studies of PDGF releasing in the exudates at same time point of this study.

Time (min)	Mean Cumulative conc.(ng/ml)	Study	Present studyMean Cumulative conc.(ng/ml)
10	1.419	Dohan et al 2006	0.585 ±0. 228
20	2.1± 0.4	Dohan et al 2009	0.964 ± 0.347
60	3.8± 0.4	Dohan et al 2009	1.963 ±0. 380
60	34.13± 22.12	Su et al 2009	1.963 ± 0.380

The accumulation volume of leaked PDGF-BB from PRF clot in 60 minutes was not comparable to the total volume extracted by streptokinase or by a freezer mill. Therefore PRF should be used promptly after transferred from the glass tube to preserve most of the growth factors as much as possible. However the leaked exudate or the squeezed exudate from PRF should be used to mix with graft material to aid handling of the graft and utilize the growth factors into the grafted area.

Unlike PRP, PRF is in a clot form after centrifugation. Measuring a quantity of growth factor in fibrin matrix cannot be done directly but the matrix fibrin has to be lysed or dispersed to expose all of growth factors in situ. We tried to extract PDGF-BB from PRF clot by using chemical lysis with streptokinase and physical dispersed with a freezer mill. Streptokinase is a thrombolytic drugthat has been used in several in vitro model studies for clot lytic activity([28, 29]). Prased S. et al used streptokinase as a thrombolytic agent on clot lysis at four different dilutions;30,000 I.U, 22,500 I.U., 15,000 I.U., and 7,500 I.U.) and got the percentage of clot lysis 70.80%, 69.68%, 68.93% and 62.16% respectively. The dilution of 15,000 I.U of streptokinase has been used in this study since it enhanced similar percentage to the higher concentration. Although PRF was incubated with streptokinase for 120 minutes, the PRF clot still remained and was not completely lysed. It is estimated that not more than 70% had been lysed, so there was approximately 30% residual fibrin. Therefore there should be some residual PDGF-BB in the remaining PRF clot after lysis in the same proportion of residual fibrin. The total quantity of growth factors should be more than that had been measured in this study for another 30%.All of incubated time 60, 90 and 120 minutes, the level of PDGF-BB extracted were similar.

This present study had extracted growth factors from frozen PRF by using a freezer mill. By this method, the PRF was frozen and milled in liquid nitrogen then left it thawed. The thawedsolution was not homogeneous but mix with thecolloid of PRF.The previous study used homogenizer extraction-dispersing machine(20). The amount of growth factor, PDGF-AB, was 32000±3500pg/ ml that seemed to be more than our study, 2492.20±199.78 pg/ml of PDGF-BB. Our method might not be able todispersed the PRF clot totally.

According to PDGF-BB level from all methods in this study, there was no difference among slow releasing in the exudates, chemical, physical extraction of PRF and whole blood serum. Therefore PDGF-BB was presented in both platelets and serum. This observation was confirmed by analysis of correlation between PDGF-BB amount from all experiments and the platelet count. It was found that there was no correlation between PDGF-BB level and platelet count in all subject.Thus PDGF-BB might not be a good representative growth factor for studying, other growth factor such as TGF-β and VEGF should be explored.

Chapter 5

Part II

In vivo : Materials and methods

Animal model

This study was performed in accordance with the regulations and approval of the animal experiment ethic committee of Prince of Songkla University. Four minipigs aged 18 months, weighted 50-60 kgs. were used and the surgeries were conducted at the Animal Science Unit, Faculty of Natural Resources, Prince of Songkla University HatYai, Songkhla, Thailand.

Group of the study

Four minipigs were divided in 2 groups of follow-up time 2- and 6- week period, two minipigs per each group. There were 3 experimental sinuses that used platelet rich fibrin and 1 control sinus that used blood clot in sinus augmentation procedure (figure 28).

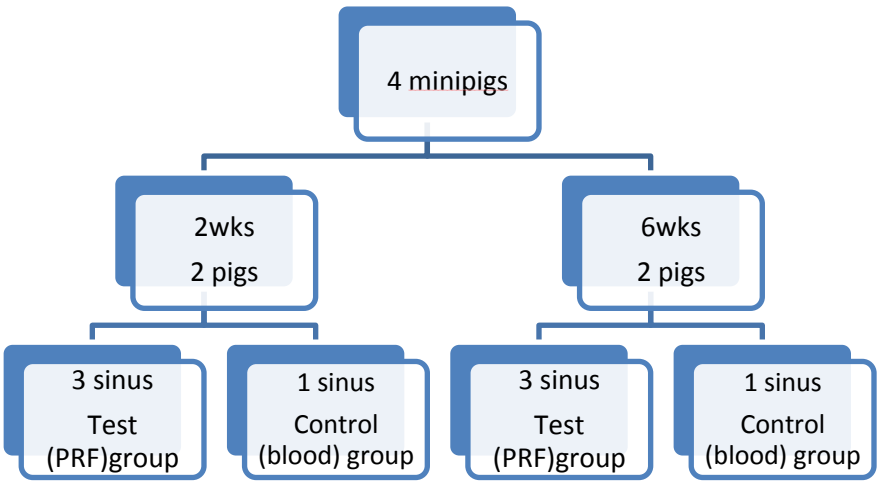


Figure 21: In vivo groups of study

Surgical technique

The minipig was placed on the surgical table in a prone position. Anesthesia was induced with intramuscular administration of azaperone, (Stresnil®), 1mg/10kg by weight and then intravascularly with combination of the tranquilizer, zolazepam, with the dissociative anesthetic, tiletamine (Zolitil®)1ml/20kg by weight. Before surgery antibiotic, amoxyxillin (Vertrimoxin®) was given 1g intramuscularly for prophylaxis of infection. General anesthesia was supplemented by local administration of 4% articaine containing epinephrine (1:100,000). All surgical procedures were performed under aseptic conditions by the same surgical team. When the animal was in the level of anesthesia, before surgery, the skin was cleaned with providone iodine. One sinus was picked up. The facial maxillary sinus was exposed bilaterally through an approximately 5 cm-long extraoral skin incision below the lower eyelid (Figure 22). The periosteal flap was raised to expose the anterior sinus wall, the zygomaticoalveolar crista and the malar prominence. A15 mm. in diameter window was outlined with template and drilled with round bur below the malar prominence (Figure 23). The bone window was carefully detached from the sinus membrane and kept in moisten gauze soaked with normal saline (Figure 24). Then the Schneiderian's membrane was carefully elevated from the underlying bony sinus wall by blunt instrument to avoid perforation. On the test sinus, platelet-rich fibrin,as space maintainer material, was put into the created subantral cavity then the bone window was re-positioned on platelet-rich fibrin. On the control site, the same volume of autogeneous blood clot was placed in the created cavity in the same manner as in the test side (Figure 25). Finally the periosteum and skin flap was reposition and sutured in layer by layer.

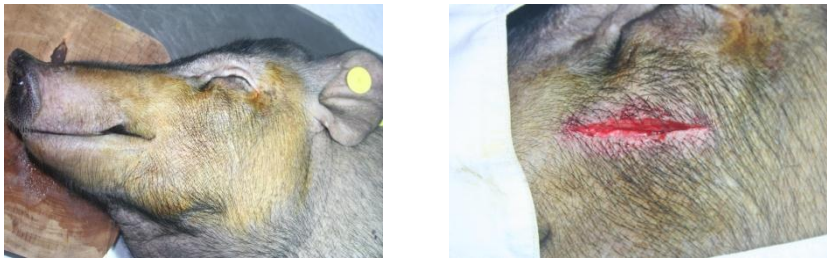


Figure 22: Incision line 5 cm-long extraoral below the lower eyelid

Figure 23: Bone window diameter 15mm. was outlined and drilled with round bur

Figure 24: The bone window was detached from the sinus membrane

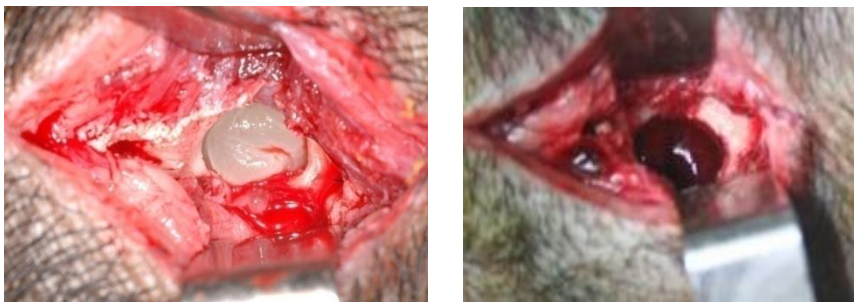


Figure 25: The sinus membrane was elevated from the underlying bony sinus wall. Autogeneous blood clot was used to fill the lifted space as a control whereas the platelet-rich fibrin was used as an experiment.

Platelet-rich fibrin preparation

The 10 ml of autologous whole blood was drawn from the ear vein or the radial vein and put in a sterile glass tube without anticoagulant. Then the whole blood was immediately centrifuged at 3000 rpm for 10 minutes. A fibrin clot obtained in the middle of the tube was used. The volume of PRF was measured by replacement of known volume of water in the same glass tube.

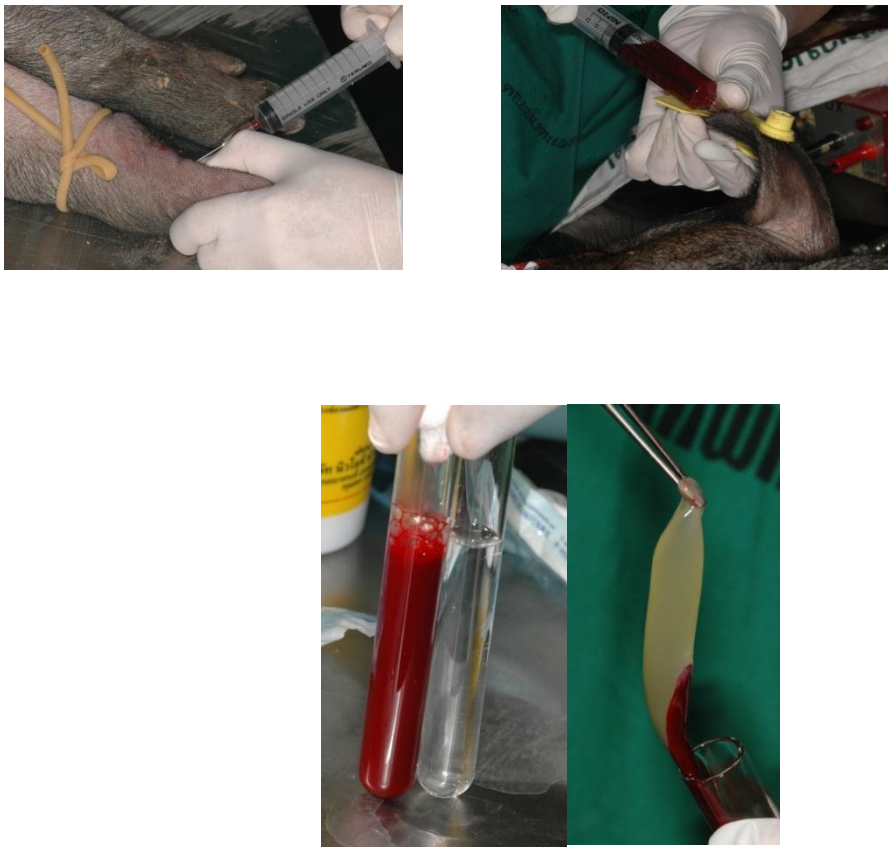


Figure 26: PRF preparation of minipigs

Follow-up

The animals were sacrificed by a natural way at 2 and 6 weeks after surgery.

Tissue processing

Gross evaluation

After sacrifice, the maxillas were resected. The soft tissue was carefully removed and then the maxilla was separated at midline by electric saw. Gross observation was done and focused on the bony window of the maxillary sinus augmentation (figure 27). Then the specimens were fixed in 10% formalin solution and submitted to radiographic and microscopic analysis for histomorphometric analysis.

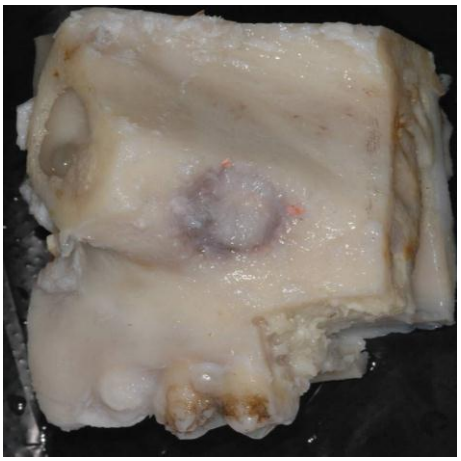


Figure 27: Gross specimen

Cone beam CT

Each sinus was examined by using cone beam CT (Veraviewepocs3D, X550,J.morita,Japan) in size 40 x 80 mm.80kV,5mA.9.4 min. with high-resolution protocol (slice thickness,1.5 mm.) Each file of sinus was used to calculated the volume of content in the created space underneath the sinus membrane.

Figure 28: Cone beam CT of each sinus

Histologic analysis

Following radiography, the specimen was cut through the highest point of augmentation site. Then the specimen was decalcified with EDTA which was subsequently changed every 2 days and agitated at 160 rpm on an Orbital shaker (KS 130 Basic Orbital Shaker,IKA Works,USA). Complete decalcification was confirmed by

the absence of a precipitation in the supernatant when a solution of saturated ammonium oxalate was added. After complete decalcified, the specimen was placed into disposable plastic cassette. An automatic tissue processor (Lipshaw automatic tissue processor model 2500A, Lipshaw, USA) was used to provide programs for fixing, dehydrating, clearing and paraffin impregnating. Serial sections of 5 μm were cut using a microtome (Leica Model RM2135, Leica Microsystems,Germany), then stained with hematoxylin and eosin (H&E) using with an automated tissue stained (Shandon LinistainTM GLX Linear Stainer, Thermo Shandon Inc, USA.). All slides were examined descriptively for detection of new bone formation in the lifting space before histomorphometric analysis.

Histomorphometric analysis

Computer-assissted histomorphometry was performed in order to measure the amount of newly formed bone in the augmented area. The entirely augmented area at the middle section of the specimen was used for histomorphometric analysis. All specimens were evaluated in a blinded, nonbiased using and image analysis system by measuring the percentages of newly deposited bone. The entire slide was automate scan by ScanScope® CS system (Aperio, Vista, CA). These were digitized and transferred into computer software,ImageScope™, for image processing and analyzing the quantify fraction of the total area in the experimental and control sites. The percentage of bone area in the experimental and control site were calculated as follow

Percent bone area = (Mineralized bone area X 100) / Total augmented area

Data analysis

The mean volume of content in created space (ml) from cone beam CT was compared to the volume of PRF and blood clot used in the lifted sinus. The mean total graft area in histomorphometry analysis was described with area of new bone formation.

Chapter 6

Part II

In vivo : Result

Four minipigs (eight maxillary sinuses), 18 months of age, weighing 50-60 kgs were used in the present study. All animals were well tolerated with the surgical procedure and the anesthesia. They recovered after surgery without any infection or wound complication in the surgical area. No sign of acute and chronic sinusitis was observed.

The volume of PRF used to lift sinus membrane was 4.5±0.5 ml in the test group and the volume of blood clot was 4 ml in the control sinus in 2 week group

The volume of PRF used to lift sinus membrane was 4.27±0.25 ml for test group and blood clot 4 ml for control sinus in 6 week group

Gross specimen

In 2-week group: Both control and test groups, the bone windows were filled with rubbery tissue. No any hole or perforation at the bone window. The size of bone windows was not different from the original size. The border of bone windows were able to define as a continuing line. The window bones were presented in the cavities and fused with surrounding soft tissue (Figure 29).

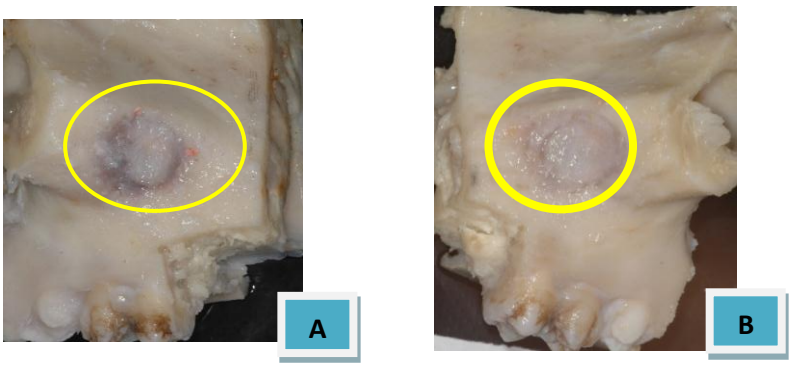


Figure 29: Bone window area of 2 weeks group, blood clot (A) and PRF (B)

In 6- week groups: Both PRF group and blood clot group presented the same features. The border of bone window cannot be defined in the lower part of the window. At these areas bone were bridged but not evenly all over the window. The area of interest was hard in consistency and was not able to be pressed. The color were homogenized with the surrounding bone. (Figure 30).

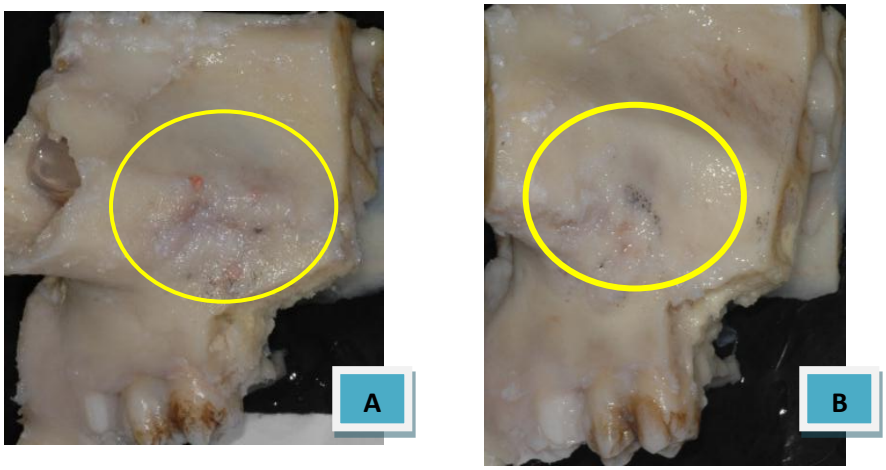


Figure 30: Bone window area of 6 weeks group, blood clot (A) and PRF (B)

Cone beam CT examination

After specimens were dissected, all of maxilla were examined with cone beam CT at the same condition.

2-week group

Platelet-rich fibrin still remained in the lifted space. The volume of PRF was decreased 25.11% (from 4.5±0.5 ml. to 3.37±1.16 ml).The density of the content was similar to infraorbital nerve density .For blood clot group, the same feature was presented as in the PRF group. The volume of blood was decrease 25% (from 4ml to 3.02ml). The volume and density of both PRF group and blood clot group were not different. (Figure 31).

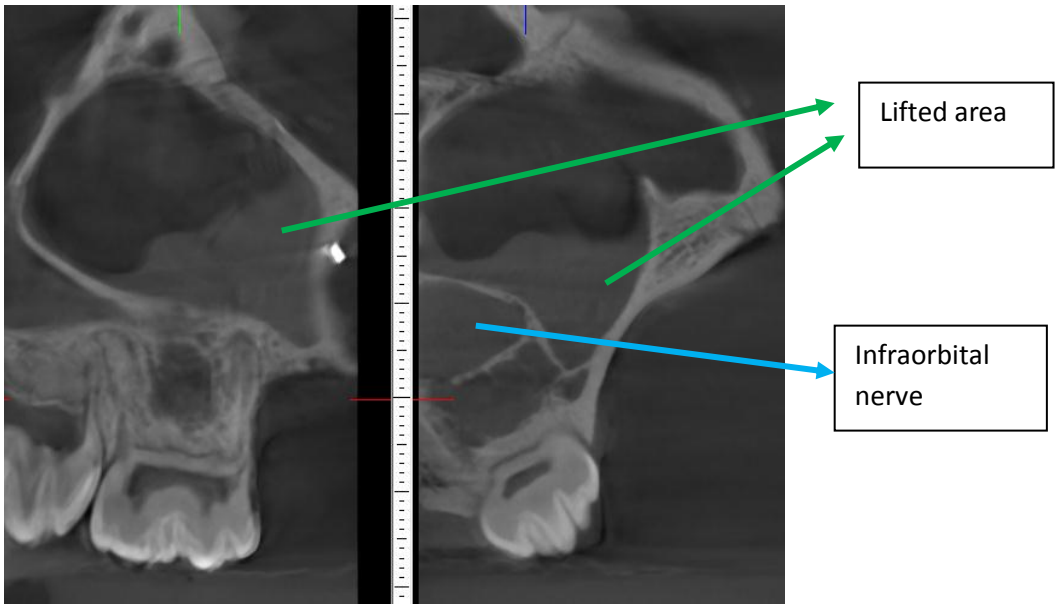


Figure 31A: CBCT of sinus lift with blood clot in sagital and coronal view in 2-week group

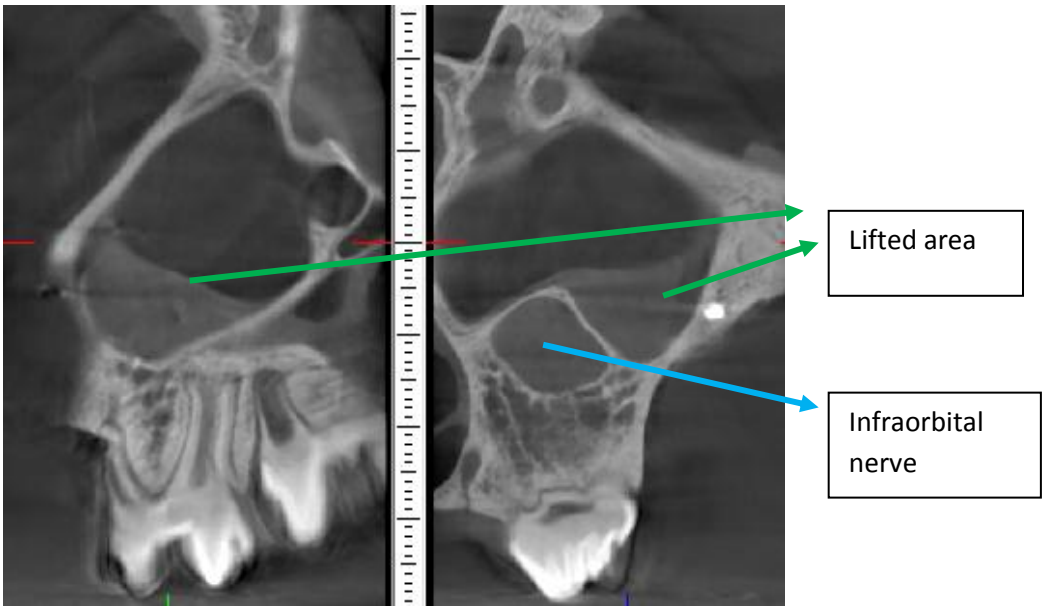


Figure 31B: CBCT of sinus lift with PRF in sagital and coronal view in 2-week group

6-week group

The volume of PRF in the lifted space was decreased markedly when compared to the original size, from 4.27±0.25 ml to 0.33±0.02 ml.(93.01%) Density of the content was similar to the surrounding bone. In blood clot group, present the same feature as PRF group. The volume of blood was decrease 92% (from 4ml to 0.38 ml). The volume and density of both PRF group and blood clot group were not different (Figure 32).

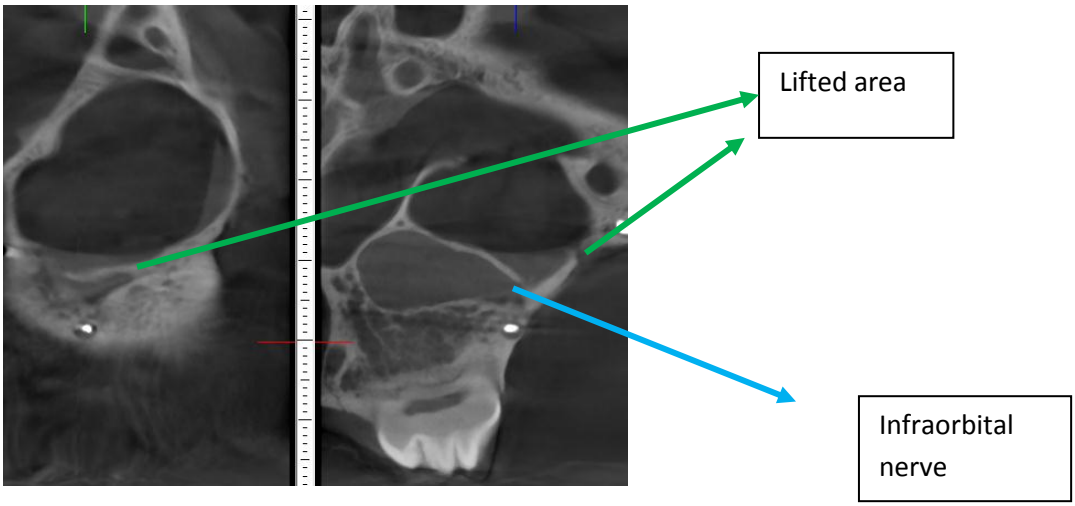


Figure 32A: CBCT of sinus lift with blood clot in sagital and coronal view in 6-week group

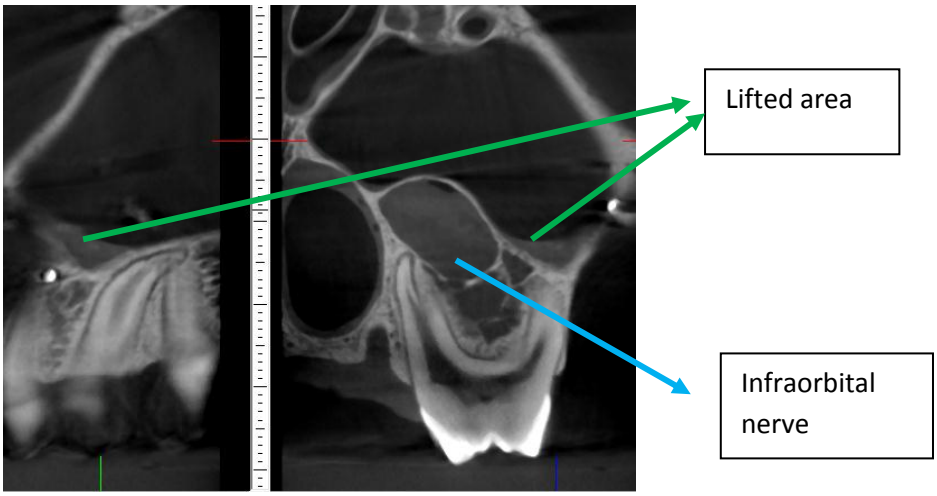


Figure 32B: CBCT of sinus lift with PRF in sagital and coronal view in 6-week group

Histology observation

The normal sinus lifted cavity was surrounded by respiratory mucosa and a cortical bone of sinus floor. The mucosa was composed of ciliated stratified cuboidal epithelium, submucosal tissue

In 2 weeks group

A large area of content underneath the sinus membrane blood clot group ($67.32x10^6$ μm^2) was presented . There was few newly formed bone projected from the bone of maxillary sinus floor. The area of new bone formation was $0.72\ x10^6$ μm^2. It was 1.07% of total lift area. The height of new bone from native bone was 220.70±28.55 µm. The cement line was observed between the newly form bone and floor of maxillary sinus. Woven bone with many osteoblast were observed.

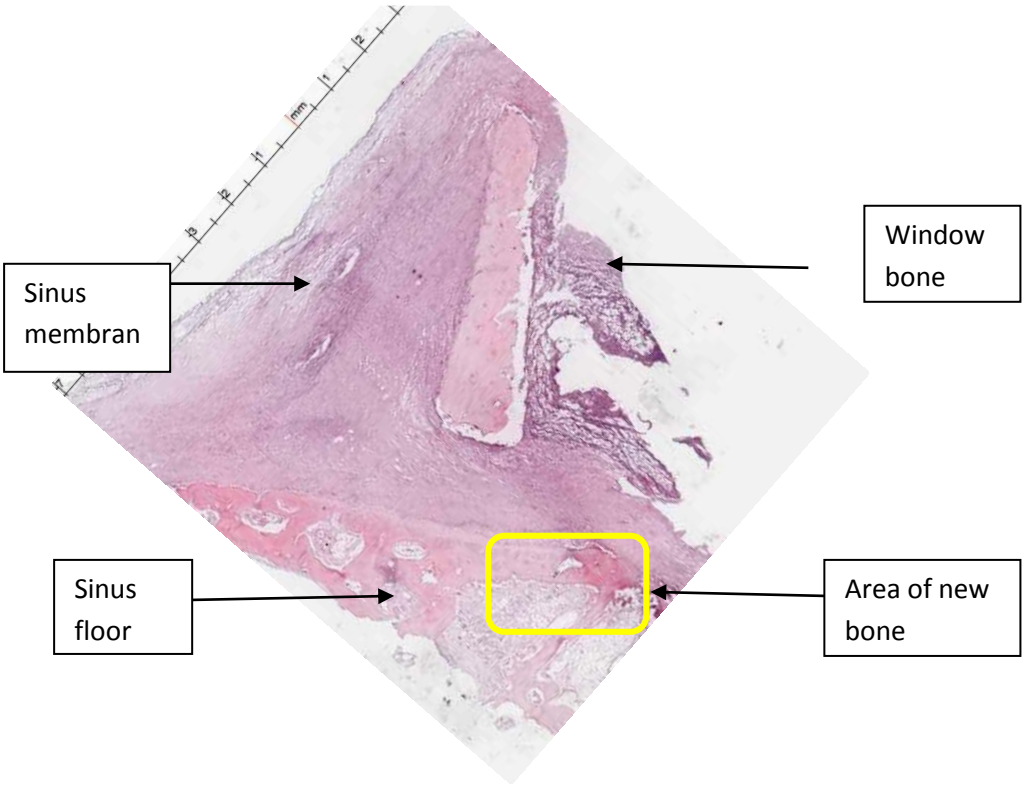


Figure 33: Histological finding of lifted sinus with blood clot at 2 weeks (1x)

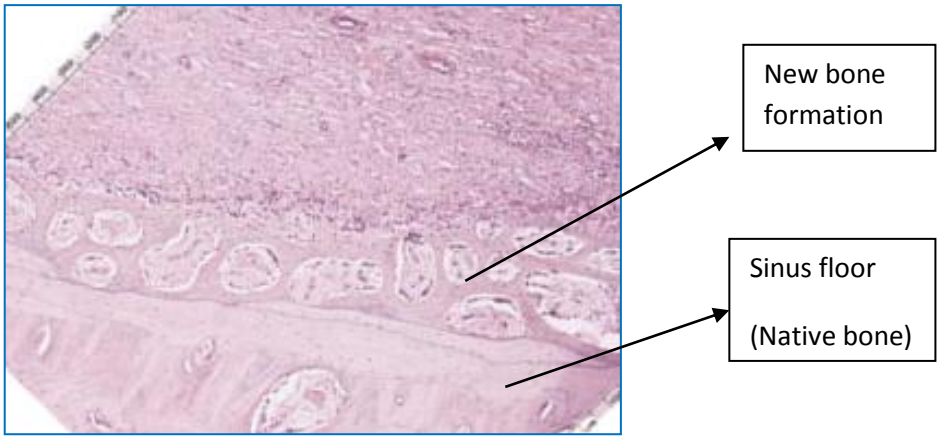


Figure 33A: New bone formation area of blood clot at 2 weeks (4x)

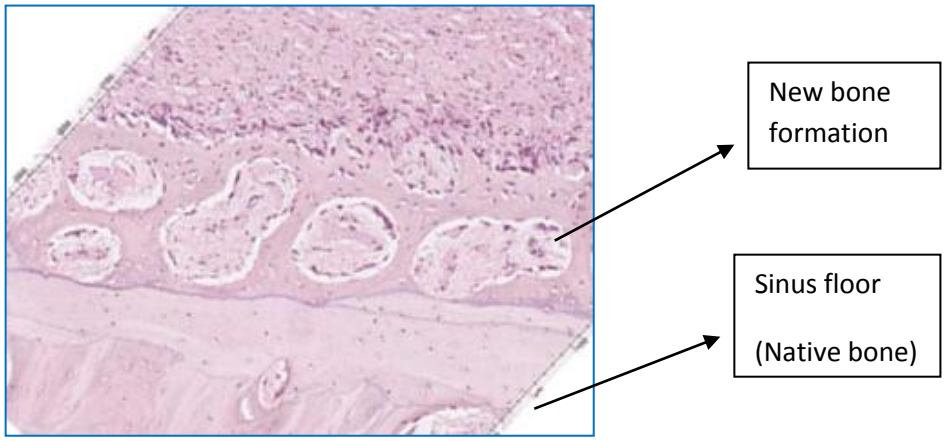


Figure 33B: New bone formation area of blood clot at 2weeks (8x)

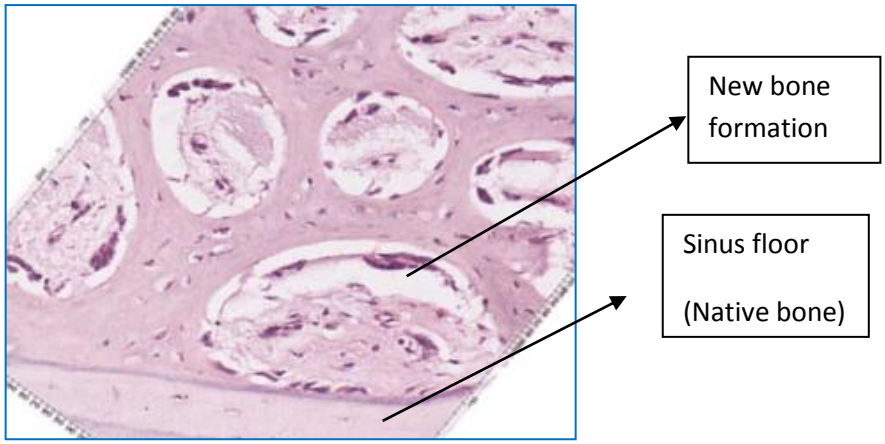


Figure 33C: New bone formation area of blood clot at 2 weeks (20x)

In PRF group, the mean of total area under the sinus membrane was 113.18±58.81 x10^6 μm^2. The mean of height of new bone from native bone was 911.66±343.42 μm. The area of newly form bone was more than in blood clot group. There was newly formed bone projected from bone of maxillary sinus floor. The area of new bone formation was 7.72 ±0.19x10^6 μm^2. It was 6.82% of total lift area, more than blood clot group(1.07%) (Figure 42). Statistic analysis cannot be performed because there was only 1 control sinus.

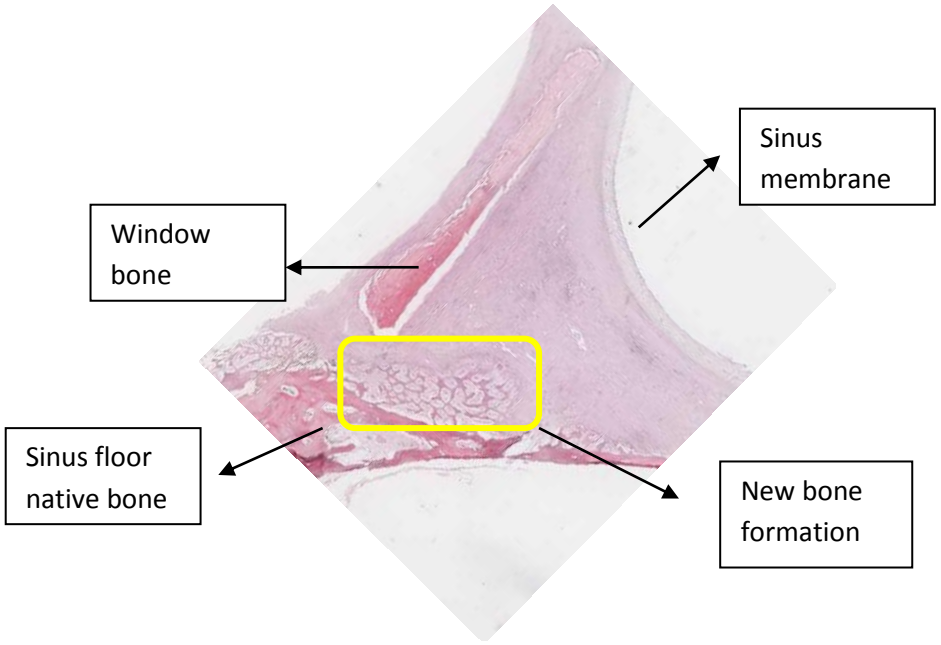


Figure 34: Histological finding of lifted sinus with PRF at 2 weeks (1x)

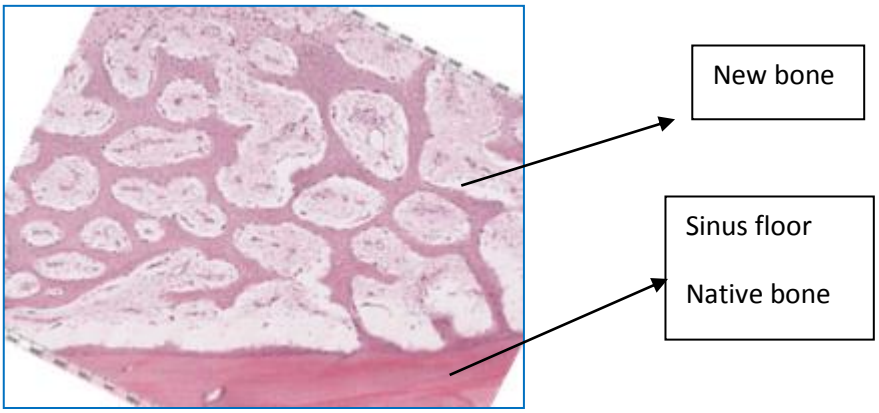


Figure 34A : New bone formation area of PRF at 2 weeks (4x)

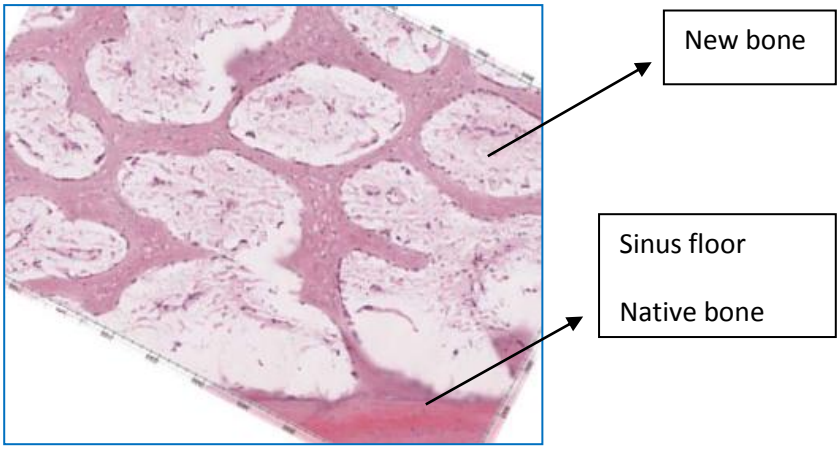


Figure 34B: New bone formation area of PRF group at 2weeks (8x)

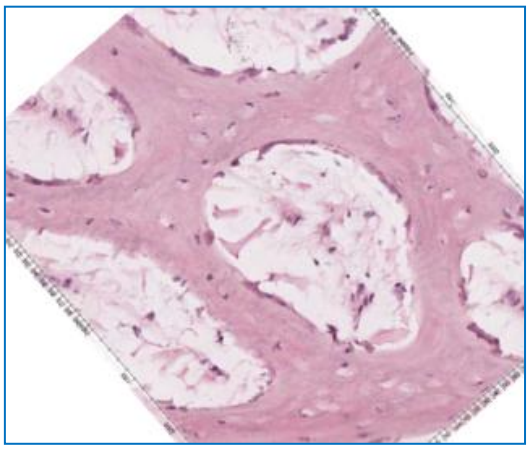


Figure 34C: New bone formation area of PRF group at 2weeks (20x)

In 6 weeks group:

The area under the elevated sinus membrane was decrease 36.36% in blood clot group, ($42x10^6$ μm^2). The newly formed bone was found adjacent to sinus floor and remodel to form the lamella bone. The area of new bone formation was 13.55 $x10^6$ μm^2. It was 31.62% of total lift area. The bone formation area was increase 178% from 2 weeks period.

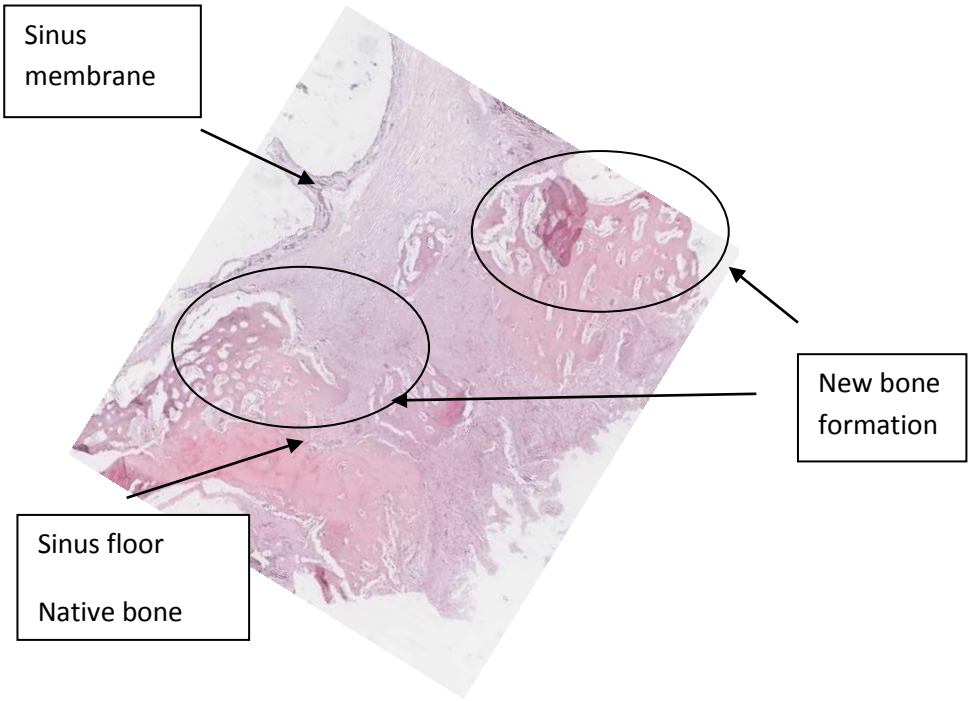


Figure 35: Histological finding of lifted sinus with blood clot at 6 weeks

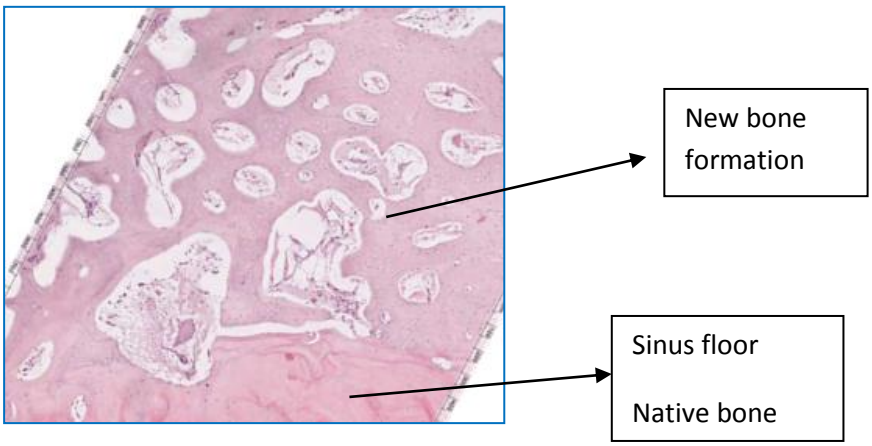


Figure 35A : New bone formation area of blood clot at 6 weeks (4x)

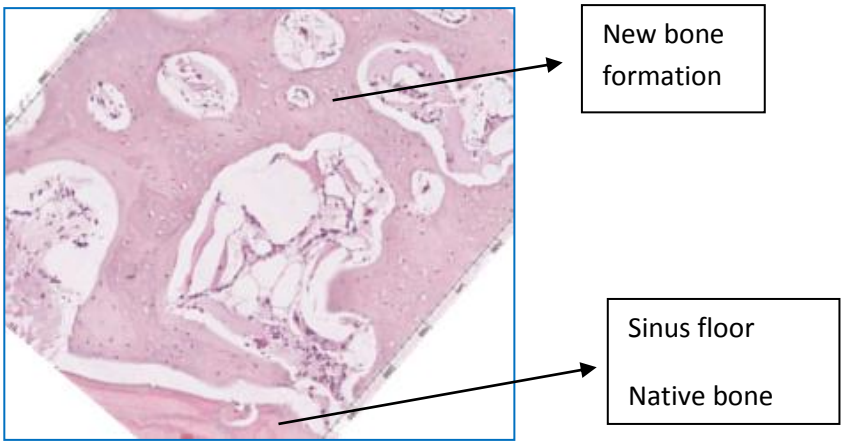


Figure 35B : New bone formation area of blood clot at 6 weeks (8x)

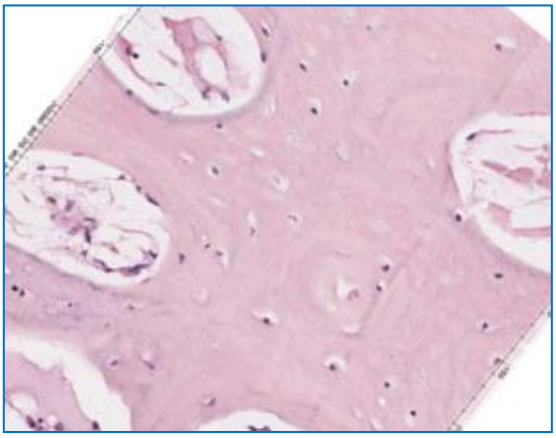


Figure 35C : New bone formation area of blood clot at 6 weeks (20x)

In PRF group, the total lift area was decrease 47.35% (51.84±12.56 $x10^6$ μm^2) from 2-week group. The newly formed bone was found adjacent to the sinus floor and remodelled to form the lamella bone. The area of new bone formation was 24.55±9.76 $x10^6$ μm^2. It was 47.36% of total lift area. The bone formation area was increase 217.85 % from 2 weeks period.

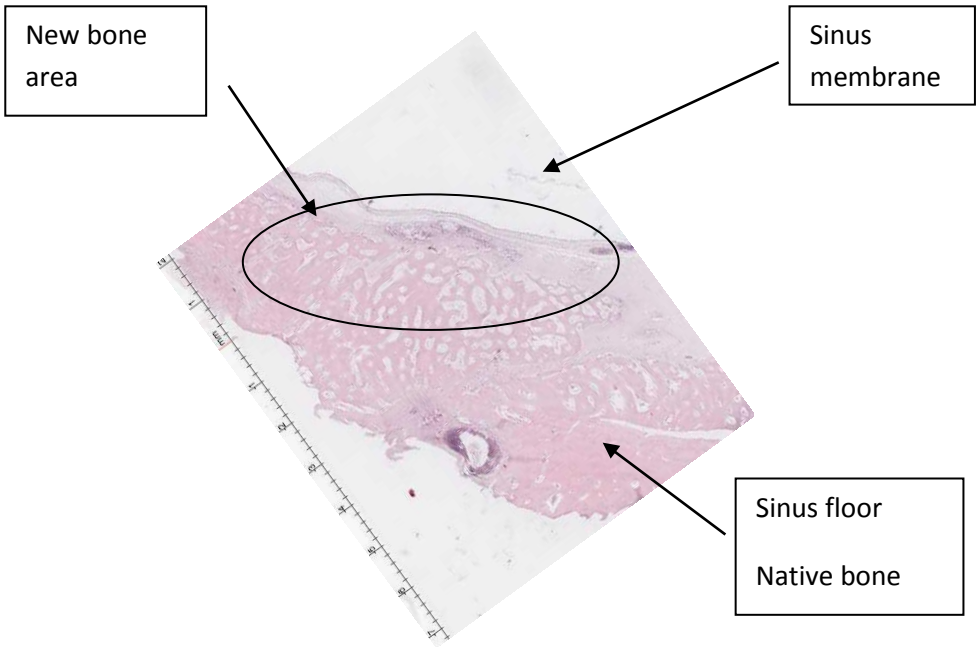


Figure 36: Histological finding of lifted sinus with PRF at 6 weeks (1x)

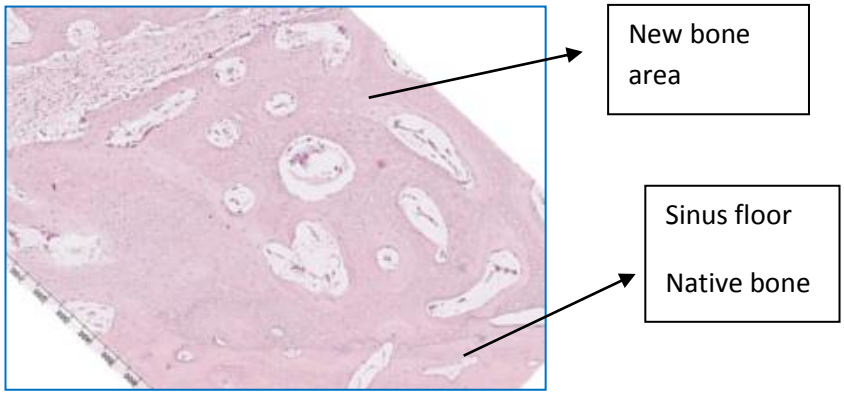


Figure 36A : New bone formation area of PRF group at 6 weeks (4x)

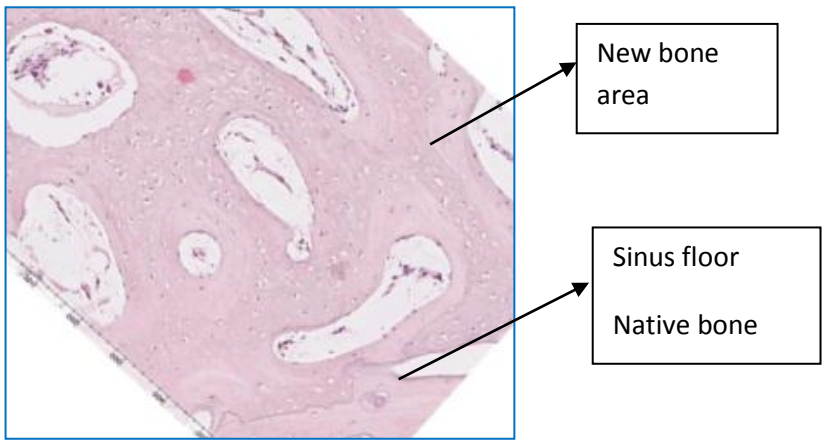


Figure 36B : New bone formation area of PRF group at 6 weeks (8X)

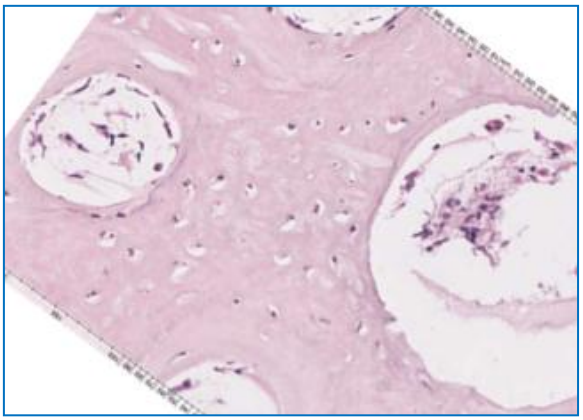
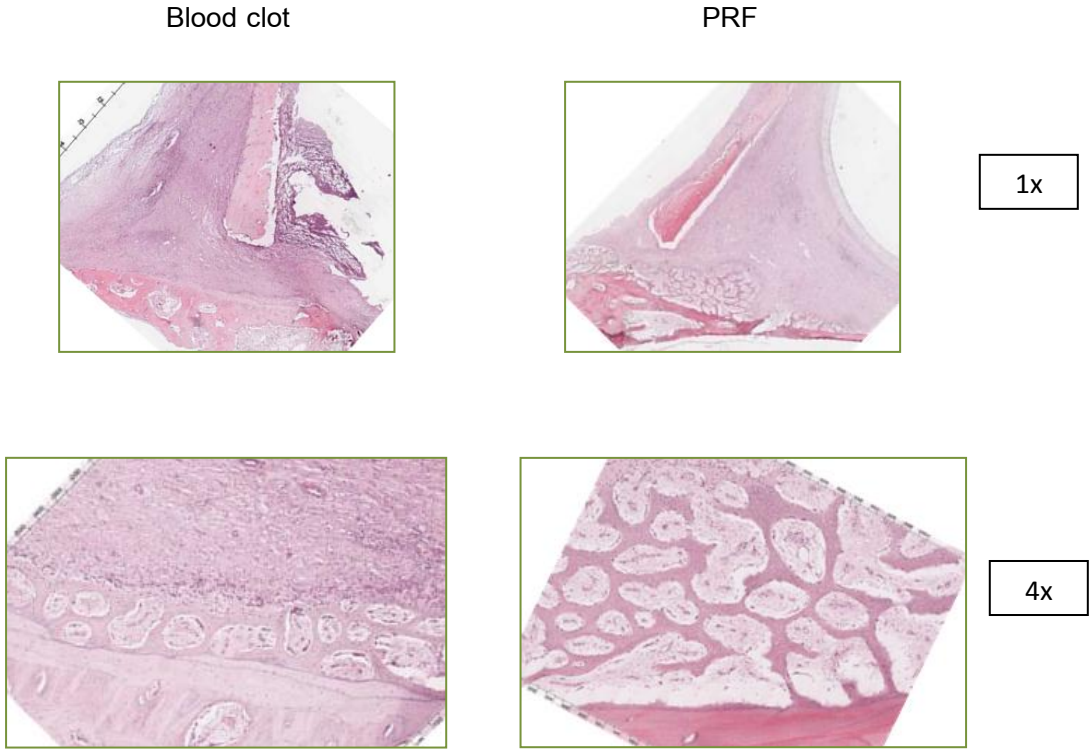


Figure 36C : New bone formation area of PRF group at 6 weeks (20x)



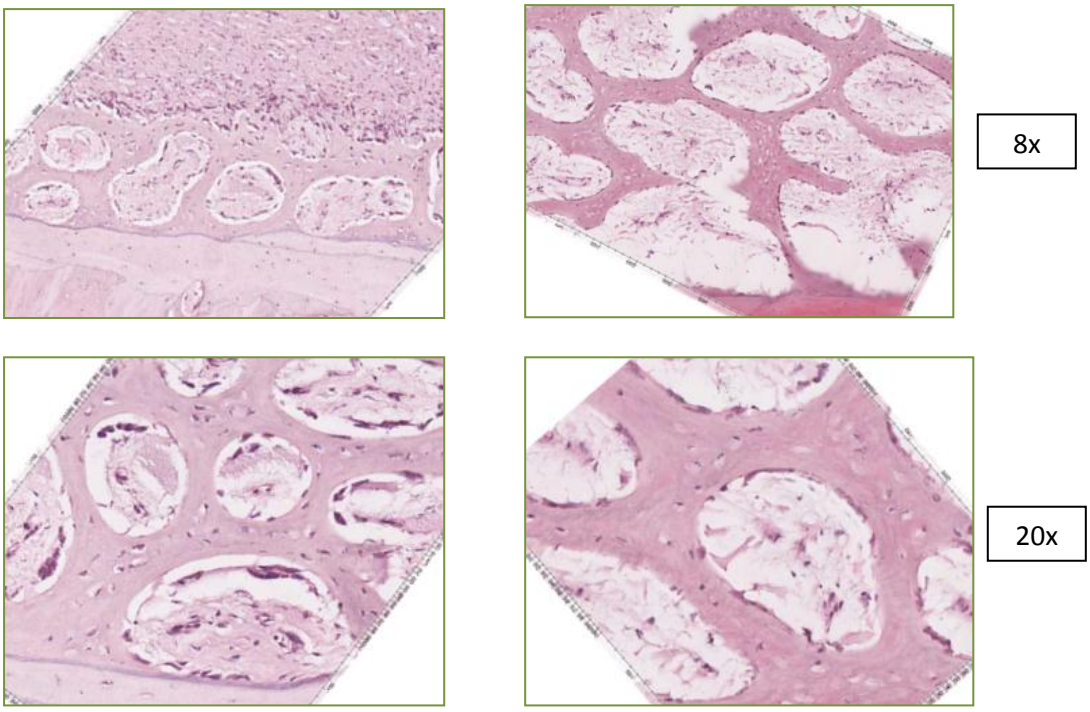
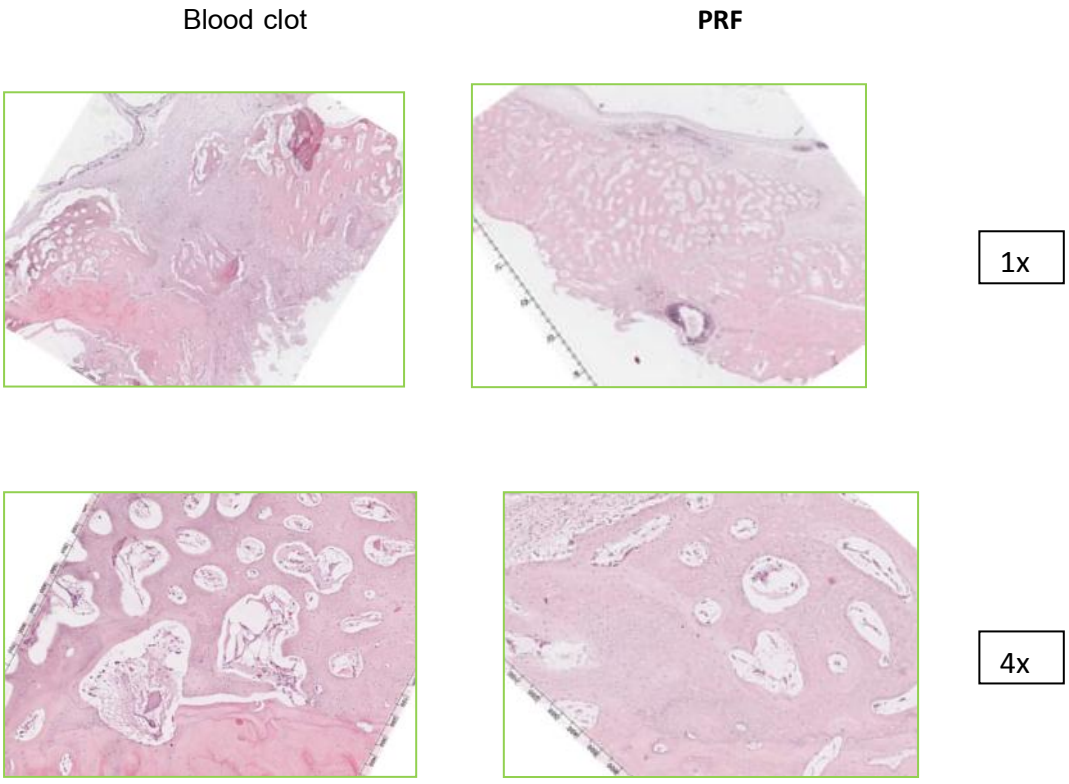


Figure 37 : Compare histologic feature of 2-week group



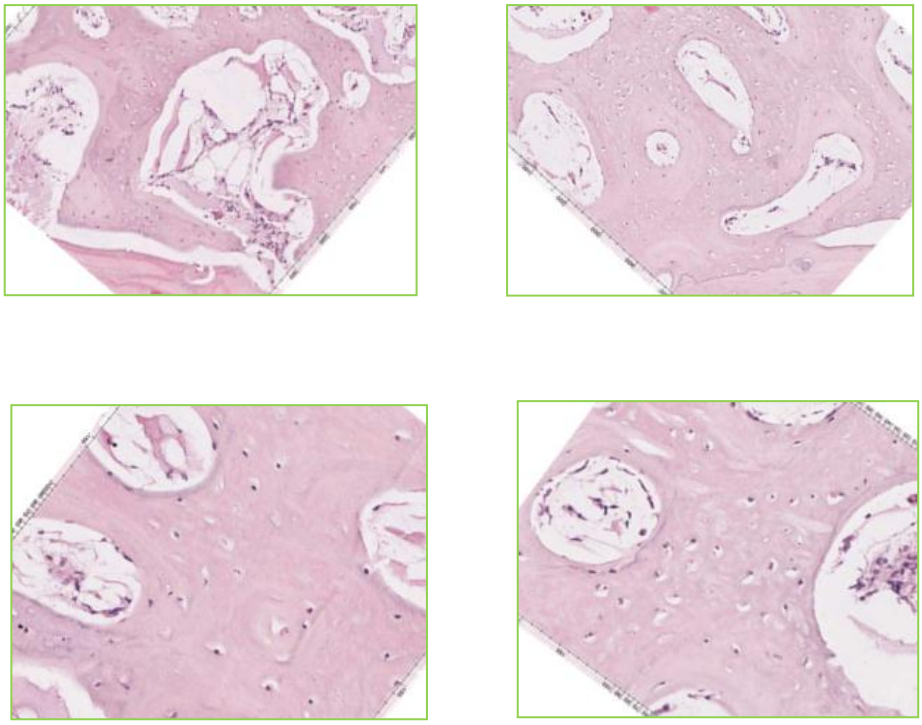


Figure 38: Compare histologic feature of 6-week group

Chapter 7

Part II

In vivo : Discussion

To determine the effect of sinus augmentation on bone formation in sinus cavity, an acceptable animal model was necessary. Few animals possess a maxillary sinus as large as or an osseous antral wall as thick as that of human. The minipig is chosen as a model for this experiment because its bone regeneration rate (1.2-1.5 μm/day) is comparable to that of human being([30]). Minipigs were first used as an animal model in 1993. They found them to be quite similar to human in terms of platelet count, clotting parameters and long bone structure. The structure of maxillary bone was also reported to be quite similar to that in human([31]).Previous studies have advocated the use of minipigs for studying the sinus augmentation([32-34])

In maxillary sinus augmentation, the cambium layer of periosteam under sinus epithelium presentedmesenchymal stem cells that had potential to bone regeneration in lifted area underneath the sinus membrane ([35]). Many studies reported bone formation around implants when simultaneous installed without grafting material or filled with blood clot ([23-25, 36]).After PRF was introduced, there were studies about the effect of PRF in sinus augmentation that PRF alone without bone substitutes could produce bone formation around the implant ([18, 37]).This condition required an implant to stabilize the lifted space by pushing the sinus membrane to the highest position and using implant tip as a tent poles. Our study did not use any material Ito be the tent pole except PRF then the result of bone formation came from PRF solely.

The bone window of maxillary sinus in 2-week group were similar in both blood clot group and PRF group. Well definite border was found and window bone can be retained on blood clot and PRF and acted like a barrier membrane.PRF clot still remained in the lifted space of minipigs' maxillary sinuses in the distinct volume in the first 2 weeks. The volume of both blood clot and PRF was decreased only 25 % in 2 weeks after surgery. No hard tissue regeneration was found in the lifted space by naked

eyes observation nor in cone beam CT. This result was similar to histomorphometric study. In H&E staining, there were a large area of content in both PRF and blood clot group. The previous report showed the SEM evaluation of blood clot and PRF clot that fibrin of PRF was clearly organized in parallel strands and appear thicker and denser than blood clot([16]). Therefore the resorption time of PRF might be longer than blood clot and it could maintain the space for a longer time. Our study shows that in 1 month period (from week-2 to week-6), area of lifted sinus in blood clot group was reduced 36.36% and in PRF group reduced47.35%. Even though PRF group might resorb more than blood clot group, the blood clot group had only 1 sinus that statistic analysis cannot be performed.

The initial period of 14 days is crucial for neovascularization to take place and leads to initial bone healing process. Furthermore previous study of Dohan et al showed that PRF clot released high quantities of three main growth factors, transforming growth factorb-1(TGFb-1), platelet derived growth factor AB (PDGF-AB), vascular endothelial growth factor (VEGF) and thrombospondin-1(TSP-1)during7days([20]). PRF clot demonstrated the increasing of proliferation and differentiation of rat osteoblats (38)and human oral bone mesenchymal stem cells in a dose dependent way ([39]). Bone formation in our study was found in both blood clot and PRF group in the early stage in 2-week group and more mature bone in 6-week group particularly in PRF group. The areas of bone formation in PRF group were more than in blood clot group in both 2 and 6 weeks period. Bone formation was increase 217.85% in blood clot and 417.8% in PRF group. The height of new bone projected from native sinus bone was used to evaluate the rate of bone formation. PRF group showed higher rate, 65.12 μm/day, in first 2 weeks than in blood clot group, 15.76μm/day. According to bone regeneration rate of minipig,1.2-1.5 μm/day([30]), in this study showed more regeneration rate even in blood clot group. It might be because of the high efficacy of maxillary sinus chamber in bone regeneration.

In theory, new bone formation could be generated underneath the sinus membrane because there are mesenchymal stem cells from the membrane. Our study found that bone formation started at the basal bone or the sinus floor. This feature was

found in gross specimen that presented bone bridging with homogeneous texture at the bottom of bone window in 6-week group. Histomophologic feature confirmed this observation that new bone formation was projected from the sinus floor bone not from sinus membrane. The basal bone without periosteam might have high potential to produce new bone formation.

The sinus lift without grafted bone material with simultaneous implant is a very simple and an attractive approach ([24, 40]). The true final bone gain at the implant end may be covered with a thick sinus connective tissue without osteointegration [41, 42]). To increase the predictability and security of this procedure, PRF was advocated to use as the graft material for protection of sinus membrane collapse and improve osteointegration around the fixture.

Chapter 8

Conclusion

In conclusion, PRF sustained slow releasing of growth factor, PDGF-BB, during ex-vivo period for 60 minutes and the cumulative amount of PDGF-BB was comparable to the total amount extracted from streptokinase lysis, frozen mill and in the serum. PRF was not resorbed and still retained in the body for at least 2 weeks period of initial wound healing and lost its volume in 6 weeks period. PRF is able to promote bone regeneration in sinus augmentation procedure more than blood clot. PRF could be used in sinus grafting wherever simultaneous implant installation can be done and space could be maintained during bone healing is in progress.

Reference list

1. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003 Jul; 83(3): 835-70.
2. Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. *Plast Reconstr Surg* 2004 Nov; 114(6): 1502-8.
3. Hooshmand-Rad R, Lu L, Heldin CH, Claesson-Welsh L, Welsh M. Platelet-derived growth factor-mediated signaling through the Shb adaptor protein: effects on cytoskeletal organization. *Exp Cell Res* 2000 Jun 15; 257(2): 245-54.
4. Harrison P, Cramer EM. Platelet alpha-granules. *Blood Rev* 1993 Mar; 7(1): 52-62.
5. Marx RE. Platelet-rich plasma: evidence to support its use. J *Oral Maxillofac Surg* 2004 Apr; 62(4): 489-96.
6. Grageda E. Platelet-rich plasma and bone graft materials: a review and a standardized research protocol. *Implant Dent* 2004 Dec; 13(4): 301-9.
7. Andrew JG, Hoyland JA, Freemont AJ, Marsh DR. Platelet-derived growth factor expression in normally healing human fractures. *Bone* 1995 Apr; 16(4): 455-60.
8. Gibble JW, Ness PM. Fibrin glue: the perfect operative sealant? *Transfusion* 1990 Oct; 30(8): 741-7.
9. Liu Y, Kalen A, Risto O, Wahlstrom O. Fibroblast proliferation due to exposure to a platelet concentrate in vitro is pH dependent. *Wound Repair Regen* 2002 Sep-Oct; 10(5): 336-40.
10. Anitua E, Sanchez M, Nurden AT, Nurden P, Orive G, Andia I. New insights into and novel applications for platelet-rich fibrin therapies. *Trends Biotechnol* 2006 May; 24(5): 227-34.
11. Mehta S, Watson JT. Platelet rich concentrate: basic science and current clinical applications. *J Orthop Trauma* 2008 Jul; 22(6): 432-8.
12. Boyapati L, Wang HL. The role of platelet-rich plasma in sinus augmentation: a critical review. *Implant Dent* 2006 Jun; 15(2): 160-70.

13. Sunitha Raja V, Munirathnam Naidu E. Platelet-rich fibrin: evolution of a second-generation platelet concentrate. ***Indian J Dent Res*** 2008 Jan-Mar; 19(1): 42-6.

14. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2006 Mar; 101(3): e37-44.

15. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part II: platelet-related biologic features. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2006 Mar; 101(3): e45-50.

16. Dohan Ehrenfest DM, Del Corso M, Diss A, Mouhyi J, Charrier JB. Three-dimensional architecture and cell composition of a Choukroun's platelet-rich fibrin clot and membrane. ***J Periodontol*** 2010 Apr; 81(4): 546-55.

17. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, 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 Mar; 101(3): e51-5.

18. Diss A, Dohan DM, Mouhyi J, Mahler P. Osteotome sinus floor elevation using Choukroun's platelet-rich fibrin as grafting material: a 1-year prospective pilot study with microthreaded implants. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2008 May; 105(5): 572-9.

19. Oprea WE, Karp JM, Hosseini MM, Davies JE. Effect of platelet releasate on bone cell migration and recruitment in vitro. ***J Craniofac Surg*** 2003 May; 14(3): 292-300.

20. Dohan Ehrenfest DM, de Peppo GM, Doglioli P, Sammartino G. Slow release of growth factors and thrombospondin-1 in Choukroun's platelet-rich fibrin (PRF): a gold standard to achieve for all surgical platelet concentrates technologies. ***Growth Factors*** 2009 Feb; 27(1): 63-9.

21. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part V:

histologic evaluations of PRF effects on bone allograft maturation in sinus lift. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006 Mar; 101(3): 299-303.

22. Linde A, Thoren C, Dahlin C, Sandberg E. Creation of new bone by an osteopromotive membrane technique: an experimental study in rats. *J Oral Maxillofac Surg* 1993 Aug; 51(8): 892-7.
23. Hatano N, Sennerby L, Lundgren S. Maxillary sinus augmentation using sinus membrane elevation and peripheral venous blood for implant-supported rehabilitation of the atrophic posterior maxilla: case series. *Clin Implant Dent Relat Res* 2007 Sep; 9(3): 150-5.
24. Lundgren S, Andersson S, Gualini F, Sennerby L. Bone reformation with sinus membrane elevation: a new surgical technique for maxillary sinus floor augmentation. *Clin Implant Dent Relat Res* 2004; 6(3): 165-73.
25. Sohn DS, Lee JS, Ahn MR, Shin HI. New bone formation in the maxillary sinus without bone grafts. *Implant Dent* 2008 Sep; 17(3): 321-31.
26. Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost* 2004 Jan; 91(1): 4-15.
27. Su CY, Kuo YP, Tseng YH, Su CH, Burnouf T. In vitro release of growth factors from platelet-rich fibrin (PRF): a proposal to optimize the clinical applications of PRF. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009 Jul; 108(1): 56-61.
28. Hardig BM, Carlson J, Roijer A. Changes in clot lysis levels of reteplase and streptokinase following continuous wave ultrasound exposure, at ultrasound intensities following attenuation from the skull bone. *BMC Cardiovasc Disord* 2008; 8: 19.
29. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Development of an in vitro model to study clot lysis activity of thrombolytic drugs. *Thromb J* 2006; 4: 14.
30. Klongnoi B, Rupprecht S, Kessler P, Zimmermann R, Thorwarth M, Pongsiri S, et al. Lack of beneficial effects of platelet-rich plasma on sinus augmentation using

a fluorohydroxyapatite or autogenous bone: an explorative study. *J Clin Periodontol* 2006 Jul; 33(7): 500-9.

31. Buser D, Hoffmann B, Bernard JP, Lussi A, Mettler D, Schenk RK. Evaluation of filling materials in membrane--protected bone defects. A comparative histomorphometric study in the mandible of miniature pigs. *Clin Oral Implants Res* 1998 Jun; 9(3): 137-50.
32. Furst G, Gruber R, Tangl S, Zechner W, Haas R, Mailath G, et al. Sinus grafting with autogenous platelet-rich plasma and bovine hydroxyapatite. A histomorphometric study in minipigs. *Clin Oral Implants Res* 2003 Aug; 14(4): 500-8.
33. Schlegel KA, Zimmermann R, Thorwarth M, Neukam FW, Klongnoi B, Nkenke E, et al. Sinus floor elevation using autogenous bone or bone substitute combined with platelet-rich plasma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007 Sep; 104(3): e15-25.
34. Liu Y, Springer IN, Zimmermann CE, Acil Y, Scholz-Arens K, Wiltfang J, et al. Missing osteogenic effect of expanded autogenous osteoblast-like cells in a minipig model of sinus augmentation with simultaneous dental implant installation. *Clin Oral Implants Res* 2008 May; 19(5): 497-504.
35. Kostopoulos L, Karring T. Role of periosteum in the formation of jaw bone. An experiment in the rat. *J Clin Periodontol* 1995 Mar; 22(3): 247-54.
36. Chen TW, Chang HS, Leung KW, Lai YL, Kao SY. Implant placement immediately after the lateral approach of the trap door window procedure to create a maxillary sinus lift without bone grafting: a 2-year retrospective evaluation of 47 implants in 33 patients. *J Oral Maxillofac Surg* 2007 Nov; 65(11): 2324-8.
37. Mazor Z, Horowitz RA, Del Corso M, Prasad HS, Rohrer MD, Dohan Ehrenfest DM. Sinus floor augmentation with simultaneous implant placement using Choukroun's platelet-rich fibrin as the sole grafting material: a radiologic and histologic study at 6 months. *J Periodontol* 2009 Dec; 80(12): 2056-64.
38. He L, Lin Y, Hu X, Zhang Y, Wu H. A comparative study of platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) on the effect of proliferation and

differentiation of rat osteoblasts in vitro. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2009 Nov; 108(5): 707-13.

39. Dohan Ehrenfest DM, Doglioli P, de Peppo GM, Del Corso M, Charrier JB. Choukroun's platelet-rich fibrin (PRF) stimulates in vitro proliferation and differentiation of human oral bone mesenchymal stem cell in a dose-dependent way. ***Arch Oral Biol*** Mar; 55(3): 185-94.
40. Thor A, Sennerby L, Hirsch JM, Rasmusson L. Bone formation at the maxillary sinus floor following simultaneous elevation of the mucosal lining and implant installation without graft material: an evaluation of 20 patients treated with 44 Astra Tech implants. ***J Oral Maxillofac Surg*** 2007 Jul; 65(7 Suppl 1): 64-72.
41. Sul SH, Choi BH, Li J, Jeong SM, Xuan F. Histologic changes in the maxillary sinus membrane after sinus membrane elevation and the simultaneous insertion of dental implants without the use of grafting materials. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2008 Apr; 105(4): e1-5.
42. Sul SH, Choi BH, Li J, Jeong SM, Xuan F. Effects of sinus membrane elevation on bone formation around implants placed in the maxillary sinus cavity: an experimental study. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2008 Jun; 105(6): 684-7.

Appendix

ที่ ศธ 0521.1.03/**1063**

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
ตู้ไปรษณีย์เลขที่ 17
ที่ทำการไปรษณีย์โทรเลขคอหงส์
อ.หาดใหญ่ จ.สงขลา 90112

หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง "การหลั่งโกรทเฟคเตอร์ของเพลตเลตริชไฟบรินในห้องทดลอง และผลต่อการสร้างกระดูกระยะแรกในโพรงอากาศแม็กซิลลา การศึกษาในสัตว์ และ ทางคลินิก"

หัวหน้าโครงการ รองศาสตราจารย์ ทพญ.ปริศนา ปริพัฒนานนท์

สังกัดหน่วยงาน ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ แล้ว ในคราวประชุมครั้งที่ 4/2553 เมื่อวันที่ 10 กันยายน 2553

ให้ไว้ ณ วันที่ 17 พ.ย. 2553

(รองศาสตราจารย์ ดร.รวี เถียรไพศาล)
รองคณบดีฝ่ายวิจัย
ประธานกรรมการ

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ทพ.นพ.สุรพงษ์ วงศ์วัชรานนท์)

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ทพญ.สรียา ศรีสินทร)

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ดร.ทพญ.อังคณา เชียรมนตรี)

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ นพ.พรชัย สถิรปัญญา)

.....กรรมการ
(อาจารย์วิจารณ์ ทอประยูร)

.....กรรมการ
(อาจารย์วศิน สุวรรณรัตน์)

ที่ ศธ 0521.11/๖๙๐

สำนักวิจัยและพัฒนา
มหาวิทยาลัยสงขลานครินทร์
อ.หาดใหญ่ จ.สงขลา 90110

Ref. 21/53

หนังสือรับรอง

โครงการวิจัย เรื่อง **การหลั่งโกรทเฟคเตอร์ของเพลตเลตริชไฟบรินในห้องทดลองและผลต่อการสร้างกระดูกระยะแรกในโพรงอากาศแม็กซิลลา การศึกษาในสัตว์และทางคลินิก**

หัวหน้าโครงการ **รศ.ดร.ปริศนา ปริพัฒนานนท์ คณะทันตแพทยศาสตร์**

ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง มหาวิทยาลัยสงขลานครินทร์

ให้ไว้ ณ วันที่ ๓๑ สิงหาคม 2553

(ผู้ช่วยศาสตราจารย์ ดร.กิจจา สว่างเจริญ)
ประธานคณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง
มหาวิทยาลัยสงขลานครินทร์

PRINCE OF SONGKLA UNIVERSITY
15 Karnjanawanij Road, Hat Yai, Songkhla 90110, Thailand
Tel (66-74) 286958 Fax (66-74) 286961
Website : www.psu.ac.th

MOE 0521.11/695 Ref. 21/2010

August 31, 2010

This is to certify that the research project entitled "In vitro release of growth factors from platelet rich fibrin and its early effect on bone regeneration in maxillary sinus augmentation, an animal and clinical trial study" which was conducted by Assoc.Prof.Dr.Prisana Pripatananont, Faculty of Dentistry, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

Vitae

Name Miss Narumol Pattaraporncharoen

Student ID 5110820008

Educational Attainment

Degree	Name of institute	Year of Graduate
Doctor of Dental Surgery	Chaingmai University	2002
Diploma in Comprehensive Dentistry	Mahidol University	2005