Evaluation of different scaffolds for bone regeneration in rat calvarial bone defects

Mr. Jeerawit Sirakittiworapong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Periodontics
Department of Periodontology
FACULTY OF DENTISTRY
Chulalongkorn University
Academic Year 2022

การศึกษาการใช้โครงร่างเลี้ยงเซลล์ชนิดต่างๆ เพื่อคืนสภาพเนื้อเยื่อกระดูกในโมเดลหนูแรทที่ทำให้เกิดความวิการบริเวณกะโหลกศีรษะพารัยทอล

นายจีราวิชช์ ศิรกิตติวรพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาปริทันตศาสตร์ ภาควิชาปริทันตวิทยา
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	Evaluation of different scaffolds for bone regeneration in rat calvarial bone defects
By	Mr. Jeerawit Sirakittiworapong
Field of Study	Periodontics
Thesis Advisor	WICHAYA WISITRASAMEEWONG, D.D.S., M.Sc., D.M.Sc.

Accepted by the FACULTY OF DENTISTRY, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF DENTISTRY

(Professor Pornchai Jansisyanont, D.D.S., M.S., Ph.D.)

THESIS COMMITTEE

..... Chairman

(Professor RANGSINI MAHANONDA, D.D.S., M.D.Sc., Ph.D.)

..... Thesis Advisor

(WICHAYA WISITRASAMEEWONG, D.D.S., M.Sc., D.M.Sc.)

..... Examiner

(Assistant Professor Peerapat Thongnuek, B.Sc., M.Res., Ph.D.)

..... External Examiner

(Emeritus Professor Stitaya Sirisinha, B.S., M.Sc., D.M.D., Ph.D.)

จีราวิชช์ ศิรกิตติวรพงษ์ : การศึกษาการใช้โครงร่างเลี้ยงเซลล์ชนิดต่างๆ เพื่อคืนสภาพเนื้อเยื่อกระดูกในโมเดลหนูแรทที่ทำให้เกิดความวิการบริเวณกะโหลกศีรษะพารัยทอล. (Evaluation of different scaffolds for bone regeneration in rat calvarial bone defects) อ.ที่ปรึกษาหลัก : อ. ทพญ. ดร.วิชญา วิศิษฐ์รัศมีวงศ์

โครงร่างเลี้ยงเซลล์มีบทบาทสำคัญสำหรับงานวิศวกรรมเนื้อเยื่อ โดยนอกจากจะช่วยทำหน้าที่เป็นโครงร่างสนับสนุนเซลล์แล้ว ยังช่วยสร้างสภาวะแวดล้อมที่เหมาะสมต่อการอาศัยอยู่ของเซลล์ที่ถูกเหนี่ยวนำเข้ามา ช่วยส่งเสริมประสิทธิภาพในการรักษาโรคโดยอาศัยเซลล์มาช่วย รวมถึงช่วยในการควบคุมการปลดปล่อยสารตัวนำทางชีวภาพ เช่น โกรทแฟคเตอร์ต่างๆ ซึ่งคอลลาเจนถือเป็นวัสดุที่ถูกนำมาใช้เป็นโครงร่างเลี้ยงเซลล์อย่างแพร่หลายในทางทันตกรรม โดยเฉพาะอย่างยิ่งในการเกิดการคืนสภาพเนื้อเยื่อกระดูกและอวัยวะปริทันต์ อย่างไรก็ตาม ในปัจจุบันได้มีความพยายามในการสร้างและพัฒนาพอลิเมอร์ธรรมชาติอีกหลายชนิดเพื่อนำมาใช้ในการรักษามากขึ้น โดยในการศึกษานี้มีจุดประสงค์เพื่อศึกษาความสามารถของวัสดุที่นำมาใช้เป็นโครงร่างเลี้ยงเซลล์ 4 ชนิด ได้แก่ คอลลาเจน ไคโตซาน ไหมไฟโบรอิน และไหมไฟโบรอินรวมกับเจลาตินไฮโดรเจล ในการเกิดการคืนสภาพเนื้อเยื่อกระดูกในโมเดลหนูแรทที่ทำให้เกิดความวิการบริเวณกะโหลกศีรษะพารัยทอล โดยหนูแรทสายพันธุ์วิสตาร์อายุ 8 สัปดาห์ จะถูกนำมาสร้างความวิการบริเวณกะโหลกศีรษะพารัยทอล จำนวน 2 ตำแหน่งต่อหนูแรท 1 ตัว แต่ละตำแหน่งมีขนาดเส้นผ่าศูนย์กลาง 5 มิลลิเมตร และถูกแบ่งเป็น 5 กลุ่ม กลุ่มละ 3-4 ตัว โดยแต่ละกลุ่มจะถูกฝังด้วยคอลลาเจน ไคโตซาน ไหมไฟโบรอิน และไหมไฟโบรอินรวมกับเจลาตินไฮโดรเจล และกลุ่มสุดท้ายที่ไม่ได้ถูกฝังด้วยโครงร่างเลี้ยงเซลล์เป็นกลุ่มควบคุม หลังจากการผ่าตัด 4 สัปดาห์ หนูทั้งหมดถูกนำมาทำการุณฆาต แล้วเอาเฉพาะส่วนกะโหลกศีรษะพารัยทอลมาวัดและเปรียบเทียบร้อยละของปริมาตรการสร้างกระดูกโดยใช้เครื่องเอ็กซ์เรย์คอมพิวเตอร์ระดับไมโครเมตร ตามด้วยการตรวจเนื้อเยื่อทางจุลกายวิภาคศาสตร์ด้วยการย้อม H&E และ Masson's trichrome โดยจากผลการศึกษาพบว่า กลุ่มที่ฝังด้วยคอลลาเจนมีค่าร้อยละของปริมาตรการสร้างกระดูกมากกว่ากลุ่มอื่นอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่นร้อยละ 95 และไม่พบความแตกต่างกันของร้อยละของปริมาตรการสร้างกระดูกในกลุ่มอื่นอย่างมีนัยสำคัญทางสถิติ ดังนั้นภายใต้ข้อจำกัดของการศึกษานี้ คอลลาเจนเป็นวัสดุโครงร่างเลี้ยงเซลล์ที่มีประสิทธิภาพมากที่สุดที่ช่วยในการเกิดการคืนสภาพเนื้อเยื่อกระดูกในโมเดลหนูแรทที่ทำให้เกิดความวิการบริเวณกะโหลกศีรษะพารัยทอล

สาขาวิชา ปริทันตศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6370003832 : MAJOR PERIODONTICS

KEYWORD: bone regeneration, animal study, collagen, chitosan, silk fibroin, gelatin

Jeerawit Sirakittiworapong : Evaluation of different scaffolds for bone regeneration in rat calvarial bone defects. Advisor: WICHAYA WISITRASAMEEWONG, D.D.S., M.Sc., D.M.Sc.

Scaffold plays a key role in the context of tissue engineering. It not only provides a structural support for cells, but also creates an appropriate milieu for recruited cells, enhancing the therapeutic effects of cell-based treatments, and enabling the controlled release of biological cues such as growth factors. Collagen is widely used scaffold material in tissue engineering, particularly in periodontal and bone regeneration. Other natural polymers, however, have been developed and can be useful. Therefore, in this study, we aim to investigate the ability of four different natural polymers including collagen, chitosan, silk fibroin, and silk fibroin/gelatin hydrogel in promoting bone regeneration in vivo using rat calvarial bone defect. Two critical-sized defects (5-mm diameter) were created on the right and left calvarium of 8-week-old male Wistar rats. The rats were randomly assigned to one of the four treatment groups (n=3-4/group) and implanted with collagen, chitosan, silk fibroin, or silk fibroin/gelatin hydrogel scaffolds, respectively. Empty defect was used as a control. Four weeks after surgery, all animals were sacrificed and the calvarial bones were dissected for bone volume/total volume percentage (BV/TV%) measurement using micro-computed tomography, and subsequent histological analysis using hematoxylin & eosin and Masson's trichrome staining. The collagen scaffold resulted in significantly higher BV/TV than the other groups ($p<0.05$) with the greatest amount of new bone formation. There was no significant difference between other scaffolds and control group. Within the limitation of this study, collagen is the most effective scaffold in promoting bone regeneration in rat calvarial bone defects.

Field of Study:	Periodontics	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my deep gratitude to my advisor, Dr. Wichaya Wisitrasameewong for expert guidance, patience, motivation, suggestion and correction of this thesis and kindness given throughout the course of the Master Degree Program. I would like to express my sincere appreciation to Professer Dr. Rangsini Mahanonda for providing me a great support, valuable advices and being chairman of the committee member. I would like to thank to Dr. Sathit Pichyangul for his expert advices and Emeritus Professor Dr. Stitaya Sirisinha for valuable suggestion and being a thesis committee member. I would like to express my sincere gratitude to Assist Professor Dr. Peerapat Thongnuek for his suggestion, support for materials used in the experiments and being a thesis committee member. I would like to thank Associate Professor Dr. Ruchanee Ampornaramveth for providing materials used in the experiments. I would like to thank Mr. Theeraphat Surisaeng for great support, device and collaboration.

Furthermore, my sincere appreciation is also extended to the laboratory staff of the Center of Excellence in Periodontal Disease and Dental Implant, Chulalongkorn University for their assistance in laboratory works.

This study was supported by Thailand Research Fund and Chulalongkorn University (DIG6280002) and the Program Management Unit for Human Resources & Institutional Development, Research and Innovation - Chulalongkorn University (grant number B05F630075).

Jeerawit Sirakittiworapong

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Background and Rationale

Periodontitis is one of the most common chronic diseases in adult population worldwide. In Thailand, 35% of elderly and more than 25% of middle-aged population are diagnosed with periodontitis. The hallmark of periodontitis is a breakdown of tooth-supporting structures, in particular alveolar bone which eventually leads to early tooth loss in severe cases. The concept of tissue engineering has been apply to treat these clinical cases, with the goal of regenerating lost or damaged tissues to restore normal function and structure. The key components of current tissue engineering-based treatment approaches include signaling molecules/growth factors, scaffolds, and cells with an emphasizing on promoting osteogenesis, angiogenesis, as well as controlling inflammation.

One of key factors in successful periodontal regeneration is to induce new bone formation around affected tooth. To achieve this goal, scaffolds are often required. Scaffold can provide a favorable milieu for recruited cells and allow the controlled release of biological molecules such as growth factors. In the regenerative biomaterials research, animal models are frequently employed and remain the gold standard prior entering human clinical trials. Among various animal models, rodent is suitable for early-stage studies due to its several advantages. Rodent has a wide

availability, low-cost housing, easy handling, small size, and importantly, well-defined and described procedures.

Therefore, in this study, we aim to investigate four different natural polymers including collagen, chitosan, silk fibroin and silk fibroin/gelatin hydrogel in promoting bone regeneration in *vivo* using rat calvarial bone defects.

Objectives

To evaluate the ability of different scaffolds in promoting bone formation in rat critical-sized calvarial bone defects.

Hypotheses

The different scaffolds have different ability in promoting bone formation in rat critical-sized calvarial bone defects.

Field of research

In vivo study of bone regeneration in rat critical-sized calvarial bone defects using different scaffolds.

Limitation of research

The sample size was limited and the follow-up time was only 4 weeks due to the cost of the study. Thus, larger sample sizes and longer follow-up time should be employed in future studies.

Application and expectation of research

We expect to apply the result of this study in further experiments aiming to develop scaffolds for delivery of modified mRNA encoding growth factors for patients with periodontal and peri-implant defects.

Keywords

bone regeneration, animal study, collagen, chitosan, silk fibroin, gelatin

CHAPTER II

REVIEW LITERATURE

Periodontitis

Periodontitis is a chronic inflammatory disease that affecting the periodontal tissues including gingiva, periodontal ligament, cementum, and alveolar bone. Dental plaque is required but not sufficient to induce the periodontal disease. It was suggested that the microbial dysbiosis, rather than single microbe, drives a host inflammatory response. When the host-microbial homeostasis is disrupted and inflammation goes awry, periodontal tissues are destroyed and clinical signs of periodontal diseases can be observed (Hajishengallis, 2014). While gingivitis is characterized by the inflammation of gingiva without affecting the underlying bone (Trombelli et al., 2018), the loss of alveolar bone is the pathognomonic sign of periodontitis, which is the major cause of tooth loss in severe cases (Duong & Schmid, 2014).

The goal of periodontal treatment is to control the inflammation and to regenerate damaged tissues in an attempt to restore their normal functions and structures. Due to limited regenerative potential of our body, periodontal regenerative procedures are needed. Not only soft tissue and bone defects around tooth, bone deficiencies in edentulous ridge and around dental implant are a great challenge to regenerate. The gold standard procedure for periodontal and bone regeneration is guided tissue regeneration (GTR) and guided bone regeneration (GBR)

by using a barrier membrane with or without grafting materials to selectively exclude the downgrowth of epithelial and fibroblast cells, while promoting the proliferation and differentiation of progenitor cells from periodontal ligament, alveolar bone and cementum (Karring et al., 1993; Nakae et al., 1991). Nevertheless, the current outcome is unpredictable, in particular, when a complete regeneration is anticipated (Trombelli, 2005; Bosshardt & Sculean, 2009; Villar & Cochran, 2010). A successful periodontal regeneration remains challenging due to the complexity of periodontal tissues that involve both soft tissues and bone. Therefore, new regenerative approaches are in search (Han et al., 2014).

The field of tissue engineering/regenerative medicine has emerged and its concept has been applied to periodontal regenerative therapy. The major components of tissue engineering-based approaches include cells, signaling molecules/growth factors, and scaffolds. These approaches can be used either alone or in combination with one another (Duong & Schmid, 2014).

Animal models of bone regeneration

Animal model is the transitional experiment of many therapeutics to clinic. Animal models in bone regeneration studies remain the gold standard of testing (Peric et al., 2015). Therefore, they are routinely used in the development of novel bone regenerative therapies and materials. Compared to other animal species, mice and rats are considered to have skeletons and bone biology that are least similar to humans because of their permanently open growth plates and low cancellous bone

content at the epiphyses of long bones, and a lack of the Haversian system (Mills & Simpson, 2012; Kalu, 1991; Miller et al., 1995). These characteristics may be unfavorable for studies focusing on the adult skeleton. However, bone cells (osteoblasts, osteocytes and osteoclasts) in rats have similar receptors to human bone cells and, therefore react to drug challenge in a similar way to human bone (Jee & Yao, 2001). Rodent models of skeletal diseases are very predictive of drug efficacy and safety in humans. Despite some deficiencies, studies in rodent are both informative and cost-effective. Rodents also have a wide availability, low-cost housing, easy handling, small size (relatively small amounts of test product required), well-defined and described procedures. Thus, they are suitable for early-stage studies such as for rapid comparison of different osteoinductive responses. For the advanced testing or a final step before initiating clinical trials, non-human primates, canine and sheep may be necessary (Stokovic et al., 2021). Large animal models such as macaque, beagle, sheep and pig will provide a more human-like bone defect, but the disadvantages include higher housing cost, longer bone maturation time, difficulty handling, and higher operating cost (Murphy et al., 2017).

Rodent models of bone regeneration can be divided into two groups, including ectopic models and bone defect models or orthopedic models (Peric et al., 2015).

(1) Ectopic models

This is primarily used for initial evaluation of novel osteoinductive capacity of new products. The implantation sites can be done under skin (intradermal) or into muscle (intramuscular). Compared to orthopedic models, ectopic models are relatively simple, less costly and less invasive.

(2) Orthotopic models or bone defect models

Bone defect models can be used for initial evaluation of novel osteoinductive properties, efficacy and safety of new products and/or procedures. In addition, the osseointegration of newly formed bone with adjacent native bone can be tested since experimental procedures are performed in or around the bone itself. Defects can be created under various loading conditions depending on objective of studies.

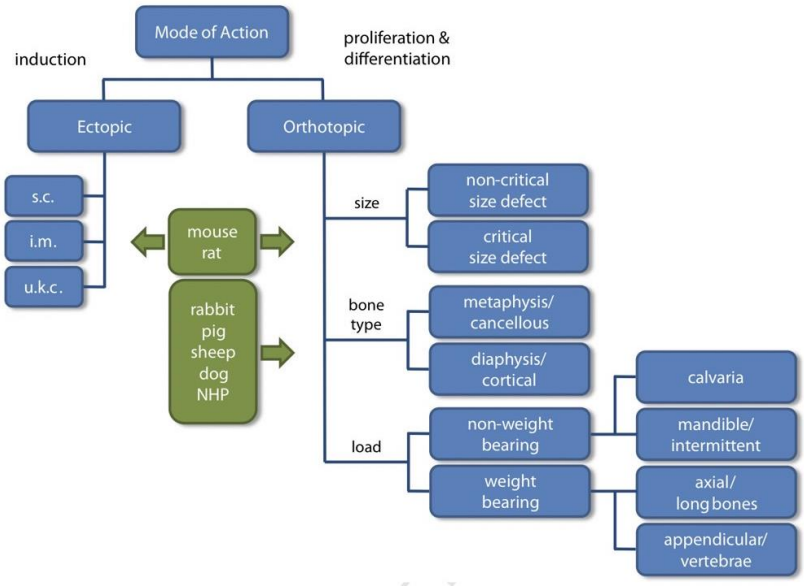

Figure 1. Graphical presentation of animal bone models based on the investigated effect of the therapy (Peric et al., 2015).

Rat critical-sized calvarial bone defects

Rat calvarial defect was first described by Freeman and Turnbull in 1973 but not truly established until Takagi and Urist in 1982 (Spicer et al., 2012). Compared to femoral bone defect, bone regeneration in calvarial defect is faster and the stabilization of the bone is not required. Because the calvarial defect represents intramembranous bone production, it cannot be used to depict endochondral bone formation (Lim et al., 2013). In addition, there is no pathological process presented and limited tissue for evaluation.

The critical size defect in rat calvarial defect is vary from <5 mm to 9 mm. The 5 mm defect has several advantages, including the ability to design both experimental and control sizes in the same animal, therefore, could reduce number of animal use in an experiment, a lower risk of sagittal sinus injury, which can lead to hemorrhage and neurological complications (Vajgel et al., 2014). In a Wistar rat model with a one 5mm calvarial defect, 16 percent of the rats had completed bone fill in 30 days, and 33 percent had completed bone fill in 60 days (Porto et al., 2012). However, systematic review and meta-analysis found that 5 mm rat calvarial defects in both central and bilateral defects had not significant different bone formation between 1 and 3 months, suggesting that calvarial defect with a diameter of 5 mm could be considered as critical size defect (Vajgel et al., 2014).

Original term “critical size defect” is defined as a defect that will not heal within the lifetime of an animal (Schmitz & Hollinger, 1986). However, most studies

are short-term, the critical-size defect in animal research thus refers to size of a defect that will not heal over the study's period (Gosain et al., 2000). Cooper et al. 2010 suggested that researchers should determine a defect size based on their study design. In other words, if analysis is performed in a short period of time, a small defect may be sufficient. Therefore, researchers can utilize their own defects to test bone healing strategies in their area of clinical interest (Cooper et al., 2010).

The experimental outcome in calvarial defect should be determined by micro-computed tomography (micro-CT) and histology. Micro-CT analyses provide information on newly formed bone volume expressed as bone volume (BV) or bone volume/tissue volume ratio (BV/TV). Furthermore, micro-CT analyses allow the calculation of trabecular parameters (trabecular number, trabecular thickness, trabecular separation) to determine the structural properties of newly formed bone. Histology and histomorphometry should also be used to confirm the results from micro-CT (Spicer et al., 2012).

It should be noted that the age and gender of the research animals can have an impact on bone healing through the action of calciotropic hormones (Bergman et al., 1996; Vajda et al., 2001). For example, aged, thyroparathyroidectomized and ovariectomized (OVX) animals are known for delayed fracture healing and reduced bone mineral density; therefore, OVX animals are frequently utilized to study osteoporotic fractures because these models mimic postmenopausal women (Lelovas et al., 2008; Dumic-Cule et al., 2014). Furthermore, alterations in Wnt

signaling have also been linked to age-related reductions in bone formation, with studies in mice showing reduced Wnt expression and canonical Wnt signaling within cortical long bones and vertebral bodies with increasing age (Boskey & Coleman, 2010; Holguin et al., 2014). While the periosteum is widely known for promoting bone regeneration in long bones, the dura mater has also been demonstrated to play a role in calvarial regeneration, providing both osteoprogenitors and important pro-osteogenic growth factors, such as TGF-b, BMP-2 and FGF-2 along with several osteoblast differentiation markers (osteopontin, RUNX2, and FGFR-1) (Slater et al., 2009; Smith et al., 2012; Spector et al., 2002; Li et al., 2007). In young mice, markers for osteoclast activity, including acp5, matrix metalloprotease-9, and cathepsin K have been found with the localization of osteoclasts within the dura mater (Wan et al., 2008). These findings suggest the coordination between osteoblasts and osteoclasts in maintaining bone homeostasis in the calvarium. Therefore, any alteration or damage to this tissue may impair any reconstructive attempt.

Scaffold in bone regeneration

Scaffolds are often required in bone regeneration to fill defects and serve as a template, providing a framework and optimal milieu for cells infiltration and subsequent tissue formation (Galli et al., 2021). In addition to space filling, scaffold can be used as a delivery platform for cells, bioactive molecules, and genes into the body (Hollister, 2009).

Scaffolds should ideally meet a number of important characteristics in order to serve these functions. They should be biocompatible, bioactive, osteoconductive and osteoinductive as well as maintaining mechanical stability during the healing process (Filippi et al., 2020). In a physiologic bone healing, granulation tissues formed in an initial phase are gradually replaced by soft and hard callus formation. During the bone remodeling phase, hard callus remodels into the lamellar bone and the cancellous bone trabeculae through an orderly bone resorption and formation process mediated by a complex network of cells and cytokines (Zhu et al., 2021). However, bone cannot heal properly when the damaged bone tissues are quite extensive. The use of scaffolds has become a potential solution to this challenge (Stevens, 2008).

Therefore, another important feature of scaffolds upon *in vivo* implantation is a degradation rate. Scaffolds provide a 3D framework for cells and tissues infiltration. If biomaterials degrade too rapidly, newly formed tissues will not have enough time to remodel. Slow-degrading scaffolds, on the other hand, will provide long-term structural support while causing fibrosis, which impedes regeneration. Therefore, it is crucial to balance a scaffold's resorption rate with the formation of new tissues in order to achieve structural and functional regeneration (Gaharwar et al., 2020).

There are variety of scaffolds used for tissue regeneration includes monolithic, microporous, nanoparticles, fibrous, hydrogels and 3D- printed scaffolds. Polymers, ceramics, metals, and composites can all be utilized to fabricate scaffolds.

These materials come from a variety of sources, including synthetic, natural, and a combination of both. Through their biophysical and biochemical properties, these biomaterials can modulate the immune system and control the kinetics and degree of healing from endogenous cells (from scarring to total regeneration) (Gaharwar et al., 2020). The scaffold material and qualities required will vary greatly depending on the tissue of interest and the specific application. In the case of periodontal and bone regeneration, injectable and soft materials are preferred due to the difficulties of reaching the target areas.

The polymers are widely use as scaffolds for bone tissue engineering. The polymers are divided into 2 main types including natural and synthesized polymers. The natural polymers can be classified as: 1) proteins such as collagen, gelatin, keratin, actin, myosin, fibronectin and elastin; 2) polysaccharides such as cellulose and chitin; and polynucleotide such as DNA and RNA (Damiati & El-Messeiry, 2021). The synthesized polymers are such as polylactic acid (PLA), polyurethane (PU), poly (lactide-co-glycolide) (PLGA), and polycaprolactones (PCL) (Sun et al., 2021). The benefits and drawbacks of the natural and synthetic polymers are presented in Table 1 (Filippi et al., 2020). Natural polymers have several advantages, including high biodegradability, cytocompatibility, and low immunogenicity. In our study, we are interested in collagen, chitosan, silk fibroin, and silk fibroin-gelatin. The advantages and disadvantages of these natural polymers are presented in Table 2 (Filippi et al., 2020).

Table 1. Advantages and disadvantages of natural and synthetic polymers used in scaffold fabrication for bone tissue engineering (Filippi et al., 2020).

	ADVANTAGES	DISADVANTAGES	References
Synthetic polymers	- Defined purity and reproducible chemical/mechanical properties - Appropriate mechanical properties - Low immune response - Low production costs - Off-the-shelf availability and production in large uniform quantities - Opportunity to tailor material properties during manufacturing	- Poor biocompatibility - Risk of biodegradation side effects (nanotoxicity, inflammation, etc.) - Difficult 3D printing - Questionable cell-matrix interaction - Loss of mechanical strength after degradation (biodegradable polymers) - Low ductility - Effects of long-term permanence in the body (non-degradable polymers) - Uncontrollable shrinkage effects	(Gunatillake et al., 2006; BaoLin and Ma, 2014; Bhatia, 2016)
Natural polymers	- Natural origin - Biocompatibility - Presence of cell recognition and adhesion sites - Similarity with native ECM - Biodegradability - Not require harsh chemicals for processing - Bioresorbability - Bioactivity	- Properties dependence on extraction and processing procedures - Inadequate mechanical properties - Expensive productive methods - Susceptibility to cross-contamination - Difficult processing - Low stability	(Bhatia, 2016; Salehi-Nik et al., 2017)

Table 2. Advantages and disadvantages of various natural polymers used in scaffold fabrication for bone tissue engineering (Filippi et al., 2020).

	ADVANTAGES	DISADVANTAGES	References
Protein-based polymers			
Collagen	- Biocompatible - Biodegradable - Fibrous morphology - Non-toxic - Non-antigenic - Mimic native bone ECM topography - Biologically renewable - Bioadhesive - Biofunctional - Ability to be cross-linked	- Poor mechanical properties - Low stability - Fusion of nanofibers in aqueous environment - Low melting point - Viral and prion contamination - Difficult processing - Difficult control over extent and rate of degradability - Potentially damaged by sterilization methods - Expensive if produced by recombinant technologies	(Whang et al., 1999; Dong and Lv, 2016; Zhang et al., 2017)
Gelatin	- Biocompatible - Biodegradable - Anti-thrombogenic - Good cell recognition properties - Low antigenicity - Easy to mold into a range of shapes (injectable hydrogels and sponges)	- Low stability - Chemical cross-linking needed - Poor mechanical properties - Brittleness	(Garg et al., 2012; Echave et al., 2017)
Silk fibroin	- Biocompatible - Biodegradable - Slow degradation - Excellent mechanical properties - High thermal stability - High mechanical strength	- Reduced availability (*e.g.* low production from spiders) - High brittleness - Residue contaminants	(Shi et al., 2016; Kowalczewski and Saul, 2018)
Polysaccharide-based polymers			
Chitosan	- Biocompatible - Biodegradable - Biologically renewable - Non-toxic - Non-antigenic - Inexpensive - Positively charged - Antibacterial properties	- Difficult processing by electrospinning - Immunogenicity - Long delay in bone formation (after several months or years) - Relatively weak mechanical strength and stability	(Garg et al., 2012; Levengood and Zhang, 2014; Shi et al., 2016)

Collagen

The collagens are animal-derived fibrous glycoproteins. Collagen is the most abundant protein in the human body, as it is a major component of the extracellular matrix in various connective tissues, especially in tendon, bone, skin and cartilage (Filippi et al., 2020). Among different types of collagen, type I is the most abundant which represents more than 90% of the organic mass of bone. Collagen presents as a template and may initiate and propagate the mineralization during bone formation process (Ferreira et al., 2012). Undoubtedly, collagen-based biomaterials have been probably the most widely used for bone tissue engineering and also other biomedical applications (Miyata et al., 1992). Various forms of collagen-based biomaterials for several tissue engineering applications have been reported including powders/particles, fibers, gels, sponges, and membranes (Rico-Llanos et al., 2021).

Collagen scaffolds have good osteoconductive properties. They not only serve as a physical support for cells to attach and grow on, but also influence cell behavior and fate via receptor-mediated interactions. However, there is no evidence of osteoinductive activity in a non-osseous environment. Nevertheless, collagen-based biomaterials can be modified to include other bioactive domains to direct cells that interact with them towards certain differentiation pathways. The so-modified collagen biomaterial could be considered as osteoinductive if the proper biological cues are added. Disadvantages of collagen are due to its high biodegradability and low mechanical strength. Collagen can be easily degraded by

several collagenase enzymes prior to completion of osteogenesis (Rico-Llanos et al., 2021).

Collagen has been used as a scaffold for bone regeneration in a number of studies. According to certain studies, collagen scaffolds alone are incapable of forming new bone. Elangovan et al. evaluated the bone regeneration of the collagen matrices implant in rat calvarial defects compared with empty defects in 4 weeks. The empty defects and the collagen implanted defects showed similar bone volume/total volume% in micro-CT and histological analysis (Elangovan et al., 2015). Zhang et al. also examined the bone regeneration of the collagen sponges implanted in rat femural defects compared with empty defects in 8 weeks. There are negligible bone volume/total volume% and bone formation in both empty and collagen sponge groups (Zhang et al., 2019). On the contrary, Song et al. examined bioabsorbable collagen wound dressing (CollaTape®) in rat calvarial defect compared with empty defects in 8 weeks. In this study, collagen scaffold showed more defect closure and new bone area than empty defects (Song et al., 2007). Due to the conflicting results regarding an ability of collagen scaffold in inducing bone formation, further studies are needed.

Chitosan

Chitosan and its derivative are another group of natural biopolymers that have been explored for bone tissue engineering. Chitosan is a fully or partially deacetylated form of chitin, which can be found in fungi, exoskeleton of insects and

shells of sea crustaceans. It is the most abundant polysaccharide in natural after cellulose. Chitosan also contains positively charged amino group, which contribute to its hemostatic and mucoadhesive capacity. Moreover, it has a chemical structure similar to glycosaminoglycans (GAGs) which are the major component of bone extracellular matrix. Their hydrophilic surfaces favorably facilitate cell adhesion, proliferation and differentiation. Additionally, chitosan is biodegradable, biocompatible, nontoxic, and has bacteriostatic properties (Lauritano et al., 2020). Chitosan can be easily shaped into many forms such as fibers, hydrogels, sponges and films (Filippi et al., 2020). Despite its excellent properties for tissue engineering, chitosan has poor water solubility, which is a significant drawback. The use of acid and/or chemical reagents as solvents is required to facilitate solubility of chitosan, although this may result in toxic byproducts (Sukpaita et al., 2019). The other disadvantages of pure chitosan include its low mechanical strength and fast degradation rate, particularly in acidic environments or in the human body where lysozymes are presented. Chitosan may be improved mechanically by the addition of other biomaterials, making it a suitable scaffold for bone regeneration. Chen et al. implanted pure chitosan in rat calvarial defects and compared with empty defects. In 4 weeks, it was found that there is greater bone regeneration in chitosan-treated group (Chen et al., 2018). In study of Sukpaita et al., chitosan/dicarboxylic acid (CS/DA) scaffold was testes *in vitro* and in mice calvarial defects. CS/DA scaffold significantly promotes osteoblast related gene expression *in vitro*, and bone

regeneration *in vivo* at 6 and 12 months. The addition of human periodontal ligament cells (hPDLCs) in scaffold also accelerated the early onset of osteogenesis but did not affect bone regeneration in later time (Sukpaita et al., 2019).

Silk fibroin

Silk fibroin is a natural polymer produced from silkworms, spiders, mites, and flies. The most famous silk fibroin derived from mulberry silkworm, *Bombyx mori L,* which mainly comprises of proteins, minor lipids, and polysaccharides (Choi et al., 2018). Besides its biocompatibility and high oxygen permeability, it has been shown to promote an alkaline phosphatase activation and collagen synthesis (Kweon et al., 2010). It also has excellent mechanical properties in tensile strength, toughness, and young's modulus (Choi et al., 2018). Silk fibroin can be fabricated in different types of scaffolds, including film, mat, artificial fiber, hydrogel, sponge, 3D-structure design, printed scaffold, and Inkjet-printed silk pattern. Lee et al. implanted silk fibroin scaffold in rat calvarial defect for 8 weeks. Compared to empty defect, silk fibroin scaffold showed more new bone formation. This demonstrated that pure silk fibroin scaffold has the ability to regenerate bone *in vivo*. Moreover, silk fibroin combined with other materials or cells exhibited better properties for bone regeneration (Lee et al., 2017). Manissorn et al. found that by crosslinking silk fibroin with bioactive glass GPTMS during sol-gel process, enzymic degradation decelerates. Greater mechanical stability and greater levels of osteogenic markers than silk fibroin scaffolds alone were also shown (Manissorn et al., 2021). Interestingly, silk fibroin scaffolds exhibits

beneficial qualities as a tissue engineering scaffold for mesenchymal stem cells (MSCs), for its ability to differentiate, secrete extracellular matrix and mineralize. Li et al. showed that the silk fibroin scaffolds seeded with human amniotic mesenchymal stem cells (hAMSCs) not only enhanced the osteogenic differentiation of hAMSCs and potentiated angiogenic differentiation *in vitro* when compared with hAMSCs alone but also enhanced new bone formation in mice calvarial bone defects compared with silk fibroin scaffolds alone (Li et al., 2020).

Silk fibroin/gelatin hydrogel

Silk fibroin has appropriate characteristic for tissue engineering as discussed above. Its bioinertness, which delays cell adhesion and limits cell growth, as well as its high water solubility and poor physiological stability, remain its main drawbacks (Duangpakdee et al., 2021). Previous studies have suggested combining silk fibroin with other materials, such as gelatin, to improve its properties. Regenerated silk fibroin can be dissolved in water unless its structures turn from to beta sheet or covalent cross-linking (Van Vlierberghe et al., 2011). Therefore, The cross-linking between silk fibroin and gelatin were significantly more stable as they could retained its weight for 10 days (almost 100% remaining weight) after immersion in PBS 37 °C (Duangpakdee et al., 2021). Gelatin is a collagen-derived natural polymer formed through hydrolysis of acid and alkaline. Gelatin has been used in drug delivery in tissue engineering applications. Gelatin was applied as carrier to deliver active molecules and cells. The active molecule of gelatin is arginine-glycine-aspartate

(RGD) peptides which are cell adhesion factors that can enhance cell proliferation. Silk fibroin in combination with gelatin was developed and fabricated in a hydrogel form. A hydrogel is a three-dimensional network formed by the physical and chemical cross-linking of hydrophilic polymers via covalent bonds or physical intramolecular and intermolecular interactions. Without dissolving in water, hydrogel absorbs water and becomes inflated with soft and rubbery characteristics comparable to living tissue. In tissue engineering, hydrogel has been used as scaffolds due to its properties that resembles extracellular matrix, providing structural integrity, housing and delivering cells, and serving as tissue barriers and bioadhesives. In addition, hydrogel has the ability to absorb and deliver drugs and other bioactive molecules to enhance regeneration process (El-Sherbiny & Yacoub, 2013).

In many studies, the addition of gelatin and hyaluronic acid exhibits greater bone regeneration to silk fibroin-based scaffolds. Lamlerd et al. compared many Thai silk fibroin-based scaffolds in rat radius bone defects and found that hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold (CGSF4) promoted significant new bone formation compared to other combinations (Lamlerd et al., 2017). Duangpakdee et al. showed that crosslinked silk fibroin/gelatin/hyaluronan blended scaffolds had high porosity, ability to support cell adhesion and proliferation into higher cell numbers than other groups (Duangpakdee et al., 2021). Therefore,

these scaffolds could provide a suitable microenvironment for cell-based tissue engineering.

CHAPTER III

MATERIALS AND METHODS

Animals

All rat experimental protocols in this study were reviewed and approved by Institute of Animal Care and Use Committee of Faculty of Tropical Medicine, Mahidol University (FTM-IACUC) and the Ethics committee of the Faculty of Dentistry, Chulalongkorn University. Wild-type Wistar male rats, aged 7-week old were purchased from Nomura Siam International Co., Ltd. (Bangkok, Thailand) and adopted in individually ventilated cages with 12-hours light/dark cycle for a week before beginning of the experiment. The rats were randomly divided into 5 groups of 3 to 4 rat each (total of 16 animals). The number of rats per group was determined with G power software, based on Elangovan, 2015 (Elangovan et al., 2015).

Scaffolds

Collagen sponge (CollaTape®) was purchased from Zimmer Dental (Carlsbad, CA, USA). The 5% w/v. freeze-dried chitosan (85% degree of deacetylation) was fabricated at Petroleum and Petrochemical Collage, Chulalongkorn University, Thailand, and provided by Assoc. Prof. Dr. Ruchanee Ampornaramveth (Sukpaita et al., 2019). The 6.5 wt.% freeze-dried silk fibroin and silk fibroin/gelatin (30:70) hydrogel were fabricated at Faculty of Engineering, Chulalongkorn University, Thailand, and provided by Asst. Prof. Dr. Peerapat Thongnuek (Okhawilai et al., 2010).

The physiochemical properties of these scaffolds used in this study are presented in Table 3.

Table 3. The physiochemical properties of natural polymers used in this study.

physiochemical properties	Collagen sponge (CollaTape®)	Chitosan	Silk fibroin	Silk fibroin/gelatin hydrogel
Pore size	54.4±10.6 μm (Liu et al., 2019)	160.36±68.36 μm (Suwattanachai et al., 2019)	247±53 μm (Manissorn et al., 2021)	About 165 μm (Duangpakdee et al., 2021)
Porosity	86.7±3.4 % (Liu et al., 2019)	N/A	91.62±1.59 % (Manissorn et al., 2021)	N/A
Degradation	Resorption rate 10-14 days (Dental Zimmer)	*In vitro* Quickly degradation in 1st week which residual weight 68.86 ±2.23 wt% and retained in 6th weeks which residual weight 66.57±3wt% in PBS with lysozyme. (Suwattanachai et al., 2019) *In vivo* The scaffold material has been lost in 6th weeks. (Sukpaita et al., 2019)	*In vitro* Remaining weight was 19.87% after immersion in de-ionized water 24 hours. (Manissorn et al., 2021)	*In vitro* SFG retained its weight for 10 days (almost 100% remaining weight) after immersion in PBS 37 °C (Duangpakdee et al., 2021)
Mechanical properties	Compression test 0.105±0.075 N (Liu et al., 2019)	Compressive modulus in dry state 0.70±0.16 MPa and wet state 0.0070± 0.0001 MPa (Suwattanachai et al., 2019)	Compressive modulus 0.015± 0.004 MPa Compressive stress 0.295±0.119 MPa (Manissorn et al., 2021)	N/A

Preparation of rat calvarial bone defects and *in vivo* implantation of scaffolds

All rats were housed and cared in Mahidol University. Before starting the experiment, they rested for one week. Preoperatively, the 8 weeks old animals were anesthetized intraperitoneally (IP) by Zoletil (Virbac®) (40 mg/kg) and Xylazine (X-Lazine®) (5 mg/kg). They were disinfected with 2% Chlorhexidine and 70% Ethanol, followed by shaving from the bridge of the snout between the eyes to the caudal end of the skull/calvarium using electric clippers. Local anesthetic injection was performed with 2% Lidocaine with 1:100,000 Epinephrine (0.2 ml). Their eyes were protected with eye ointment. Sagittal incision in the scalp was made from the nasal bone to middle sagittal crest or bregma. Reflect full thickness flap was performed by using blunt instrument to separate bone from the underlying dura. Bone was excised and defects were created by generated two critical-sized defects on the right and left from midline (5 mm diameter, through to through) using trephine bur under continuously sterile saline irrigation. The rats were randomly divided into 5 groups.

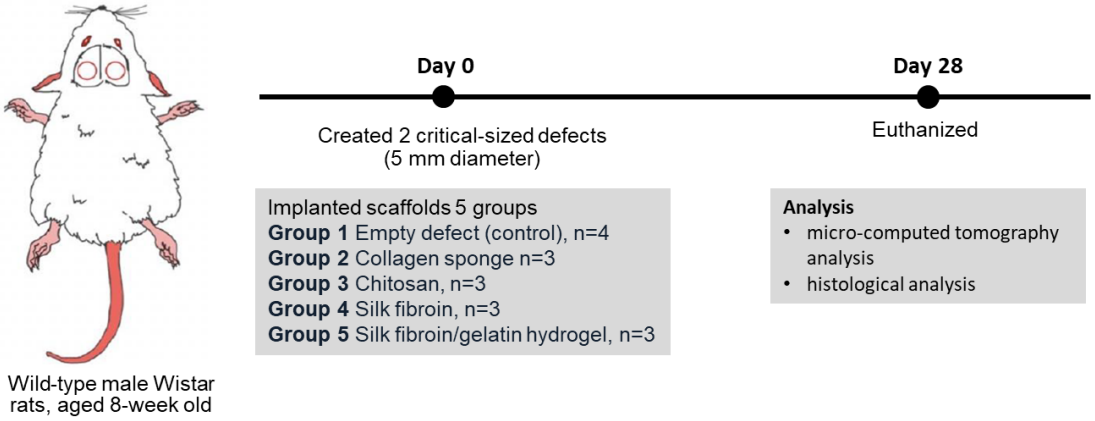

Figure 2. Diagram of the experimental timeline of this study.

Group 1 Empty defect were used as control. (n=4)

Group 2 Implanted bilaterally with collagen sponge. (n=3)

Group 3 Implanted bilaterally with chitosan. (n=3)

Group 4 Implanted bilaterally with silk fibroin. (n=3)

Group 5 Implanted bilaterally with silk fibroin/gelatin hydrogel. (n=3)

The periosteum and scalp was closed from one layer at a time with interrupted 5-0 Vicryl resorbable sutures. Additional, Carprofen (Rimadyl®) (2.2 mg/kg) was injected to have an analgesic effect in all the rats. They were sacrificed at 4 weeks respectively after surgery. The calvarial bone was dissected for micro-CT and histological analysis.

Micro-computed tomography (micro-CT) analysis

Calvaria, including the defect areas were dissected out and fixed in 10% buffered formalin for 48 hours, before being scanned with X-ray micro-CT apparatus (SCANCO Medical AG, μCT 35, Switzerland). Scanned CT images were processed to quantify bone volume per total volume within the defect. 3D images were constructed using instrumentation software for calvarial defect, a cylindrical region of interest (ROI), and a 3.5-diameter cylinder of sufficient height to cover the entire thickness of the calvarial bone (Elangovan et al., 2015).

Histological analysis

Following micro-CT analysis, calvarial bones were fixed in 10% buffered formalin for 24 hr and demineralized in 10% ethylenediaminetetraacetic acid (EDTA)

solution. Paraffin blocks were sectioned with 5 μm thickness, and the sections were stained with H&E to analyze new bone formation and bridging of the created defects (Elangovan et al., 2015). To confirm the presence of collagen in newly formed bone, the sections were also stained with Masson's Trichrome staining (Lim et al., 2013).

Statistical analysis

The volume/total volume percentage (BV/TV%) were analyzed using SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL, USA). The normality distribution was determined by Shapiro-Wilk test. Parametric one way ANOVA test with Dunn's correction (followed by pairwise comparisons) were performed for multiple group comparisons, respectively. For all statistical analysis, p-value less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

Micro-computed tomography (micro-CT) analysis

Micro-CT analysis was done to display bone regeneration in rat calvarial bone defects by analyzing the mineralized bone quantity and calculating the percentage of mineralized bone volume divided by the total tissue volume of interest (BV/TV%). The collagen group exhibited the most mineralized bone quantity among all groups at 4 weeks after scaffold implantation (Fig. 3A). The %BV/TV was also significantly higher in the collagen group ($p<0.05$) when compared with other groups, while no significant difference were found among other scaffolds and empty defects (Fig. 3B).

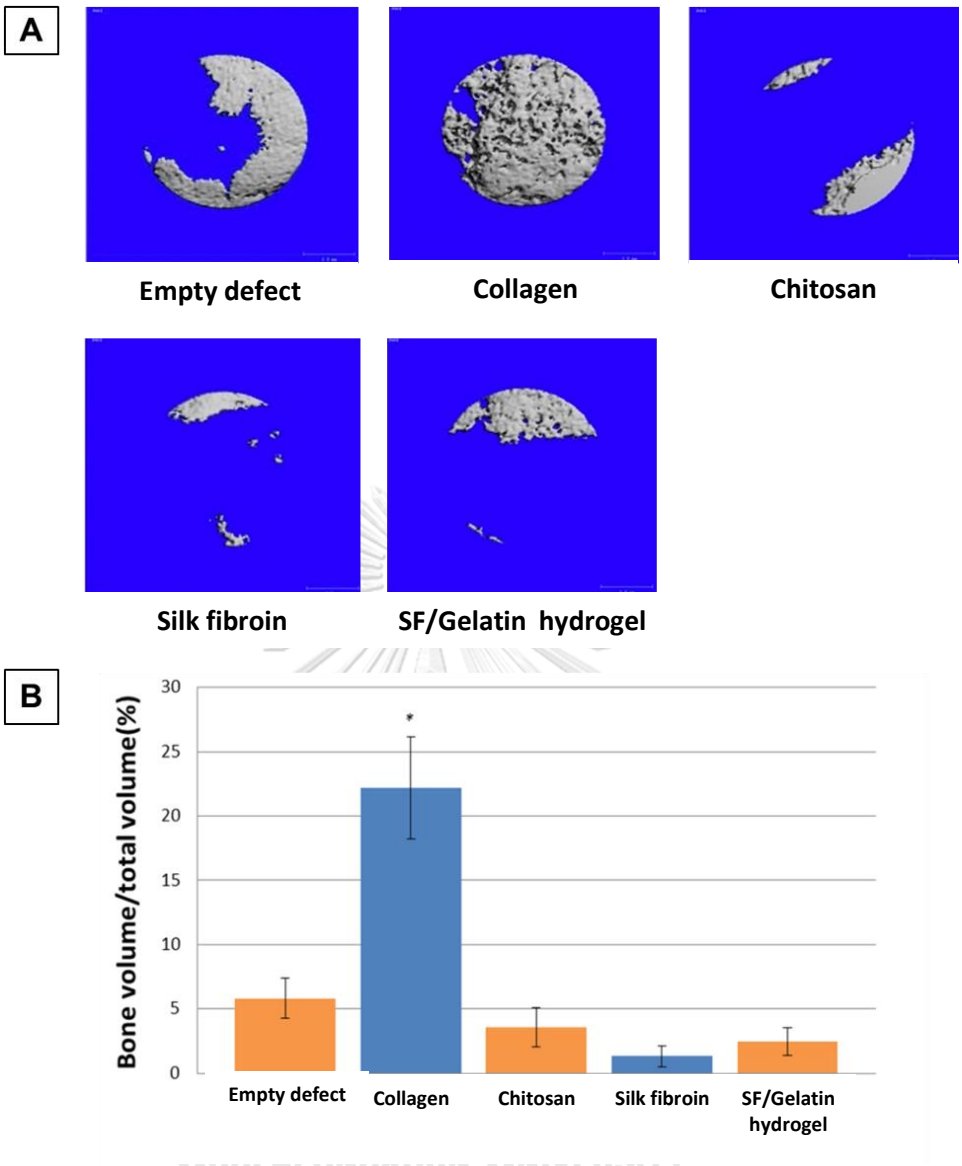

Figure 3. (A) Representative micro-CT scans showing the level of regenerated bone after 4 weeks in empty defects (control group) and defects implanted with collagen, chitosan, silk fibroin and silk fibroin/gelatin hydrogel. (B) Bone volume fraction (BV/TV%) of regenerated bone after 4 weeks of implantation with different scaffolds (Data are presented as mean ± SD, n = 3-4, * $p < 0.05$ compared to other groups).

Histological analysis

The results from micro-CT were confirmed by histological analysis. In H&E staining, the new bone was significantly formed in the defects implanted with collagen scaffold. The defect bridging was also observed. Other groups showed only a small amount of new bone formation, with no significant difference from empty defects (Fig. 4). In defects implanted with silk fibroin, inflammatory cell infiltration was observed between the fibrous tissue and scattered scaffolds remnants (Fig. 5). In defects implanted with silk fibroin/gelatin hydrogel, the substantial amounts of residual scaffolds were observed surrounded with intense plasma cell and lymphocyte infiltration. A large number of neutrophils and a few eosinophils were identified among scaffold remnants (Fig. 6). Results from Masson's Trichrome staining confirmed the significant amount of collagen formed within newly regenerated bone (blue color) in the defects implanted with collagen scaffold when compared to the other groups (Fig. 7).

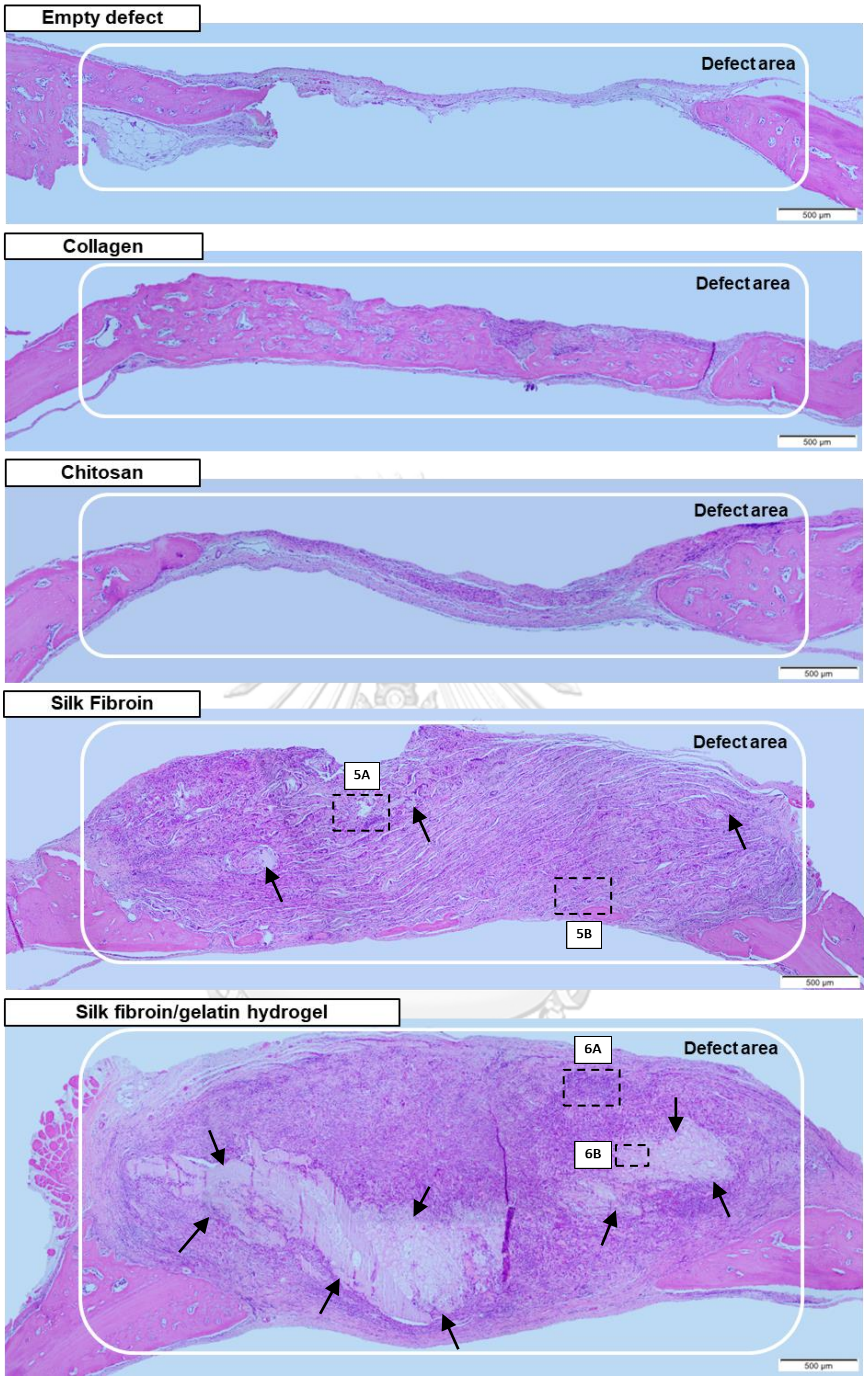

Figure 4. Illustrative histologic sections (4x) with H&E staining demonstrating the extent of new bone formation in the empty defect (control group) and defects implanted with collagen, chitosan, silk fibroin and silk fibroin/gelatin hydrogel. The scaffold remnants are indicated by the black arrow. High-magnification images (20x and 40x) of the black square of silk fibroin and silk fibroin/gelatin hydrogel are shown in figure 5 and 6, respectively.

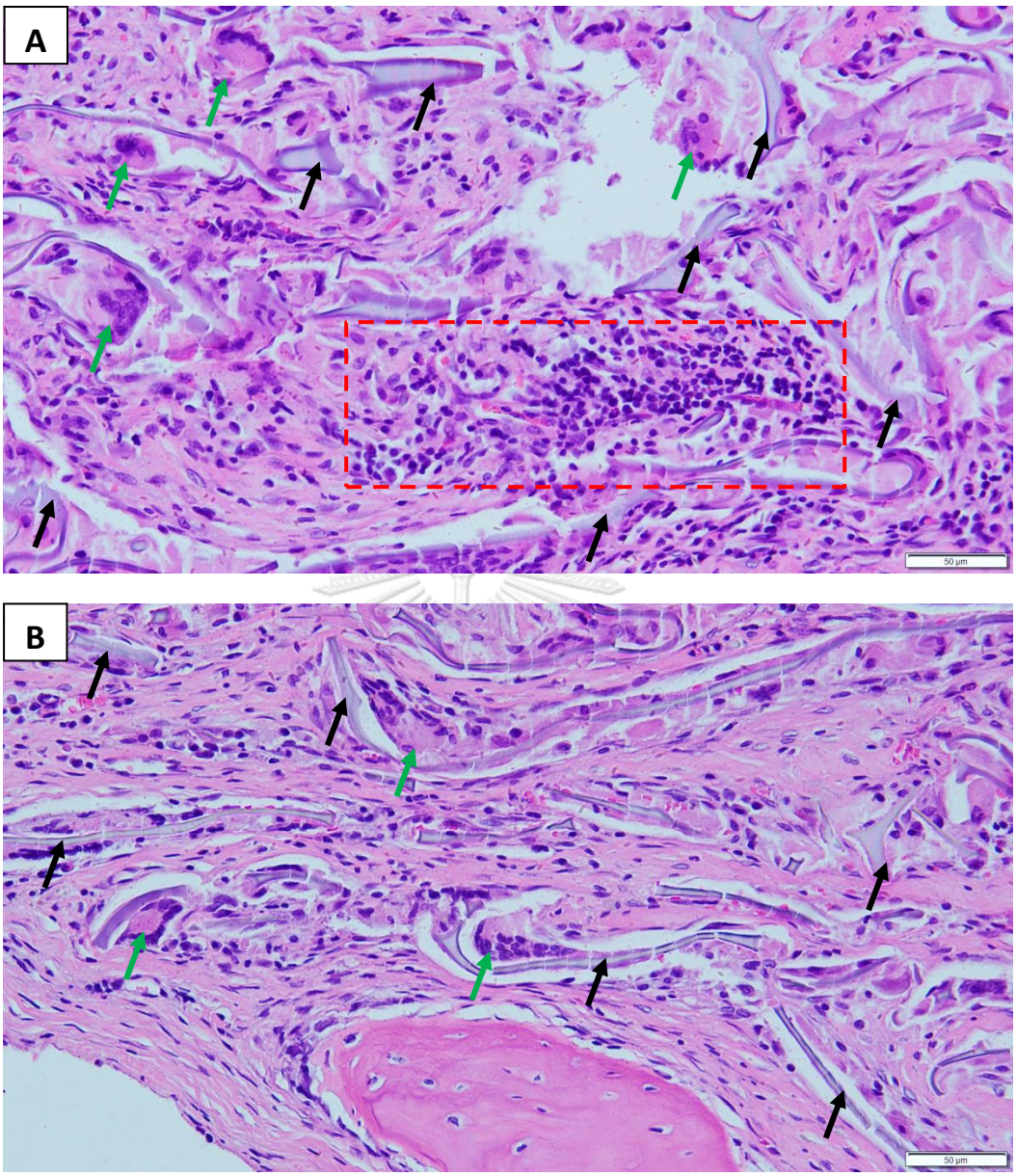

Figure 5. Illustrative histologic sections (20x) with H&E staining of the defects implanted with silk fibroin demonstrating (A) the infiltration of some plasma cells, lymphocytes and multi nucleated giant cells and (B) the infiltration of multi nucleated giant cells between the fibrous tissues and the scaffold remnants. The scaffold remnants are indicated by the black arrow. The intense infiltration of plasma cells and lymphocytes is indicated in the red square. And the multinucleated giant cells are indicated by the green arrow.

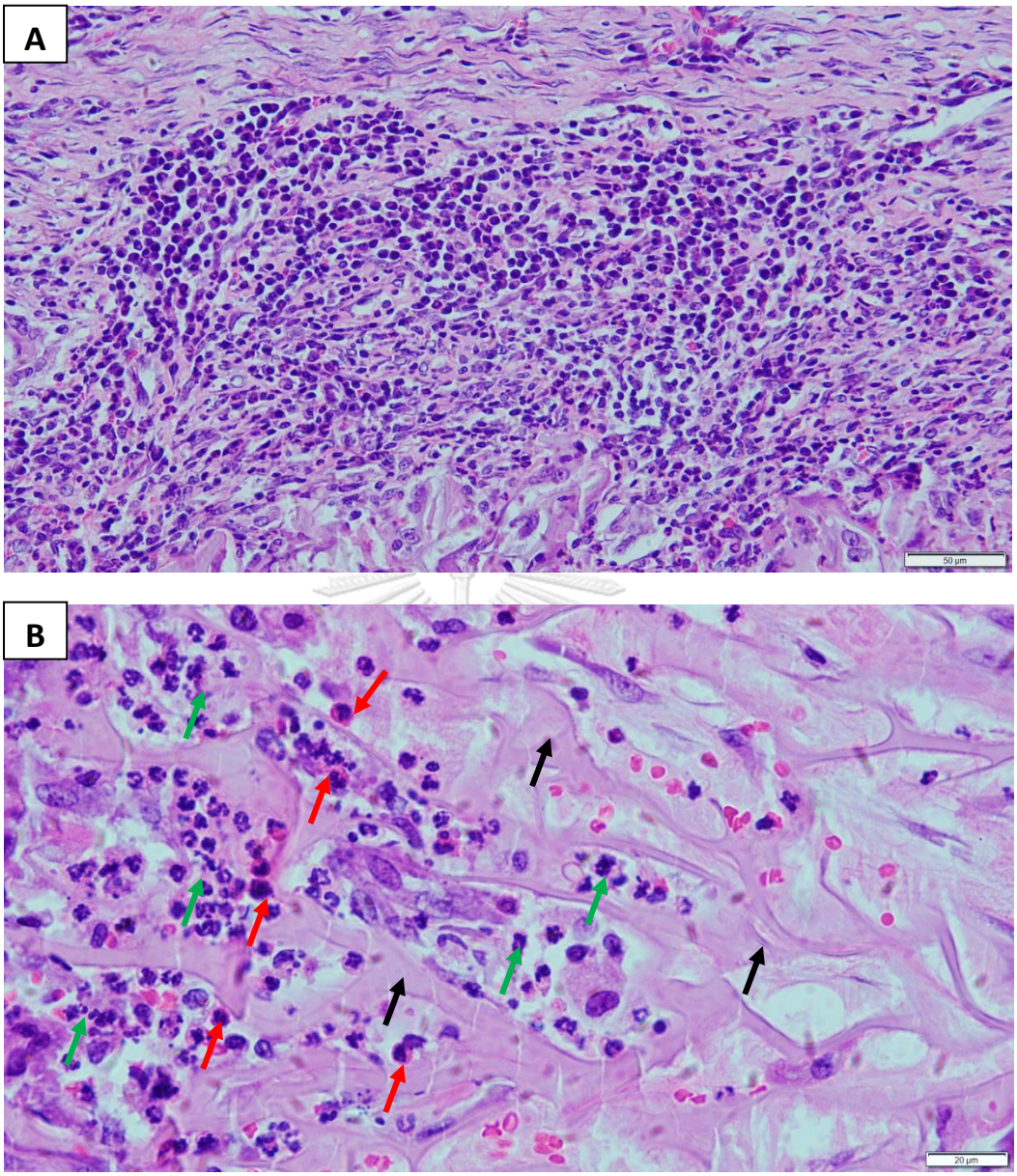

Figure 6. Illustrative histologic sections (6A: 20x and 6B: 40x) with H&E staining of the defects implanted with silk fibroin/gelatin hydrogel demonstrating (A) the infiltration of abundant plasma cells and lymphocytes around the scaffold remnants and (B) the infiltration of abundant neutrophils and some eosinophils between the scaffold remnants. The residual scaffolds are indicated by the black arrow. The infiltration of neutrophils is indicated by the green arrow. The infiltration of eosinophils is indicated by the red arrow.

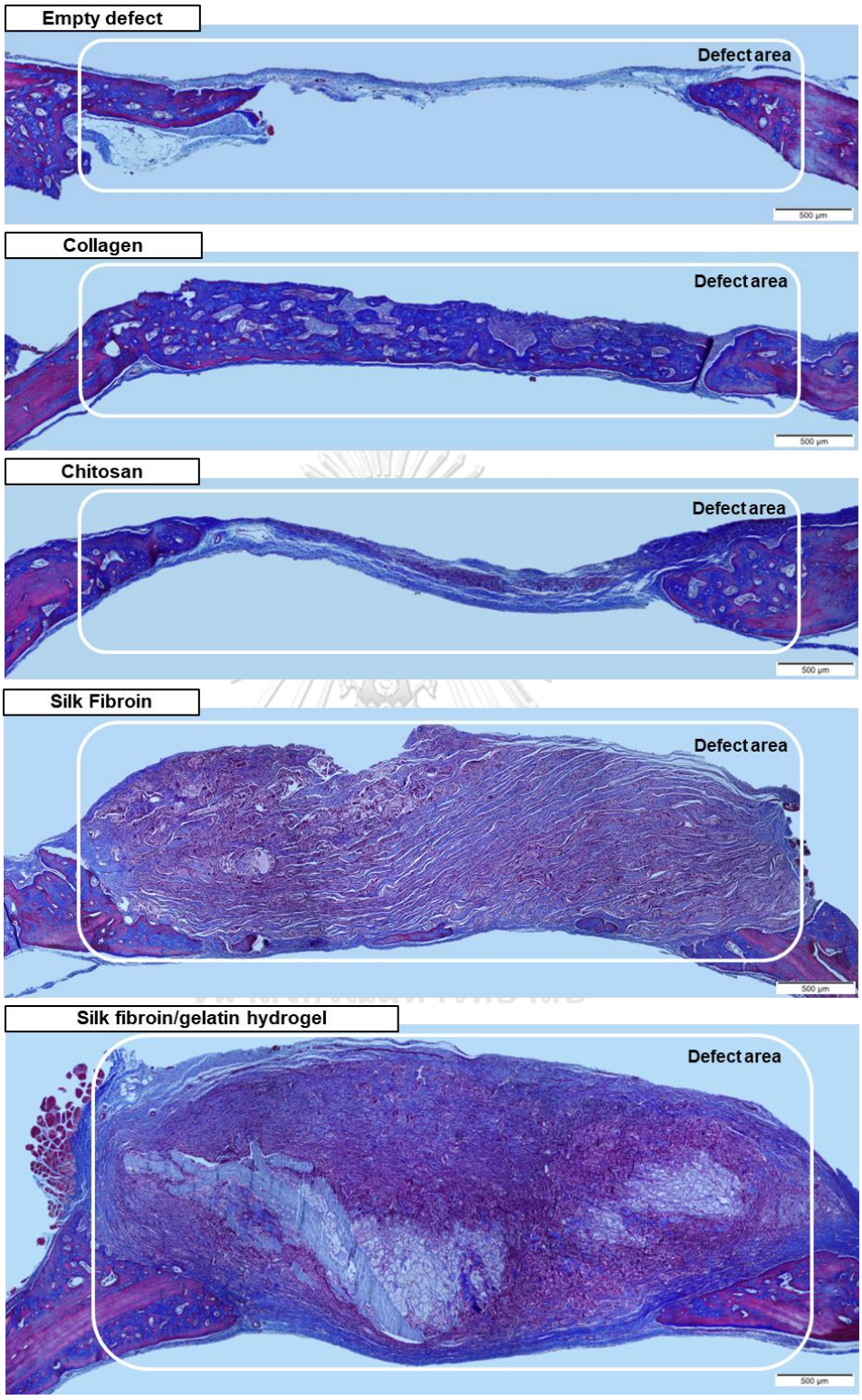

Figure 7. Illustrative histologic sections (4x) with Masson's Trichrome staining highlighting the presence of collagen in the empty defect (control group) and defects implanted with collagen, chitosan, silk fibroin and silk fibroin/gelatin hydrogel.

CHAPTER V

DISCUSSION AND CONCLUSION

There is a need in the field of regenerative medicine for the development of novel scaffolding biomaterials, particularly for bone regeneration. Many scaffolds have been introduced and studied such as polymers, ceramics, metal and composite (Sheikh et al., 2015). None of these, however, are referred to as gold standards. Among different materials, collagen scaffold is one of the most widely used and commercially available for clinical application in various forms including sponge, membrane, powders/particles, fibers and gels (Rico-Llanos et al., 2021). In this study, non-cross-linked type I collagen was compared with chitosan, silk fibroin and silk fibroin/gelatin in hydrogel. It was showed that collagen resulted in greatest new bone formation in rat calvarial bone defects.

Our findings were in accordance with Song et al. that compared the same type of collagen (CollaTape®) in rat calvarial defects to empty defects. In their study, collagen scaffold was implanted for 8 weeks and showed more defect closure with greater bone formation than empty defects (Song et al., 2007). However, Elangovan et al. found that collagen matrices implanted in rat calvarial defects resulted in little or no bone formation similar to empty defects after 4 weeks (Elangovan et al., 2015). This finding was similar to another study that tested collagen sponge in rat femoral defects. There were negligible bone volume/total volume% and bone formation in

both empty and collagen sponge groups at 8 weeks after implantation (Zhang et al., 2019). The discrepancies in the ability of collagen scaffold to induce bone regeneration in rat experiment could be attributed to the heterogeneity of the animal used. Different strains and age were showed to have different osteogenic potential (Hudieb et al., 2021; Li et al., 2003).

Chitosan has been also investigated for its potential in inducing bone regeneration in many studies both *in vitro* and *in vivo*. The results were, however, inconsistent (Sukpaita et al., 2019; Shinohara et al., 2016). Despite its attractive properties for tissue engineering, chitosan has limited water solubility, which is a significant disadvantage. To facilitate chitosan solubility, acid and/or chemical reagents must be used as solvents, which may result in toxic byproducts (Sukpaita et al., 2019). Furthermore, pure chitosan may have inadequate mechanical strength and high degradation rate, especially in acidic environments or in the human body where lysozymes are present. In our study, chitosan alone was not able to induce bone formation. However, chitosan might be used in combination with other growth factors (Shinohara et al., 2016).

Silk fibroin has gained its interest as a potential material for bone scaffolding. Pure silk fibroin was investigated in rat calvarial defects and significantly induced new bone formation as compared to empty defects. However, in our investigation, there was no bone formation in silk fibroin group, and the remnant of scaffold was observed. In this study, silk fibroin structures were converted from random coil to

beta sheet. Thus, its high water solubility was decreased which resulted in some residual scaffold in the defect area (Duangpakdee et al., 2021). Its low stability and bioinertness may also have a significant role that negatively impacts the amount of bone regeneration. This could be improved by incorporating with other molecules (Lee et al., 2017). It was demonstrated that the ability of silk fibroin in regenerating bone *in vivo* was enhanced when combine with betatricalciumphosphate (β-TCP) (Lee et al., 2017). In addition, silk fibroin cross-linked with gelatin demonstrated increased stability and cell adhesion *in vitro* (Duangpakdee et al., 2021). Hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold (CGSF4) promoted significant new bone formation compared to other combinations *in vivo* (Lamlerd et al., 2017). In our study, however, silk fibroin/gelatin (30:70) hydrogel did not induce bone regeneration, and the residual scaffolds were found at the defect area. Future research should be done on other formulations of silk fibroin, such as silk fibroin/bioactive glass scaffold (Duangpakdee et al., 2021; Manissorn et al., 2021), since they may provide different results in *in vivo* model.

Scaffolds used in this study have various pore diameters and resorption rates. Therefore, the results should be interpreted with caution. Rather than type of scaffolds, their overall physical and chemical properties should not be overlooked. Pore sizes and resorption rates, for example, may have an impact on bone regeneration (Zhu et al., 2021). Although macropores (pore size larger than 50 μm)

has advantage for osteogenic quality and cell infiltration, micropores (pore size less than 10 μm) provide greater surface area that leads to better ion exchange and bone protein absorption (Abbasi et al., 2020). At present, the optimal pore size of bone tissue engineering scaffolds is still inconclusive, because different experimental settings (e.g., scaffold materials and bone defect site) often yield different results (Zhu et al., 2021). In our study, collagen has the smallest pore size, while silk fibroin has the largest pore size. Scaffolds should also survive environmental stress to act as a template during bone formation, and then degraded during bone remodeling phase. Previous studies have demonstrated that collagen sponge (CollaTape®) resorbed quickly within 10 to 14 days (Dental Zimmer). Chitosan completely degraded in the sixth weeks *in vivo* (Sukpaita et al., 2019). While silk fibroin rapidly degraded and had just 19.87 percent remaining weight after immersion in de-ionized water for 24 hours (Manissorn et al., 2021), silk fibroin/gelatin hydrogel had almost 100 percent remaining weight even after 10 days *in vitro* (Duangpakdee et al., 2021). The scaffolds should have proper resorption rate that matches to the replacement of new bone growth (Sheikh et al., 2015), which is approximately 2 to 3 weeks in rodents and 6 to 12 week in human (Einhorn & Gerstenfeld, 2015; Rios et al., 2015). According to our findings, a significant amount of silk fibroin/gelatin hydrogel was identified histologically 4 weeks after implantation. In comparison to silk fibroin and silk fibroin/gelatin hydrogel, collagen and chitosan may have an appropriate proper resorption rate. Another limitation of this study is the small sample size and the

short follow-up time which is only 4 weeks. Thus, larger sample sizes and longer follow-up time should be employed in future studies.

In conclusion, within the limitation of this study, collagen is the most effective scaffold in promoting bone regeneration in rat calvarial bone defects after implantation for 4 weeks.

APPENDEX

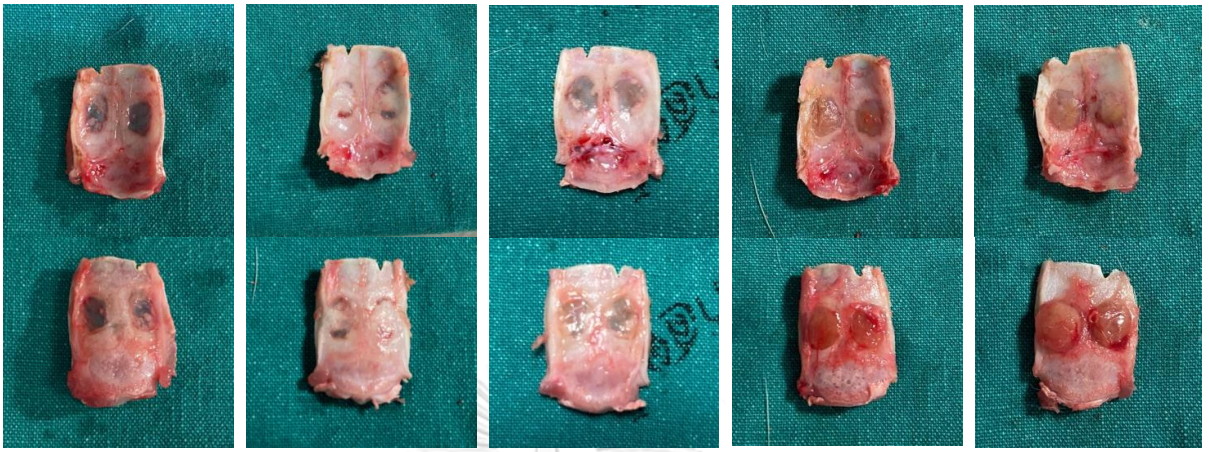

Figure 1. Representative of the calvarial bones that were dissected after 4 weeks of surgery in the empty defect (control group) and defects implanted with collagen, chitosan, silk fibroin and silk fibroin/gelatin hydrogel.

Figure 2. Representative of the scaffolds used in this study consist of collagen, chitosan, silk fibroin and silk fibroin/gelatin hydrogel.

Table 1. The bone volume and bone %volume/total volume of calvarial bones in the empty defect (control group).

Empty defect	1Left	1Right	2Left	2 Right	3 Left	3 Right	4 Left	4 Right	mean	SD
BV(mm3)	0.547	0.373	0.623	1.562	0.492	0.333	1.077	2.295	0.913	0.246
BV/TV(%)	3.480	2.370	3.960	9.940	3.130	2.120	6.850	14.600	5.806	1.563

Table 2. The bone volume and bone %volume/total volume of calvarial bones in the defects implanted with collagen.

Collagen sponge	1Left	1Right	2Left	2 Right	3 Left	3 Right	mean	SD
BV(mm3)	2.011	1.477	3.229	4.291	4.456	5.445	3.485	0.625
BV/TV(%)	12.790	9.400	20.550	27.310	28.350	34.650	22.175	3.975

Table 3. The bone volume and bone %volume/total volume of calvarial bones in the defects implanted with chitosan.

Chitosan	1Left	1Right	2Left	2 Right	3 Left	3 Right	mean	SD
BV(mm3)	1.697	0.347	0.197	0.357	0.165	0.595	0.559	0.236
BV/TV(%)	10.800	2.210	1.250	2.270	1.050	3.780	3.560	1.501

Table 4. The bone volume and bone %volume/total volume of calvarial bones in the defects implanted with silk fibroin.

Silk fibroin	1Left	1Right	2Left	2 Right	3 Left	3 Right	mean	SD
BV(mm3)	0.063	0.190	0.065	0.010	0.850	0.068	0.208	0.131
BV/TV(%)	0.400	1.210	0.420	0.060	5.410	0.430	1.322	0.832

Table 5. The bone volume and bone %volume/total volume of calvarial bones in the defects implanted with silk fibroin/gelatin hydrogel.

SF/gelatin hydrogel	1Left	1Right	2Left	2 Right	3 Left	3 Right	mean	SD
BV(mm3)	0.068	0.015	0.783	0.000	0.907	0.554	0.388	0.168
BV/TV(%)	0.430	0.100	4.980	0.000	5.770	3.520	2.467	1.067

REFERENCES

Abbasi N, Hamlet S, Love RM, Nguyen N-T. Porous scaffolds for bone regeneration. Journal of Science: Advanced Materials and Devices. 2020;5(1):1-9.

Bergman RJ, Gazit D, Kahn AJ, Gruber H, McDougall S, Hahn TJ. Age-related changes in osteogenic stem cells in mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1996;11(5):568-77.

Boskey AL, Coleman R. Aging and bone. Journal of dental research. 2010;89(12):1333-48.

Bosshardt DD, Sculean A. Does periodontal tissue regeneration really work? Periodontology 2000. 2009;51:208-19.

Chen CK, Chang NJ, Wu YT, Fu E, Shen EC, Feng CW, et al. Bone Formation Using Cross-Linked Chitosan Scaffolds in Rat Calvarial Defects. Implant dentistry. 2018;27(1):15-21.

Choi JH, Kim DK, Song JE, Oliveira JM, Reis RL, Khang G. Silk Fibroin-Based Scaffold for Bone Tissue Engineering. Advances in experimental medicine and biology. 2018;1077:371-87.

Cooper GM, Mooney MP, Gosain AK, Campbell PG, Losee JE, Huard J. Testing the critical size in calvarial bone defects: revisiting the concept of a critical-size defect. Plastic and reconstructive surgery. 2010;125(6):1685-92.

Damiati LA, El-Messeiry S. An Overview of RNA-Based Scaffolds for Osteogenesis. Frontiers in molecular biosciences. 2021;8:682581.

Dental Z. Absorbable Collagen Wound Dressings; CollaTape, CollaCote and CollaPlug [package insert]. Carlsbad, CA:Zimmer Dental.

Duangpakdee A, Laomeephol C, Jindatip D, Thongnuek P, Ratanavaraporn J, Damrongsakkul S. Crosslinked Silk Fibroin/Gelatin/Hyaluronan Blends as Scaffolds for Cell-Based Tissue Engineering. Molecules (Basel, Switzerland). 2021;26(11).

Dumic-Cule I, Draca N, Luetic AT, Jezek D, Rogic D, Grgurevic L, et al. TSH prevents bone resorption and with calcitriol synergistically stimulates bone formation in rats with low levels of calciotropic hormones. Hormone and metabolic research = Hormon-

und Stoffwechselforschung = Hormones et metabolisme. 2014;46(5):305-12.

Duong H-Y, Schmid E. Natural History of Periodontitis. Current Oral Health Reports. 2014;1.

Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. Nature reviews Rheumatology. 2015;11(1):45-54.

El-Sherbiny IM, Yacoub MH. Hydrogel scaffolds for tissue engineering: Progress and challenges. Global cardiology science & practice. 2013;2013(3):316-42.

Elangovan S, Khorsand B, Do AV, Hong L, Dewerth A, Kormann M, et al. Chemically modified RNA activated matrices enhance bone regeneration. Journal of controlled release : official journal of the Controlled Release Society. 2015;218:22-8.

Ferreira AM, Gentile P, Chiono V, Ciardelli G. Collagen for bone tissue regeneration. Acta Biomater. 2012;8(9):3191-200.

Filippi M, Born G, Chaaban M, Scherberich A. Natural Polymeric Scaffolds in Bone Regeneration. Frontiers in bioengineering and biotechnology. 2020;8:474.

Gaharwar AK, Singh I, Khademhosseini A. Engineered biomaterials for in situ tissue regeneration. Nature Reviews Materials. 2020;5(9):686-705.

Galli M, Yao Y, Giannobile WV, Wang H-L. Current and future trends in periodontal tissue engineering and bone regeneration. Plastic and Aesthetic Research. 2021;8:3.

Gosain AK, Song L, Yu P, Mehrara BJ, Maeda CY, Gold LI, et al. Osteogenesis in cranial defects: reassessment of the concept of critical size and the expression of TGF-beta isoforms. Plastic and reconstructive surgery. 2000;106(2):360-71; discussion 72.

Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. Trends in immunology. 2014;35(1):3-11.

Han J, Menicanin D, Gronthos S, Bartold PM. Stem cells, tissue engineering and periodontal regeneration. Australian dental journal. 2014;59 Suppl 1:117-30.

Holguin N, Aguilar R, Harland RA, Bomar BA, Silva MJ. The aging mouse partially models the aging human spine: lumbar and coccygeal disc height, composition, mechanical properties, and Wnt signaling in young and old mice. Journal of applied physiology (Bethesda, Md : 1985). 2014;116(12):1551-60.

Hollister SJ. Scaffold design and manufacturing: from concept to clinic. Advanced materials (Deerfield Beach, Fla). 2009;21(32-33):3330-42.

Hudieb M, Haddad A, Bakeer M, Alkhazaaleh A, AlKhader M, Taani D, et al. Influence of Age on Calvarial Critical Size Defect Dimensions: A Radiographic and Histological Study. The Journal of craniofacial surgery. 2021;32(8):2896-900.

Jee WS, Yao W. Overview: animal models of osteopenia and osteoporosis. Journal of musculoskeletal & neuronal interactions. 2001;1(3):193-207.

Kalu DN. The ovariectomized rat model of postmenopausal bone loss. Bone and mineral. 1991;15(3):175-91.

Karring T, Nyman S, Gottlow J, Laurell L. Development of the biological concept of guided tissue regeneration--animal and human studies. Periodontology 2000. 1993;1:26-35.

Kweon H, Kim S-G, An J, Shim H, Yang B, Kim J, et al. Silk Fibroin Membrane as Guided Bone Regeneration in Rat Calvarial Defects. Int J Indust Entomol. 2010;Vol. 21, No. 2:175-9.

Lamlerd T, Soontornvipart K, Kesdangsakonwut S, Kanokpanont S, Damrongsakkul S. In vivo bone regeneration of thai silk fibroin scaffolds with gelatin, hydroxyapatite and hyaluronic acid. Thai Journal of Veterinary Medicine. 2017;47:165-72.

Lauritano D, Limongelli L, Moreo G, Favia G, Carinci F. Nanomaterials for Periodontal Tissue Engineering: Chitosan-Based Scaffolds. A Systematic Review. Nanomaterials (Basel, Switzerland). 2020;10(4).

Lee DH, Tripathy N, Shin JH, Song JE, Cha JG, Min KD, et al. Enhanced osteogenesis of β-tricalcium phosphate reinforced silk fibroin scaffold for bone tissue biofabrication. International journal of biological macromolecules. 2017;95:14-23.

Lelovas PP, Xanthos TT, Thoma SE, Lyritis GP, Dontas IA. The laboratory rat as an animal model for osteoporosis research. Comparative medicine. 2008;58(5):424-30.

Li JZ, Li H, Dunford B, Holman D, Beres B, Pittman DD, et al. Rat strain differences in the ectopic osteogenic potential of recombinant human BMP adenoviruses. Molecular therapy : the journal of the American Society of Gene Therapy. 2003;8(5):822-9.

Li S, Quarto N, Longaker MT. Dura mater-derived FGF-2 mediates mitogenic signaling in calvarial osteoblasts. American journal of physiology Cell physiology. 2007;293(6):C1834-42.

Li Y, Liu Z, Tang Y, Fan Q, Feng W, Luo C, et al. Three-dimensional silk fibroin scaffolds enhance the bone formation and angiogenic differentiation of human amniotic mesenchymal stem cells: a biocompatibility analysis. Acta biochimica et biophysica Sinica. 2020;52(6):590-602.

Lim J, Lee J, Yun H-S, Shin H-I, Park EK. Comparison of bone regeneration rate in flat and long bone defects: Calvarial and tibial bone. Tissue Engineering and Regenerative Medicine. 2013;10(6):336-40.

Liu Y, Nelson T, Chakroff J, Cromeens B, Johnson J, Lannutti J, et al. Comparison of polyglycolic acid, polycaprolactone, and collagen as scaffolds for the production of tissue engineered intestine. Journal of biomedical materials research Part B, Applied biomaterials. 2019;107(3):750-60.

Manissorn J, Wattanachai P, Tonsomboon K, Bumroongsakulsawat P, Damrongsakkul S, Thongnuek P. Osteogenic enhancement of silk fibroin-based bone scaffolds by forming hybrid composites with bioactive glass through GPTMS during sol-gel process. Materials Today Communications. 2021;26:101730.

Miller SC, Bowman BM, Jee WS. Available animal models of osteopenia--small and large. Bone. 1995;17(4 Suppl):117s-23s.

Mills LA, Simpson AH. In vivo models of bone repair. The Journal of bone and joint surgery British volume. 2012;94(7):865-74.

Miyata T, Taira T, Noishiki Y. Collagen engineering for biomaterial use. Clinical materials. 1992;9(3-4):139-48.

Murphy MP, Quarto N, Longaker MT, Wan DC. (*) Calvarial Defects: Cell-Based Reconstructive Strategies in the Murine Model. Tissue engineering Part C, Methods. 2017;23(12):971-81.

Nakae H, Narayanan AS, Raines E, Page RC. Isolation and partial characterization of mitogenic factors from cementum. Biochemistry. 1991;30(29):7047-52.

Okhawilai M, Rangkupan R, Kanokpanont S, Damrongsakkul S. Preparation of Thai silk fibroin/gelatin electrospun fiber mats for controlled release applications. International journal of biological macromolecules. 2010;46(5):544-50.

Peric M, Dumic-Cule I, Grcevic D, Matijasic M, Verbanac D, Paul R, et al. The rational use of animal models in the evaluation of novel bone regenerative therapies. Bone.

2015;70:73-86.

Porto GG, Vasconcelos BC, Andrade ES, Carneiro SC, Frota MS. Is a 5 mm rat calvarium defect really critical? Acta cirurgica brasileira. 2012;27(11):757-60.

Rico-Llanos GA, Borrego-González S, Moncayo-Donoso M, Becerra J, Visser R. Collagen Type I Biomaterials as Scaffolds for Bone Tissue Engineering. Polymers. 2021;13(4).

Rios HF, Bashutski JD, Giannobile WV. Bone as a living organ. In: Lang NP, Lindhe J, editors. Clinical periodontology and implant dentistry. 1. 6th ed: Hoboken : John Wiley & Sons, Incorporated; 2015. p. 48-64.

Schmitz JP, Hollinger JO. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clinical orthopaedics and related research. 1986(205):299-308.

Sheikh Z, Najeeb S, Khurshid Z, Verma V, Rashid H, Glogauer M. Biodegradable Materials for Bone Repair and Tissue Engineering Applications. Materials (Basel, Switzerland). 2015;8(9):5744-94.

Shinohara Y, Nakamura T, Shirakata Y, Noguchi K. Bone healing capabilities of recombinant human bone morphogenetic protein-9 (rhBMP-9) with a chitosan or collagen carrier in rat calvarial defects. Dental materials journal. 2016;35(3):454-60.

Slater BJ, Kwan MD, Gupta DM, Lee JK, Longaker MT. The role of regional posterior frontal dura mater in the overlying suture morphology. Plastic and reconstructive surgery. 2009;123(2):463-9.

Smith DM, Cray JJ, Jr., Weiss LE, Dai Fei EK, Shakir S, Rottgers SA, et al. Precise control of osteogenesis for craniofacial defect repair: the role of direct osteoprogenitor contact in BMP-2-based bioprinting. Annals of plastic surgery. 2012;69(4):485-8.

Song KY, Um YJ, Kim CK, Choi SH, Lee YK, Jung UW. The effects of collagen membrane coated with PLGA on bone regeneration in rat calvarial defects. Key Engineering Materials. 2007;342~343:357~60-60.

Spector JA, Greenwald JA, Warren SM, Bouletreau PJ, Detch RC, Fagenholz PJ, et al. Dura mater biology: autocrine and paracrine effects of fibroblast growth factor 2. Plastic and reconstructive surgery. 2002;109(2):645-54.

Spicer PP, Kretlow JD, Young S, Jansen JA, Kasper FK, Mikos AG. Evaluation of bone regeneration using the rat critical size calvarial defect. Nature protocols.

2012;7(10):1918-29.

Stevens MM. Biomaterials for bone tissue engineering. Materials Today. 2008;11(5):18-25.

Stokovic N, Ivanjko N, Maticic D, Luyten FP, Vukicevic S. Bone Morphogenetic Proteins, Carriers, and Animal Models in the Development of Novel Bone Regenerative Therapies. 2021;14(13):3513.

Sukpaita T, Chirachanchai S, Suwattanachai P, Everts V, Pimkhaokham A, Ampornaramveth RS. In Vivo Bone Regeneration Induced by a Scaffold of Chitosan/Dicarboxylic Acid Seeded with Human Periodontal Ligament Cells. International journal of molecular sciences. 2019;20(19).

Sun W, Gregory DA, Tomeh MA, Zhao X. Silk Fibroin as a Functional Biomaterial for Tissue Engineering. International journal of molecular sciences. 2021;22(3).

Suwattanachai P, Pimkhaokham A, Chirachanchai S. Multi-functional carboxylic acids for chitosan scaffold. International journal of biological macromolecules. 2019;134:156-64.

Trombelli L. Which reconstructive procedures are effective for treating the periodontal intraosseous defect? Periodontology 2000. 2005;37:88-105.

Trombelli L, Farina R, Silva CO, Tatakis DN. Plaque-induced gingivitis: Case definition and diagnostic considerations. Journal of clinical periodontology. 2018;45 Suppl 20:S44-s67.

Vajda EG, Bowman BM, Miller SC. Cancellous and cortical bone mechanical properties and tissue dynamics during pregnancy, lactation, and postlactation in the rat. Biology of reproduction. 2001;65(3):689-95.

Vajgel A, Mardas N, Farias BC, Petrie A, Cimões R, Donos N. A systematic review on the critical size defect model. Clinical oral implants research. 2014;25(8):879-93.

Van Vlierberghe S, Dubruel P, Schacht E. Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review. Biomacromolecules. 2011;12(5):1387-408.

Villar CC, Cochran DL. Regeneration of periodontal tissues: guided tissue regeneration. Dental clinics of North America. 2010;54(1):73-92.

Wan DC, Kwan MD, Gupta DM, Wang Z, Slater BJ, Panetta NJ, et al. Global age-dependent differences in gene expression in response to calvarial injury. The

Journal of craniofacial surgery. 2008;19(5):1292-301.

Zhang W, De La Vega RE, Coenen MJ, Müller SA, Peniche Silva CJ, Aneja MK, et al. An Improved, Chemically Modified RNA Encoding BMP-2 Enhances Osteogenesis In Vitro and In Vivo. Tissue engineering Part A. 2019;25(1-2):131-44.

Zhu G, Zhang T, Chen M, Yao K, Huang X, Zhang B, et al. Bone physiological microenvironment and healing mechanism: Basis for future bone-tissue engineering scaffolds. Bioactive materials. 2021;6(11):4110-40.

VITA

NAME	จีราวิชช์ ศิรกิตติวรพงษ์
DATE OF BIRTH	23 มกราคม 2537
PLACE OF BIRTH	สุพรรณบุรี
INSTITUTIONS ATTENDED	ทันตแพทยศาสตร์บัณฑิต มหาวิทยาลัยศรีนครินทรวิโรฒ
HOME ADDRESS	144/193 ถ.มาลัยแมน ต.รั้วใหญ่ อ.เมือง จ.สุพรรณบุรี 72000
AWARD RECEIVED	เกียรตินิยมอันดับ 2