Biocompatibility and Biodegradation of Deproteinized Human Demineralized Tooth Matrix (dpDTM) in Rabbit Model

Sureerat Tanwatana

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

Prince of Songkla University

2019

Biocompatibility and Biodegradation of Deproteinized Human Demineralized Tooth Matrix (dpDTM) in Rabbit Model

Sureerat Tanwatana

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

Prince of Songkla University

2019

Thesis Title	Biocompatibility and Biodegradation of Deproteinized Human Demineralized Tooth Matrix (dpDTM) in Rabbit Model
Author	Miss Sureerat Tanwatana
Major Program	Oral and Maxillofacial Surgery

Major Advisor

……..…….....

(Assoc. Prof. Dr.Srisurang Suttapreyasri)

Co-advisors

……..…….....

(Asst. Prof. Narit Leepong)

Examining Committee :

……..…..……..Chairperson

(Assoc. Prof. Dr. Atiphan Pimkhaokham)

……...……..Committee

(Assoc. Prof. Dr. Srisurang Suttapreyasri)

……...……..Committee

(Asst. Prof. Narit Leepong)

……...……..Committee

(Asst. Prof. Dr. Bancha Samruajbenjakun)

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. Damrongsak Faroongsarng)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

..……

(Assoc. Prof. Dr. Srisurang Suttapreyasri)

Major Advisor

...……

(Miss Sureerat Tanwatana)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

..……

(Miss Sureerat Tanwatana)

Candidate

ชื่อวิทยานิพนธ์ การศึกษาคุณสมบัติความเข้ากันได้ทางชีวภาพและการย่อยสลายทางชีวภาพของดีมินเนอราไลซ์ทูธเมทริกที่ผ่านกระบวนการสกัดโปรตีนออกในกระต่าย

ผู้เขียน นางสาวสุรีรัตน์ ตันวัฒนะ

สาขาวิชา ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล

ปีการศึกษา 2561

บทคัดย่อ

ที่มาและความสำคัญ: ฟันมนุษย์เป็นอวัยวะที่ประกอบด้วยสัดส่วนของอินทรีย์สารและอนินทรีย์สารใกล้เคียงกับกระดูกมนุษย์ ชิ้นส่วนฟันได้ถูกพัฒนาและนำมาใช้เป็นวัสดุทดแทนกระดูก โดยนำมาใช้ทางคลินิกในรอยวิการรูปแบบต่างๆอย่างแพร่หลาย อย่างไรก็ตามข้อจำกัดหลักในเรื่องของการนำฟันตนเองมาใช้ทดแทนกระดูก คือ มีปริมาณฟันที่จำกัด ดังนั้นดีมินเนอราไลซ์ทูธเมทริกที่ผ่านกระบวนการสกัดโปรตีนออก (dpDTM) ได้ถูกพัฒนาให้เป็นวัสดุทดแทนกระดูกเอกพันธุ์ (Allograft) เพื่อนำมาใช้ทดแทนกระดูกข้ามคน โดยมีวัตถุประสงค์งานวิจัย คือ เพื่อประเมินและเปรียบเทียบความเข้ากันได้ทางชีวภาพ และการย่อยสลายทางชีวภาพของ dpDTM และกระดูกวัวสกัดโปรตีนออก (ABB) โดยฝังวัสดุทดสอบในกล้ามเนื้อส่วนหลังของกระต่ายที่ระยะเวลา 1, 2, 4, 8 และ 12 สัปดาห์

วัสดุและวิธีการ: นำฟันที่ได้จากการถอนฟัน ตัดแบ่งตัวฟันและรากฟัน นำเนื้อเยื่อโพรงประสาทฟันออก นำชิ้นส่วนฟันไปบดให้มีขนาด 500-1,000 ไมครอน จากนั้นแช่ในสารละลายกรดไฮโดรคลอริกความเข้มข้น 0.5 โมล เพื่อละลายแร่ธาตุบางส่วนออกจากฟัน นำชิ้นส่วนฟันผ่านกระบวนการสกัดโปรตีนออกโดยใช้สารเคมีและความร้อน (โซเดียมไฮดรอกไซด์, 300 องศาเซลเซียส) ชิ้นส่วนฟันจะถูกนำมาขึ้นรูปโดยใช้โพลิไวนิลแอลกอฮอล์ไฮโดรเจล ร้อยละ 5 โดยน้ำหนักต่อปริมาตร ด้วยกระบวนการฟรีซทรอล งานวิจัยในครั้งนี้ แบ่งกลุ่มการทดลองออกเป็น 3 กลุ่ม ได้แก่ กลุ่มที่ฝังวัสดุ dpDTM, ABB และ โพลิไวนิลแอลกอฮอล์ไฮโดรเจล(PVA)โดยนำวัสดุดังกล่าวไปฝังบริเวณกล้ามเนื้อส่วนหลังขอกระต่ายขาวนิวซีแลนด์ จำนวน 15 ตัว ประเมินผลความเข้ากันได้ทางชีวภาพโดยการประเมินทางคลินิกและอาศัยการส่งตรวจทางพยาธิวิทยาในส่วนของการประเมินผลในเรื่องการย่อยสลายทางชีวภาพประเมินผล โดยการส่งตรวจทางพยาธิวิทยาและการใช้เครื่องเอกเรย์คอมพิวเตอร์ระดับไมโครเมตร การประเมินผลทั้งหมดทำที่ช่วงระยะเวลา 1, 2, 4, 8 และ 12 สัปดาห์

ผลการศึกษา: กระต่ายทั้ง 15 ตัวมีสุขภาพแข็งแรงไม่พบการลดลงของน้ำหนักตัว ผลการศึกษาทางคลินิกไม่พบการติดเชื้อและการอักเสบในทุกช่วงระยะเวลาการศึกษา ผลการศึกษาทางพยาธิวิทยาในกลุ่ม dpDTM และ ABB พบการอักเสบในปริมาณมากในช่วง 1 ถึง 2 สัปดาห์ การอักเสบลดลงเมื่อระยะเวลาผ่านไปและลดลงจนอยู่ในระดับปานกลางที่ระยะเวลา 4 ถึง 12 สัปดาห์ นอกจากนั้นพบว่าค่าเฉลี่ยปริมาณเซลล์อักเสบไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่ม dpDTM และ ABB ผลการศึกษาการย่อยสลายทางชีวภาพพบว่า ค่าเฉลี่ยปริมาตรกระดูกต่อปริมาตรทั้งหมด (BV/TV) ในกลุ่ม dpDTM มีค่าสูงกว่าในกลุ่ม ABB ในทุกช่วงระยะเวลาการศึกษา โดยมีร้อยละการสลายตัวของกลุ่ม dpDTM และ ABB เท่ากับ 1.663 และ 1.897 ตามลำดับ

บทสรุป: dpDTM และ ABB มีความเข้ากันได้ทางชีวภาพกับเนื้อเยื่อโดยพบการอักเสบในระดับปานกลางที่ระยะเวลา 12 สัปดาห์ และมีความสามารถในการสลายตัวเมื่อระยะเวลาผ่านไป โดยมีอัตราการสลายตัวช้า แสดงให้เห็นว่า dpDTM สามารถนำมาใช้เป็นวัสดุทดแทนกระดูกจากคุณสมบัติที่มีความเข้ากันได้ทางชีวภาพและมีอัตราการสลายตัวที่เหมาะสม อย่างไรก็ตาม การนำวัสดุ dpDTM ไปศึกษาต่อในระดับการศึกษาทดลองในมนุษย์เป็นสิ่งจำเป็นก่อนจะสามารถนำวัสดุไปใช้เป็นวัสดุทดแทนกระดูกในมนุษย์ต่อไป

Thesis Title Biocompatibility and Biodegradation of Deproteinized Human Demineralized Tooth Matrix (dpDTM) in Rabbit Model
Author Miss Sureerat Tanwatana
Major Program Oral and Maxillofacial Surgery
Academic Year 2018

ABSTRACT

Background and Objectives: Tooth is a composite structure consisting of inorganic and organic components which are very similar to those of alveolar bone. The autologous tooth matrix has been successfully developed and has been used clinically as bone substitution in various bone defect model. However, the major drawback of the autologous tooth matrix is the limited availability of the graft. Therefore, deproteinized human demineralized tooth matrix (dpDTM) was developed into allograft materials to overcome those limitations. The aims of this study were to assess and compare the biocompatibility and biodegradation of dpDTM and anorganic bovine bone (ABB) by intramuscular implantation at 1, 2, 4, 8, 12 weeks

Materials and Methods: dpDTM was prepared by using 0.5M HCl for demineralization. Deproteinization was performed by using dual chemical-thermal treatment (NaOH 1N, 300ºC). 5%wt/v Polyvinyl alcohol (PVA) was used to fabricate scaffold using hydrogel solution as binding materials and the scaffolds were prepared by freeze-thawing method. The dpDTM, ABB and PVA scaffolds (n=6/group/time point) were implanted into paralumbar muscle of the 15 adult New Zealand White rabbits. The biocompatibility analysis was performed by clinical and histological methods. The biodegradation analysis was achieved by histological images and Micro computed tomography (Micro-CT). All the assessments were performed after healing period of 1, 2, 4, 8 and 12 weeks.

Results: All rabbits recovered uneventfully without weight loss. Clinical observation revealed the absence of infection and inflammation at all time point. Histological data of dpDTM and ABB bone indicated the moderate to intense inflammatory response at 1-2 weeks and decreased with time to moderate grade inflammatory response at 8-12 weeks. The mean inflammatory cells count was not significant different between two groups at all time point. According to the biodegradation, the relative bone volume/total volume (BV/TV) in dpDTM scaffolds was greater than that in ABB scaffolds at all time point. The percentage of reduction in dpDTM and ABB were 1.663% and 1.897% respectively.

Conclusions: In terms of biocompatibility, dpDTM and ABB demonstrated good biocompatibility with moderate degree of inflammatory cells infiltration at 12 weeks. Both materials had potential to degrade with time with a slow degradability rate. Suggesting that dpDTM was favorable for reconstructive bone graft substitution due to its good biocompatibility together with the slow degradation profiles. However, clinically relevant implantation studies should be concerned before human used of this tooth graft material.

ACKNOWLEDGEMENTS

I would like to express my appreciation to my sincere supervisor, Assoc. Prof. Dr. Srisurang Suttapreyasri and Asst. Prof. Narit Leepong who have encouraged, supported, introduced and suggested me for all the time about this research not only the experimental period of time but also the data analysis and research written.

I would also like to thank to the Department of Oral and Maxillofacial Surgery Faculty of Dentistry, Southern Laboratory Animal Facility, Scientific Equipment Center at Prince of Songkla University for supported all the research facilities.

In addition, I would like to thank you to my senior assistance Miss. Ajchara Kaewjurat who have assisted me in so many parts including the rabbit operation, histological analysis and micro-CT analysis. Moreover, I would like to thank my parents, Mr. Wachirakorn Aonsong and my research partner Dr.Anopong Jeerachaipansakul who always supported me and cheered me up when I have got some difficulty situation during the research time. This achievement would not have been possible without them.

Sureerat Tanwatana

CONTENTS

Contents **Page**

CONTENTS (CONTINUED)

Contents **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

Figure **Page**

LIST OF ABBREVIATIONS AND SYMBOLS

dpDTM = Deproteinized human demineralized tooth matrix

ABB = Anorganic deproteinized bovine bone

PVA = Polyvinyl alcohol

NaOH = Sodium hydroxide

H_2O_2 = Hydrogen peroxide

NaOCl = Sodium hyprochloride

KOH = Potassium hydroxide

HCL = Hydrochloric acid

EDTA = Ethylene diamine tetra-acetic acid

XRD = X-ray Diffractometer

XRF = X-ray Fluorescence

SEM = Scanning Electron Microscope

FTIR = Fourier Transform Infrared Spectrophotometer

MHC-II = Major histocompatibility complex

TGF = Transforming growth factor beta

CD4 = Cluster of differentiation 4

CD8 = Cluster of differentiation 8

OPN = Osteopontin

DSP = Dentin sialoprotein

DPP = Dentin phosphoprotein

IL-1 = Interleukin 1

TNF-alpha = Tumor necrosis factor- alpha

ISO = International Organization for Standardization

Ca-P = Calcium phosphate ratio

mg = Milligram

ml = Milliliter

μm^2 = Micron square

wt/v = Weight per volume

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

BV/TV = Bone Volume per Total Volume

Micro-CT = Microcomputed tomography

H&E = Hematoxylin and Eosin stainning

HA = Hydroxyappatite

β-TCP = Beta-tricalcium phosphate

Auto BT = Autogenous Tooth Bone Graft

DPSCs = Dental Pulp Stem Cells

Micro-CT = Micro- Computed Tomography

GBR = Guided Bone Regeneration

HRA = Horizontal Ridge Augmentation

EDS = Energy Dispersive X-ray Spectroscopy

Real time PCR= Real Time Polymerase Chain Reaction

ALP = Alkaline Phosphatase

OCN = Osteocalcin

APDDM = Autogenous partially demineralized dentin matrix.

DDM = Demineralized dentin matrix

CHAPTER 1

INTRODUCTION

Alveolar bone defects develop as a result of trauma, infection, tumor resection, or post-extraction. To reconstruct the bone defect, various types of biomaterials have become essential in dental and orthopedic reconstruction. The autogenous bone graft has been considered as a gold standard material which promotes osteogenesis, osteoinduction, and osteoconduction. Nonetheless, the major complications of autogenous bone graft are donor site morbidity and limited availability. The uses of allograft, xenograft and synthetic materials can overcome those limitations. But their specific concerns are disease transmission, immunogenic reaction and high cost of the materials.

Tooth is a composite structure consisting of inorganic and organic components which are very similar to those of alveolar bone. The autologous tooth matrix successfully developed and has been used clinically as bone substitution in various bone defect model such as alveolar ridge preservation, peri-implant defect augmentation and maxillary sinus augmentation. However, the major drawback of the autologous tooth matrix is the limited availability of the graft in case of large bone defect, and no donor tooth to be extracted. Therefore, deproteinized human demineralized tooth matrix (dpDTM) has been developed into allograft materials to overcome these limitations. The aims of this study were to assess and compare the biocompatibility and biodegradation of dpDTM and anorganic bovine bone (ABB) by intramuscular implantation at 1, 2, 4, 8, 12 weeks.

Bone grafting materials in oral surgery

Alveolar ridge defects develop as a result of surgery, trauma, infection, or congenital malformations. The insufficient quantity of bone after tooth loss result in rapid resorption of alveolar bone due to lack of intraosseous stimulation by periodontal ligament (PDL) fibers[1]. It is well established that tooth extraction followed by a reduction of the bucco-lingual as well as apico-coronal dimension of the alveolar ridge at the edentulous site[2].

Bone grafting is a surgical procedure that replaces missing bone using material from patient′s own body, an artificial, synthetic, or natural substitute. Bone grafting in oral surgery is obligatory to regenerate such defects to have successful rehabilitation and aims to maintenance of contour, elimination of dead space and thus enhance bony and soft tissue healing[1].

There are a variety of sources of bone grafting material used for preserving or augmenting the bone in oral and maxillofacial surgery including autogenous bone graft, allogenic bone graft, xenogeneic bone graft, synthetic/alloplastic bone graft and composite bone graft[3].

Autogenous bone is derived from patients which may come from intra-oral (symphysis, ramus) or extra-oral (hip, ileac, femur etc.) area. Several advantages found in autogenous bone graft including the biocompatibility, osteoinductive,

osteoconductive, and osteogenic properties. However due to the limited donor site and high morbidity of autogenous bone graft, other bone substitution materials have been used to replaced it. An allogenic bone graft is defined as tissue which has been harvested from one individual and implanted into another of the same species. Several processes have been used to reduce the host immune reaction and risk of disease transmission. The osteoinductive and osteoconductive properties of allograft varied depending on graft processing and methods. Xenogenic bone graft harvests from one species and implanted into another species. The deproteinization and defatted are reported to reduce immune response but these processes also destroy the osteoinductive proteins of the graft. The materials usually originated from bovine, porcine, horse etc. The bovine xenogenic bone graft is most widely used to repair the alveolar bone defect in oral and maxillofacial surgery. For synthetic bone graft, this graft is usually derived from calcium phosphate-base materials such as hydroxyapatite (HA), biphasic calcium phosphate (BCP), beta-tricalcium phosphate (β-TCP), etc. This type of grafts has both osteoconductive and osteoinductive properties (The Ca^{2+} and PO_4^{2-} ions) [1, 3-6].

Different type of bone grafting materials represents the different physical properties. The physical characteristics of materials, such as crystallinity, crystal size, particle size, porosity and surface roughness, have been reported to influence the biological performance of biomaterials[7]. Sousa *et al*[8]. reported the systematic review and meta-analysis. The results revealed no significant difference between the biomaterial and autogenous groups in terms of bone gain. Another systematic review reported the degree of bone formation within the percentage of grafted volume including 33.2 ± 14.9% (allogeneic), 45.6 ± 21.4% (xenogeneic), 48.1 ± 6.5% (synthetic), 51.5 ± 15.9 (autogenous) and 56.6 ± 24.0% for mixtures of autogenous and other grafting materials[9].

Overview of bone graft healing

Bone graft healing is a sequential process involving inflammation, revascularization, osteogenesis, remodeling, and incorporation into the host skeleton bone[10]. For the incorporation of the graft and host bone, the process of incorporation include: (1) The formation of hematoma and the releasing of cytokines and growth factors; (2) An Inflammation, migration, and proliferation of mesenchymal cells and development of fibrovascular tissue around the graft; (3) The Invasion of blood vessels into the graft, penetrating via Haversian and Volkmann canals; (4) The resorption of the graft via osteoclastic function at graft surfaces; and (5) The endochondral and/or intramembranous bone formation on graft surfaces[5].

For the biology of bone graft healing, Two-phase theory of bone healing has been described. Phase I, defined as the first stage of osteogenic process. The quantity of surviving bone mass is determined. Contribute to the second part of the two-phase theory "Phase II". Phase II consisted of classical resorptive remodeling process and no quantitatively new bone is formed[11].

Healing of allogeneic bone graft and Immunologic response to bone allograft materials

Within the limitation of autogenous bone graft including donor site morbidity, limit quantity and can cause some possible of major and minor complications.

Allograft could overcome these limitations, but it bears the risk of infections and immune response. However, disease transmission (viral or bacterial) are extremely rare. The incidence of viral transmission (hepatitis or HIV) is less than 1 in 1.6 million[12].

In allograft, organic components such as collagen and non-collagenous proteins could activate the inflammatory response, inducing unfavorable immune response and also infectivity[13]. During the early phase of allogenic bone graft healing, a nonspecific inflammatory period occurs[14-16].

Cellular response of neutrophils:

Tissue damage leads to the activation of neutrophils and macrophages[17]. Ischemia of tissue could found. This causes the metabolism of tissue to anaerobic environment. During the reperfusion, oxygen is transported to the ischemic area resulting in the present of radical oxygen species (ROS). These ROS are chemo-attractants and activators of neutrophils[18]. Moreover, polymorphonuclear granulocytes (PMNs) obviously play an important role in the debridement mechanism from the first 10 minutes after injury until 3 days[19].

Cellular response of macrophages:

Subsequent to the initial neutrophil response, monocytes/macrophages are recruited. Macrophages have a strongly influence on the phases of cell proliferation and tissue differentiation. Macrophages secrete many growth factors such as Transforming growth factor (TGF). TGF plays an important role in cell growth and tissue repair and thus essential in the wound repair after trauma[20]. Another main function of macrophages is modulation of the adaptive immune response by mediating antigen presentation to B and T-lymphocytes. Antigens are taken up and partially degraded by the macrophage and then presented to a T-lymphocytes for recognition by MHC-II molecules. Macrophages could be explained as the bridge between innate and acquire immunity in the injured tissue in human.

For the specific immune response, B and T-cells play an important role in the recognition the alloantigens by their antigen-specific receptors at the cell surface. T-cells are divided into CD4 (T-helper cells) and CD8 (Cytotoxic/suppressor cells). These are involved in the antigen recognition[21]. When the T-cells recognize alloantigens as non-self. A specific immune response is activated. This recognition activates T-cells to secrete many cytokines and chemokines resulting in the osteoclastic activity at bony environment. This situation causes the resorption of the graft follow with failure of the incorporation of the allogenic graft. The histo-incompatibility of allografts are the proteins on cell surface. The matrix proteins are may or may not elicit graft rejection. The rejection of allograft is consider to be cellular rather than humeral response[14].

Following the innate and acquire immune response, the allogenic bone graft healing continues with phase I bone formation. The initial consolidation of the graft and host bone is occurred. For phase II bone remodeling, the resorption and replacement of the graft will be occurred known as creeping substitution process. Creeping substitution begins at the graft-host junction. Graft incorporation takes the time depending on graft particle size and local/systemic condition of host response[22].

For the immunologic response of the tooth matrix proteins, Silva *et al*[23, 24]. demonstrated that dentin matrix proteins such as dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) can induce neutrophil migration via the synthesis of IL-1beta, TNF-alpha, and chemokines controlled by macrophages. Jaha *et al*[25]. reported that N-terminal Dentin Sialoprotein fragment could induces type I collagen production.

Deproteinization for allograft preparation

Deproteinization is the process for the elimination of antigenicity for preparing the allograft bone substitution. There are several deproteinization processes such as heat deproteinization, chemical deproteinization, enzymatic hydrolysis, heavy metal salts, alcohol disrupts hydrogen bonding or radiation[26,27]. Heat deproteinization breaks down the hydrogen bond of polypeptide chain[28], whereas chemical deproteinization such as sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) alter pH resulting in protein precipitation[29].

Thermal deproteinization

For thermal deproteinization, there were several *in vitro* studies preparing bone allograft materials. Murugan *et al*[29]. reported the effect of heat treatment in bovine bone using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) spectroscopy. The FTIR spectra of raw bone and bone heated at 300˚C indicated the presence of organic macromolecules whereas these disappeared in the samples heated at 500, 700, 900˚C, which suggested the removal of antigenic organic matter around 500 ˚C. Moreover, Hiller *et al*[30]. reported very little structural changed in bone samples heated for 15 mins at 500˚C, but on heating to 700˚C or higher, the mineral phase was replaced by large clumps of crystallites. Nonetheless, the thermal deproteinated bone materials showed the disadvantage properties including the low ultimate compressive strength, the more brittle and the high thermal stress to bone structure. Uklejewski *et al*[31]. demonstrated the microcracks on the trabecular surfaces of bone generated by thermal deproteinization stresses using Scanning Electron Microscope (SEM). Lambert *et al*[32]. demonstrated that the higher temperature manufacturing processes seem to alter the crystal size, the higher crystallinity and the low porosity. In the same way as Conz *et al*[33]. and Do Desterro *et al*[34]. reported that the high-temperature sintering process potentially alter the micro-porosity (pore diameter <10 μm) and the micro-roughness which significant decrease the specific surface area of the graft.

Lim *et al*[35]. reported the thermal deproteinization of tooth particle and evaluated in nude mice. The results demonstrated that the tooth powders annealed above 1,000˚C resulting in the bio-ceramic which completely removed of organic components. After implanted the 1,000˚C annealed-tooth powder in nude mice, the histological analysis indicated osteogenic lineages and tissue stroma around tooth materials without any inflammation at 4 weeks.

Chemical deproteinization

Regarding chemical deproteinization, there are several types of chemical agents used for deproteinization process in bone such as NaOH, NaOCl, and H_2O_2. In previous study, 90% of bone proteins (mainly collagen) were removed from bovine cancellous bone specimens by NaOCl and H_2O_2 solution at day 14. While, 65 and 70% of bone proteins were removed by NaOH and KOH at the day 7 of the process respectively[31]. Moreover, some study demonstrated that using the NaOCl 5.25% after 21 days, the average collagen content had decreased by 55%[36]. Long Bi *et al*[37]. studied the microstructure, mechanical strength and cytocompatibility of NaOH, NaOCl and gaseous H_2O_2 (g H_2O_2) on cancellous bone. The result illustrated that the microstructure, organic content, and mechanical strength were dramatically altered at the surface of bone in both NaOH and NaOCl treated groups, but not in the gH_2O_2-treated group. Moreover, cell attachment and proliferation were higher in gH_2O_2-treated group. According from the study, only the chemical deproteinization process could not eliminate the protein completely.[37] Therefore, the materials retain some antigenicity, which could evoke immunological response. However, there is no study reported about chemical deproteinization processing in tooth particle.

To identify the protein remaining in biomaterials, various type of protein detection methods could be used such as Fourier Transform Infrared Spectroscopy (FTIR) [38]. FTIR utilizes the infrared radiation absorbed by the sample. The specificity of this method indicated that there is no two-unique molecular structure produce the same infrared spectrum. So FTIR could Identified the functional groups of protein such as N-H band, amide band accurately[29, 39].

Biomaterial evaluations

For a newly designed biomaterial to move from the lab to the clinic, it must go through the phases of tests, after the biomaterial is screened through a series of *In vitro* biocompatibility testing and found to be non-toxic, it is recommended that such a material undergoes clinically relevant *In vivo* tests. The *In vivo* tests essentially involve implanting the material in an animal model and the evaluation of its biocompatibility, biodegradation, tissue response prior to clinical use in the human.

Biocompatibility assessments

Implantation tests are used to determine the biocompatibility of medical devices or biomaterials that directly contact living tissue. According to a practical guide of International Organization for Standardization(ISO) 10993-6[40, 41]. mentions the use of mice, rats, guinea pigs and the rabbit. The rabbit model described in the standard is similar to that called for in several national pharmacopoeias. Rabbits offers many advantages as they are cost effective, good size for easy surgical procedures and specimen handling, their physiological bone healing is similar to that of humans, but the rate of healing is 3 times faster than in humans. The rabbit model has long been the animal of choice for implant testing[42, 43]. There are many types of implantation site such as subcutaneous, intramuscular and peri-renal implantation (kidney capsule implantation). Intramuscular implantation had a native skeletal progenitor cells, pro-osteogenic cytokine elaboration from sites of muscle injury, given an

appropriate osteogenic stimulus and induce ectopic bone formation better than subcutaneous implantation due to the increased vascularity[44, 45].

Base on the ISO 10993-6[40, 41]. related to the biocompatibility study in rabbit model, the test and the control materials are recommended to be prepared into approximately 1 x 10 mm-strips. If a medical device material is in the form of a powder, particle, or paste, it may be possible to coat an inert reference plastic or stainless steel with the substance and use the standard test model. The recommendation regarding to the distance between each sample state that the distance extending between the nearby materials should be 2-4 mm to prevent the reaction among materials. According to the number of animals, there should be at least three samples per time period of assessment[41]. However, based on the number of samples for evaluation, no minimum number specified.[46]

The biocompatibility issue is evaluated according to the number of positive samples. The test sample is consider positive if more than two of four test site in each animal exhibit a significant response compared to control sites[46].

Biodegradation assessments

Degradation is a critical property of the biomaterials. An ideal biodegradable material should be comparable to the rapid replacement rate by the new bone formation, in order for the material to provide sufficient support while leaving space for tissue growth[47]. Biodegradation of biomaterial are relative to the resorption rates of bone graft substitutes which depend on composition of graft, porosity and geometry.

Biodegradation of the Calcium Phosphate (Ca-P) biomaterials may be a combination of the following processes: (1) Physical abrasion, fracture, disintegration; (2) Chemical dissolution, increases of Ca and P locally at the surface; and (3) Biological reductions in pH caused by cellular and phagocytic activities resulting in increases in the rate of biodegradation due to the dissolution of the Ca-P biomaterials[48]. Biodegradation of the Ca-P biomaterials in vivo can be caused by two processes: The first involves extracellular liquid dissolution, the second, disintegration into small particles that are either intracellularly digested or transported to neighboring tissues such as the lymph nodes[49].

Biodegradation of deproteinized bovine bone (Bio-Oss®) has been investigated in numerous clinical and experimental studies and seem to be controversy in the literature whether Bio-Oss® is resorbable, slowly degraded, or non- resorbable. One study indicated signs of resorption lacunas and the presence of osteoclasts on the Bio-Oss® surface[50]. Other studies on maxillary sinus grafting with Bio-Oss® show no signs of resorption of the material at all[51]. There was no explicit study about the biodegradation pattern of tooth-derived bone graft materials.

Several methods were used to evaluated biomaterial degradation *In vivo* such as histological, histomorphometric and micro CT evaluation. Histological evaluation indicated the semi-qualitative data, while histomorphometric and micro CT indicated the total quantitative data. To distinguish the remaining fully mineralized

tissue, a threshold of 0.6 g/cm^3 was recommended, where the mineralized material was all tissue with a density higher than the threshold[52].

Tooth as bone graft material

Tooth comprised of inorganic and organic components which almost similar to the bone. Enamel consists of 96% inorganic substances and 4% water, whereas dentin has 65% inorganic substances, 35% organic substances, and water. Cementum is made up of 45- 50% inorganic substances, 50-55% organic substances, and water. Alveolar bone has 65% inorganic and 35% organic substances[53].

The major inorganic compartments of bone and tooth are calcium phosphate substances mainly HA.[54] From X-ray Diffraction (XRD), tooth element composed 5 phases of calcium phosphate components including HA, TCP, octacalcium phosphate (OCP), amorphous calcium phosphate (ACP), and dicalcium phosphate dehydrate (DCPD). Enamel consists of HA, which is a high degree of crystallinity and hardness. While, in dentin and bone, HA is low-crystalline calcium phosphate[55]. In recent studies, CaP phase in demineralized tooth is not only in HA but also monetite and brushite phase[56, 57]. From the CaP dissolution analysis, tooth and cortical bone displayed the similar pattern of calcium and phosphorus content from the beginning[58].

The organic components of bone and tooth are comprised of 90% of type I collagen and 10% of non-collagenous proteins. Moreover, the essential proteins found in dentin includes a small amount of growth factors especially endogenous bone morphogenetic protein(BMP), phosphoproteins, osteocalcin, proteoglycans, dentin sialophosphoprotein (DSPP) which are beneficial in bone formation process[59-61]. Dentin-matrix-derived BMPs are similar to bone-matrix-derived BMPs. Both types of BMP have the same action which can induced new bone formation[62]. The expression of Dentin sialoprotein could induced osteoblastic differentiation[63], platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) could enhanced angiogenesis[64].

Morphologically, the basic unit of enamel is an enamel rod (hydroxyapatite rod) which is about 4–8 μm in diameter. Enamel crystals in the head of the enamel rod are oriented parallel to the long axis of the rod. Enamel does not contain collagen, as found in other hard tissues such as dentin and bone but it does contain two unique classes of proteins; amelogenins and enamelins[65]. For dentin structure, Phylogenetic studies have revealed that dentin were very similar to bone, with osteoblast/odontoblast-like cells located within alveoli, as it is the case for osteocytes surrounded by bone within lacunae. This organization called osteodentin[66]. The SEM photograph of tooth present dentinal tubules with possess diameters ranging from approximate 1 to 3 μm, depending on patient age[67].

In vivo studies, Claudio *et al*[68] investigated the possible use of human demineralized dentine matrix (DDM), obtained from the extracted teeth, as bone graft material in the healing process of tooth sockets of rats. Evaluation at 3, 7,14 and 21 days after surgery. This study showed DDM graft proved to be biocompatible, with negligible host immune reaction, demonstrated by the presence of relatively mild inflammatory cell infiltrate in bone repair region, the particles of the DDM graft incorporated with host bone and become the newly formed bone tissue. In other research, in rat showed DDM induces osteoblast differentiation in implanted sites mediated by BMP-2 and BMP-4, and acts as

scaffold for osteoblast differentiation, yielding active new bone formation and no inflammation process. So represented DDM had effective bone implant material[69].

Only one published animal studies of demineralized tooth material had been reported. Farzad *et al*[70]. evaluated and compared the host tissue response of autogenous and xenogenic non-demineralized dentin blocks implanted in abdominal connective tissue and femoral muscle of rabbits at 12 weeks. Dentin autografts taken from the same rabbit and dentin xenografts taken from human premolars. The clinical observation showed normal soft tissue healing without any sign of infection in all rabbits. For the histological observation, there was no significant difference (p=0.388) in fibrous tissue thickness and inflammatory cells counted between autografts and xenografts. A significant difference (p = 0.018) was seen with more inflammatory cells in abdominal grafts than muscle grafts among the autografts.

According to our research group, the tissue reaction, osteoinduction property and degradation profile of human demineralized tooth matrix (hDTM) after intramuscularly implantation in rabbit model were evaluated and compared with those of human demineralized Freeze-dried Bone Allograft (OraGRAFT®). The histological assessment demonstrated the low-grade inflammatory response of both hDTM and OraGRAFT® that decreased with time. Graft materials were degraded with time over 12 weeks which OraGRAFT® revealed faster resorption rate.

Table 1 Summarized of the *In vitro*, *In vivo* and clinical studies of tooth derived bone graft materials in different preparation form.

Authors/ Year	**Study**	**Methods**	**Evaluation methods**	**Outcomes**
Moharamzadeh *et al*, 2008.[71]	*In vitro*	- Non-demineralized dentin	Human gingival fibroblast cell-biocompatibility	Excellent biocompatibility
Murata *et al*, 2012.[72]	*In vitro*	- Auto BT (demineralized in 0.6M HCl, and freeze-dried)	Histological, Histomorphometric evaluation	Auto BT was an effective carrier for delivering BMP-2 and superior scaffold for bone-forming cells.
Liu *et al*, 2016. [73]	*In vitro*	- Auto BT + DPSCs (pig)	SEM, Transwell migration assays, ALP and alizarin red staining	Demineralized dentin matrix induces DPSC odontoblastic differentiation
Bormann *et al*, 2012. [74]	*In vitro and In vivo*	- Auto BT from mice (unspecified methods)	Intravital fluorescence microscopy	Dentin and ß-TCP are comparable in neovascularization response

Table 1 (Continued)

Gome *et al*, 2001.[75]	*In vivo* (rabbit)	- Auto BT (tooth slice8μm), (0.6N HCl at 2°C until completely demineralized)	Histological evaluation	Direct bone formation and incorporated by the newly formed bone.
Nampo *et al*, 2010. [76]	*In vivo (*Wistar rat's)	- Wistar rat's particulated tooth 500 μm	Histological evaluation, Micro-CT, Real-time PCR	Tooth graft material produced new bone formation. Osteopontin and dentin sialoprotein was expressed.
Jeong *et al*, 2011.[77]	*In vivo* (minipig)	- Auto BT (tooth block) (unspecified methods)	Histological, Histomorphometric evaluation	New bone over total area & bone to implant contact statistically significant at 12 weeks
Kim *et al*, 2014.[58]	*In vivo* (Nude Mouse)	- Auto BT (Particulated tooth),(dehydration,defatting, lyophilization)	Histological, Histomorphometric evaluation	Demineralized human dentin matrix has osteoinductive ability.
Kim *et al*, 2009, 2010, 2013. [53, 54]	*Clinical trial*	- Auto BT (Particulated tooth 500-800 μm), (dehydration, defatting, lyophilization) - GBR, HRA, VRA.	Histological and radiological evaluation.	Resorption of tooth graft and newly form bone was found.
Jeong *et al*, 2011.[77]	*Clinical trial*	- Auto BT (Particulated tooth 500-1,000 μm and 1,000-2,000 μm), (unspecified methods)	Histomorphometric evaluation, Implant survival rate.	Auto BT showed gradual resorption and new bone formation. Implant survival rate was 96.15%
Park *et al*, 2012.[78]	*Clinical trial*	- GBR, Maxillary sinus graft, Socket preservation,Ridge augmentation	Histological evaluation, Implant stability quotient (ISQ)	Formation of new bone, Providing good bone generation

Table 1 (Continued)

Minamizato *et al*, 2017.[79]	*Clinical trial*	- APDDM - GBR, Maxillary sinus graft, Socket preservation, Ridge augmentation horizontal and vertically	Histological and radiological evaluation.	APDDM showed successful alveolar bone regeneration in many clinical applications

Auto BT = Autogenous Tooth Bone Graft, DPSCs = Dental Pulp Stem Cells, Micro-CT = Micro- Computed Tomography, GBR = Guided Bone Regeneration, HRA = Horizontal Ridge Augmentation, SEM = Scanning Electron Microscope, EDS = Energy Dispersive X-ray Spectroscopy, Real time PCR = Real Time Polymerase Chain Reaction, ALP = Alkaline Phosphatase, APDDM = Autogenous partially demineralized dentin matrix.

The properties of Polyvinyl-alcohol (PVA) and Anorganic deproteinized bovine bone (Cerabone®)

Polyvinyl alcohol (PVA), a hydrophilic, biodegradable and biocompatibility synthetic polymer, has been widely used in biomedical field[80]. The basic properties of PVA are depending on many factors including concentration of PVA, degree of polymerization, degree of hydrolysis, distribution of hydroxyl groups, stereoregularity and crystallinity of PVA[81]. Demerlis *et al*[82]. strongly reported that orally administered PVA is relatively safe. The acute oral toxicity of PVA is very low, with LD50s in the range of 15–20 g/kg. In addition, PVA is known to be a biodegradable synthetic polymer. Hydrolysis is the process of degradation of PVA, thus could induce the inflammatory reaction of the implanted site[83]. Solaro *et al*[81]. reported the 88% hydrolysis degree of PVA degradation was found up to 120 days. *In vitro* study, PVA based scaffold demonstrates good biocompatibility. No sign of macroscopic inflammation. Histological evaluation shows low grade of inflammatory cell surrounding PVA implanted in rabbit tibia at 4 weeks[84].

Cerabone® is the commercial xenogenic bone graft materials fabricated from bovine bone. This raw material undergoes a step-wise heating process (up to >1,200 °C), which removes all organic components, including potential bacteria, viruses and prions. Its physical properties indicate a highly porous bone grafting material with a porosity range from 65-80% and a mean pore size of 600-900 μm. Cerabone® exhibits a very high crystallinity together with phase purity, i.e. it is 100% pure HA. Moreover, Cerabone® exhibits slow biodegradability due to the highly crystalline structure[38].

Fabrication of deproteinized tooth as grafting materials

The deproteinized human demineralized tooth matrix (dpDTM) fabrication protocol has been developed in PSU. The fabrication process includes tooth pulverization, demineralization with 0.5M HCl for 3 hours, deproteinization with 1N

NaOH for 7 days and followed by thermal deproteinization at temperature 300ºC. The calcium phosphate components and phase characteristics in dpDTM were evaluated. The result indicated the mainly components of tooth were calcium (Ca) and phosphorous(P) and the Ca/P molar ratio was about 1.5-1.8 (Table 2). and phase characteristics confirmed that there was only hydroxyapatite phase without secondary phase transformation (Fig.1). The surface characteristics of dpDTM through the scanning electron microscope (SEM) represented dentinal tubules with rough dentin surface (Fig.2). The FTIR spectra of dpDTM characterized no remarkable of protein macromolecules in tooth structure indicated by no distinct amide I and amide II absorption peak (Fig.3).

Table 2 The concentration elements of dpDTM

Elements	% By weight	Elements	% By weight
Ca	19.6	**Al**	154 ppm
P	9.87	**K**	39.3 ppm
Na	0.565	**Zn**	81.0 ppm
Mg	0.508	**Sr**	89.3 ppm
Cl	859 ppm	**Fe**	14.2 ppm
Si	844 ppm	**Cu**	11.8 ppm

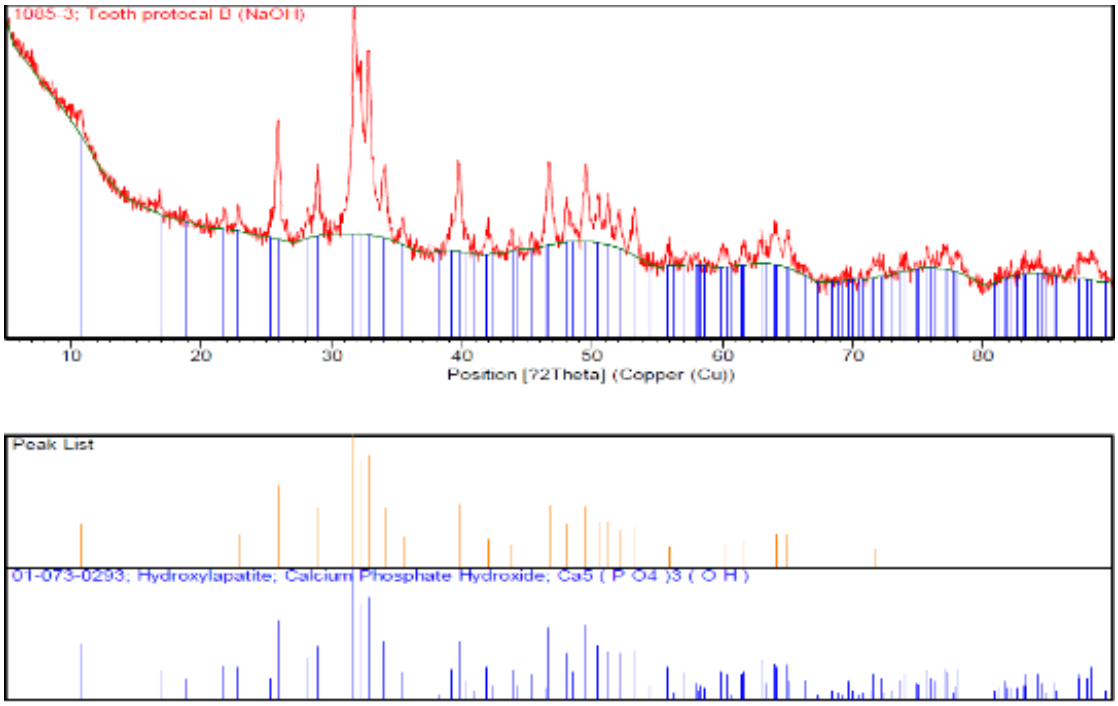


Figure 1 XRD patterns of dpDTM

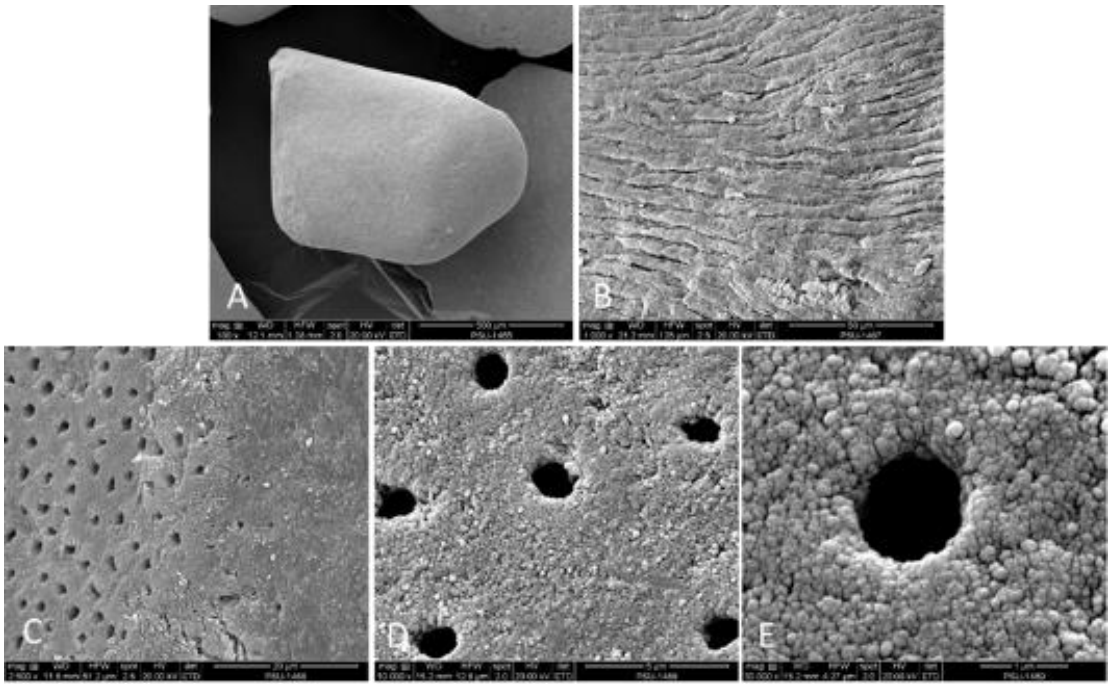


Figure 2 SEM photograph of dpDTM. (A)100X, (B)1000X, (C)2500, (D)10000X and (E)30000X magnifications.

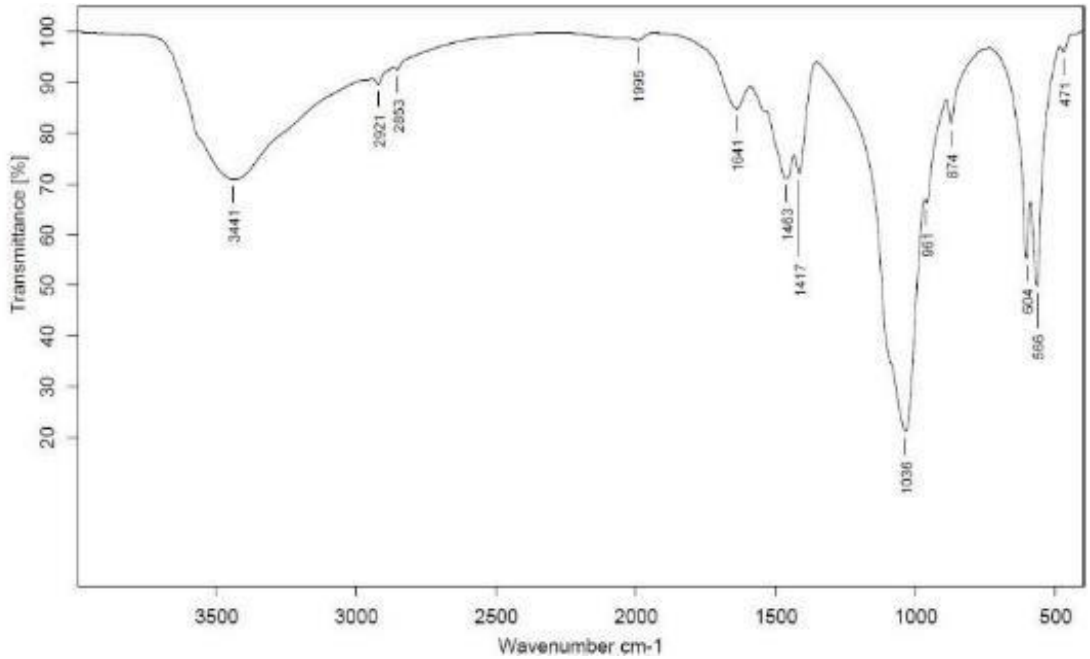


Figure 3 Transmittance FTIR spectra of dpDTM

In vitro osteoblast cell biocompatibility

As the evidence from the physicochemical properties, only the dpDTM processing with NaOH/thermal group demonstrated the most suitable for being used as allograft as evidence from the SEM, FTIR, and the degradation behavior.

Cell adhesion

SEM analysis of NaOH/thermal scaffold showed that MC3T3-E1 osteoblasts cells were able to adhere and proliferate to the surface of dpDTM at day1 and day 7. (Fig.4,5) At day 1, SEM showed the extension of pseudopodia, while at day 7 the SEM showed the cells grew in multilayers and formed dense cell sheet accumulated to the surface of the scaffold.

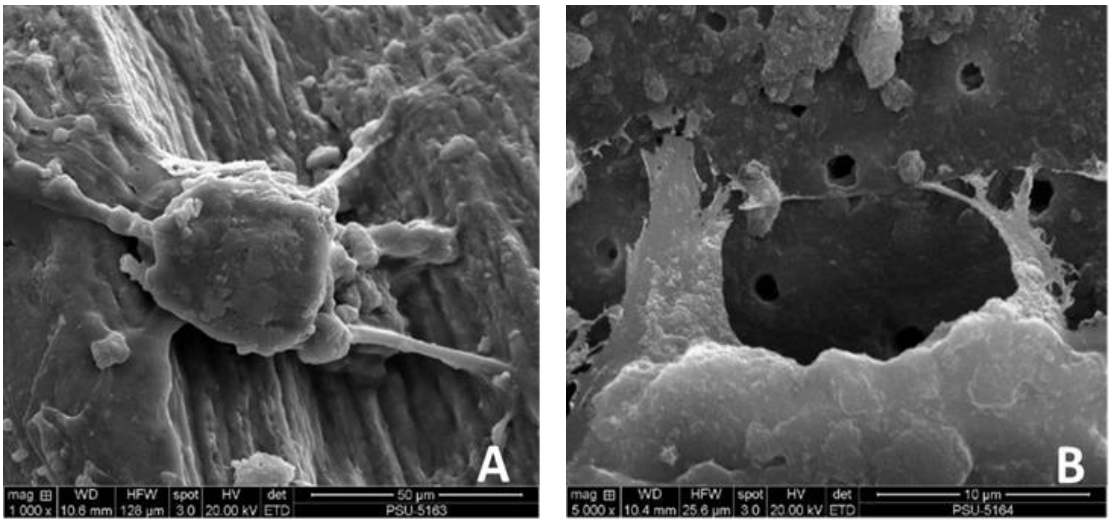


Figure 4 SEM photograph at day 1 presenting the MC3T3-E1 osteoblasts cells were able to adhere and proliferate to the surface of NaOH/thermal scaffold. (A)1000X, (B)5000X

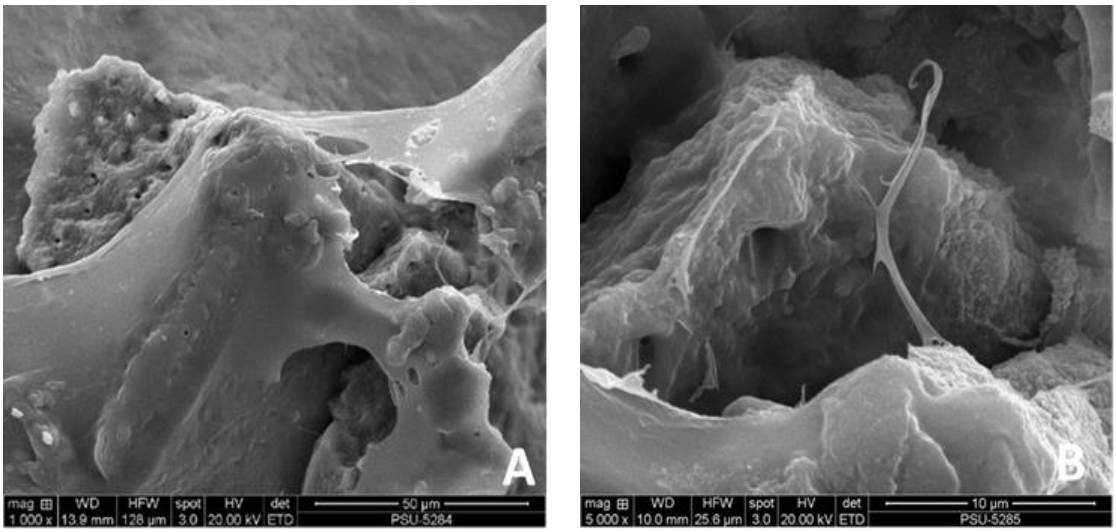


Figure 5 SEM photograph at day 7 presenting the MC3T3-E1 osteoblasts cells were able to adhere and proliferate to the surface of NaOH/thermal scaffold. (A)1000X, (B)5000X

Cell proliferation

The quantification of the cell viability of mineralized tooth, NaOH/thermal, and 5% PVA scaffold on day 1, 3, 5, 7, 14 and 21 were presented in

Figure 10. The bar graph represented the NaOH/thermal scaffolds which was increase in time from day1 to day 14 and reach the highest peak on day 14. But on day 21 the quantification of the cell was slightly decreased. (Fig.6)

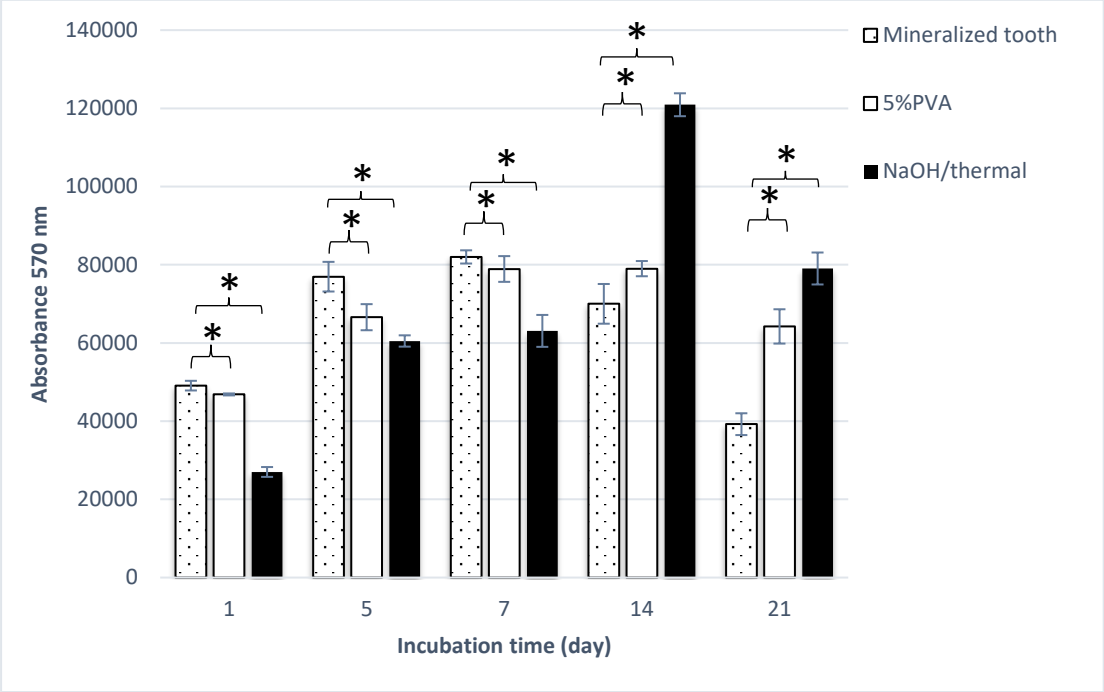


Figure 6 The quantification of the cell viability of NaOH/thermal scaffolds (opaque bar), Mineralized tooth (dot bar) and 5% PVA (blank bar) on day 1, 3, 5, 7, 14 and 21. At day 14 the NaOH/thermal scaffolds showed the highest value. (*$p < 0.05$)

As the evidence from the physicochemical characteristics and cell biocompatibility, the dpDTM demonstrated good properties and may be appropriated for being used as bone substitution material. In order to adopt dpDTM and use as allograft bone substitution, the *In vivo* tissue biocompatibility should be performed.

The objectives of the study

1. To assess and compare the biocompatibility of dpDTM and anorganic bovine bone (ABB) by intramuscular implantation at 1, 2, 4, 8, 12 weeks.

2. To estimate the biodegradation pattern of dpDTM and anorganic bovine bone (ABB) by intramuscular implantation at 1, 2, 4, 8, 12 weeks.

Research question

Can dpDTM be used as bone graft substitution which could provide biocompatible, bioresorbable and osteoconductive properties as commercially anorganic deproteinized bovine bone xenograft?

Hypothesis of the study

The used of dpDTM in terms of biocompatibility, biodegradation and osteoconductive properties would not be different from those using anorganic deproteinized bovine bone histologically and radiographically.

CHAPTER 2

MATERIALS AND METHODS

The study was performed at the Southern Laboratory Animal Facility. The study protocol was approved by the Human Research Ethics Committee of the Faculty of Dentistry, and the Animal Ethics Committee, Prince of Songkla University. (2560-03-063)

Research design

The study was the animal experimental study.

Deproteinized human demineralized tooth matrix (dpDTM) fabrication

Caries-free molar or premolar teeth were obtained from adult patients who undergo tooth extraction at Surgery clinic, Faculty of Dentistry, Prince of Songkla University. The tooth was divided into crown and root portion, whereas pulp tissue was removed by handed endodontic files. The tooth was pulverized into small particles by mixer ball mill machine (Mixer Mill M301, Retsch GmbH, Haan, Germany). Sieves with 500 μm and 1000 μm aperture (Endecotts, London, UK) was used to select desired particle size (Fig.7). The tooth matrix was pooled and demineralized using 0.5mol/L HCl for 3 hours.

The deproteinized process was performed using 1N Sodium hydroxide (NaOH) for 7 days at 37°C. Then, the tooth particles were heated at 300°C using press furnace (IPS Empress EP 600 Combi, Ivoclare vivadent, Liechtenstein) with rate 30 °C/min and holding time 10 mins (Fig.8). The bleaching method was performed using H_2O_2 for 10 mins.

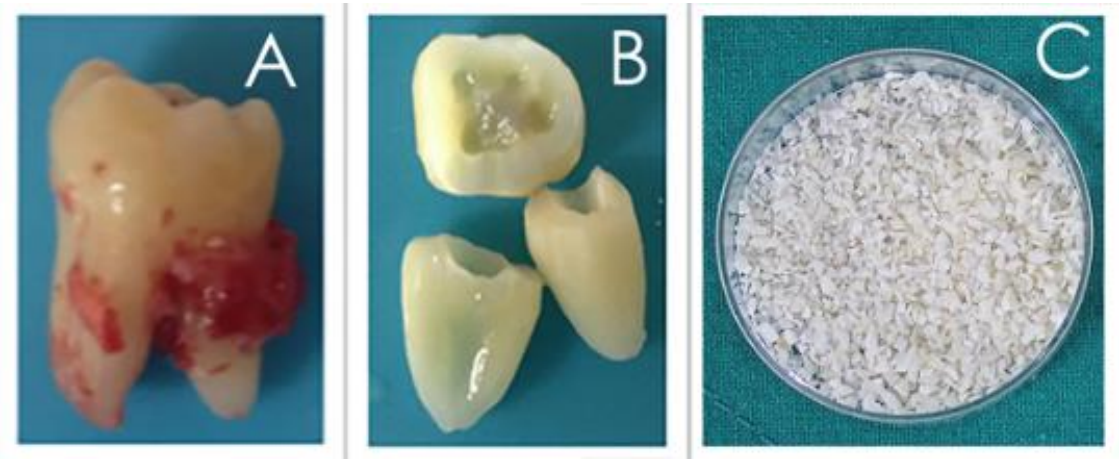


Figure 7 (A) Gross appearance of extracted human tooth. (B) Cleaned tooth after mechanically removed soft tissue. (C) Human tooth matrix sized 500-1000 μm.

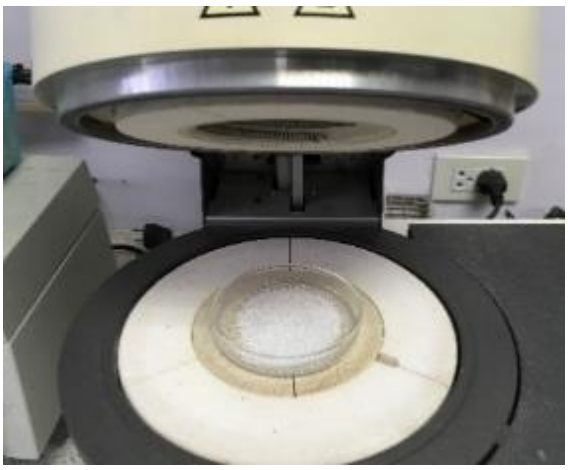


Figure 8 Thermal deproteinization 300°C using tube furnace (IPS Empress EP 600 Combi) with rate 30 °C/min, holding time 10 mins.

Anorganic deproteinized bovine bone (ABB)

The commercially anorganic deproteinized bovine bone (Cerabone®, Botiss, Zossen, Germany) was chosen as the positive control material. Sieves with 500 μm and 1000 μm aperture (Endecotts, London, UK) was used to select desired particle size.

dpDTM and ABB scaffold preparation

Polyvinyl alcohol (PVA; Mw 145,000 Da; hydrolysis; Merck, Germany) was used to fabricate scaffold using hydrogel solution as binding materials.

For test and positive control group, dpDTM and ABB scaffolds were synthesized according to previous study with modification (Gupta S *et al*[85].). 300 mg of dpDTM or ABB were added into 200 μL of 5%wt/v PVA solution, mixed thoroughly, and then perfused into a container (diameter of 10 mm and height 3 mm.) that equal to about 1.26 g/ml. The dpDTM and ABB were subjected to freeze-thaw cycles at temperature of -20°±2 °C (freezing) and 25°±2 °C (thawing) 3 cycles for 3 days. The dpDTM and ABB scaffold were sterilized by hydrogen-plasma gas before use. For the negative control group, 200 μL of 5%wt/v PVA alone was used and prepared as mention above. (Fig.9A-D)

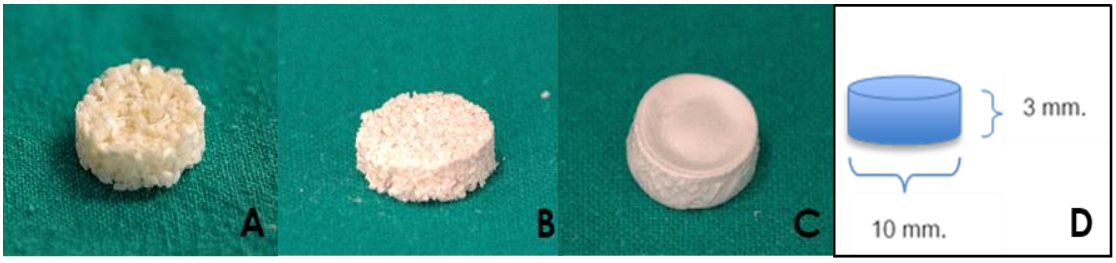


Figure 9 The pictures demonstrated the scaffold of (A) dpDTM, (B) ABB (Cerabone®), (C) PVA and (D) Schematic of cylindrical shape of samples.

Animal preparation and surgical technique.

The study was performed in 15 adult New Zealand White rabbits (mean weight 3.5-4.0 kg). The animals were fed with rabbit food in a daily amount equivalent to 2% of the animal's weight and water ad libitum.

dpDTM scaffold (n=6) were implanted in the paralumbar muscle of the rabbit. ABB and PVA were served as positive and negative control, respectively. The evaluation was performed after healing period of 1, 2, 4, 8 and 12 weeks. (Table 3, 4)

Table 3 The detail of biomaterials and number of experimental sites.

Groups	**Biomaterials**	**Number of experimental sites (n)**				
		1 wk	2 wk	4 wk	8 wk	12wk
(A) dpDTM	Deproteinized hDTM	6	6	6	6	6
(B) ABB	Anorganic deproteinized bovine bone	6	6	6	6	6
(C) PVA	PVA Hydrogel	6	6	6	6	6

Table 4 The number of rabbits. (n=15) Note* 1 rabbit including 6 sites

No. of rabbit	**Left**	**Right**	**Timing (week)**
1	B x 3 sites	A x 3 sites	1
2	B x 3 sites	A x 3 sites	1
3	C x 3 sites	C x 3 sites	1
4	B x 3 sites	A x 3 sites	2
5	B x 3 sites	A x 3 sites	2
6	C x 3 sites	C x 3 sites	2
7	B x 3 sites	A x 3 sites	4
8	B x 3 sites	A x 3 sites	4
9	C x 3 sites	C x 3 sites	4
10	B x 3 sites	A x 3 sites	8
11	B x 3 sites	A x 3 sites	8
12	C x 3 sites	C x 3 sites	8
13	B x 3 sites	A x 3 sites	12
14	B x 3 sites	A x 3 sites	12
15	C x 3 sites	C x 3 sites	12

Surgical technique

The rabbit was sedated with ketamine-xylazine anesthesia. Xylazine (3mg/kg) and ketamine (20 mg/kg) were administered intramuscularly in different muscle. During the surgery, anesthetic induction was achieved in an animal by titrated Ketamine (5 mg/kg) and diazepam (0.5 mg/ kg) every 10 minutes.

After the rabbits were anesthetized, the surgical areas were shaved and washed with iodine solution. The local anesthesia (Lidocaine hydrochloride 2% with Epinephrine 1:10^5 (Medicaine ®, Huons Co., Ltd, Korea) 1.8 ml was administered in each experimental area.

1-cm long incision was performed on either side of the paralumbar muscle with scalpel blade No.15. The intramuscular pouch was created using blunt dissection. The biomaterial scaffold was implanted in the paralumbar muscle. Each implanted site was at least 2.5 cm apart. Six implanted sites (left 3 sites, right 3 sites) were achieved. Each tissue layer, muscle, subcutaneous and skin, were closed in a layer by layer manner with vicryl® 4/0. Pethidine (10 mg/kg) was administered by intramuscular injection postoperatively 1 hour to reduce pain. (Fig.10A,B)

All rabbits were sacrificed after 1, 2, 4, 8, and 12 weeks by a pentobarbital sodium (200 mg/kg) 5-10 ml by intravenous injection.

Figure 10(A) The Diagram indicated the six implanted sites including dpDTM, ABB and PVA groups on the paralumbar muscle.

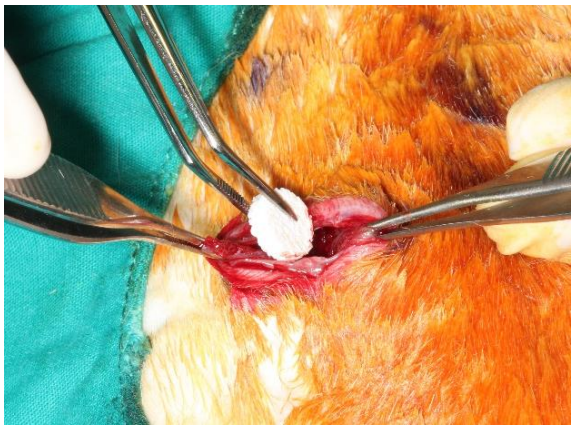


Figure 10(B) The biomaterial scaffold was implanted into the created pouch of paralumbar muscle.

Biocompatibility evaluation.

Local tissue response was assessed clinically, radiologically and histologically after healing of 1, 2, 4, 8, and 12 weeks. The specimens were cut, label for the upper right corner, and then fixed in 10% buffered formalin for 48 hours.

Regarding clinical evaluation, signs of infection and inflammation at operation site including pus and exudate, swelling, redness of skin, warmth and wound dehiscence at all time point was evaluated. Clinical evaluation was graded including:

Grade 0: Absence of inflammation and infection
Grade 1: Only sign of inflammation: swelling, redness of skin, warmth
Grade 2: Both sign of inflammation and infection
Grade 3: Both sign of inflammation and infection + wound dehiscence

Micro computed tomography (Micro-CT)

High resolution Micro-CT (Scanco Medical AG, Basserdorf, Switzerland) was used to evaluate the biodegradation pattern of dpDTM. Three-dimensional images were reconstructed using 3D Creator software. Three-dimensional measurements of the amount of bone volume per total volume (BV/TV) in the implantation site was also calculated using the analysis software at 1, 2, 4, 8, and 12 weeks[86].

Histological evaluation

The specimens were decalcified in 10% ethylene diamine tetracetic acid (EDTA PH 7.4) and then were embedded in paraffin. The serial of 5 µm-thick sections were performed in a position of 500 µm from the midline. The sample was stained with hematoxylin and Eosin (H & E). The specimens were evaluated histological analysis by light microscope at 1, 2, 4, 8, and 12 weeks.

Inflammation evaluation: Grid pattern diagram 10x10 μm^2 was used to evaluate the present of inflammation cell (neutrophil, macrophage, lymphocyte and plasma cell), as well as the present of fibrous capsule surrounding the graft.

Biodegradation evaluation: Grid pattern diagram 10x10 μm^2 was used to evaluate the present of osteoclast, and macrophage cell around graft materials. (Fig.11)

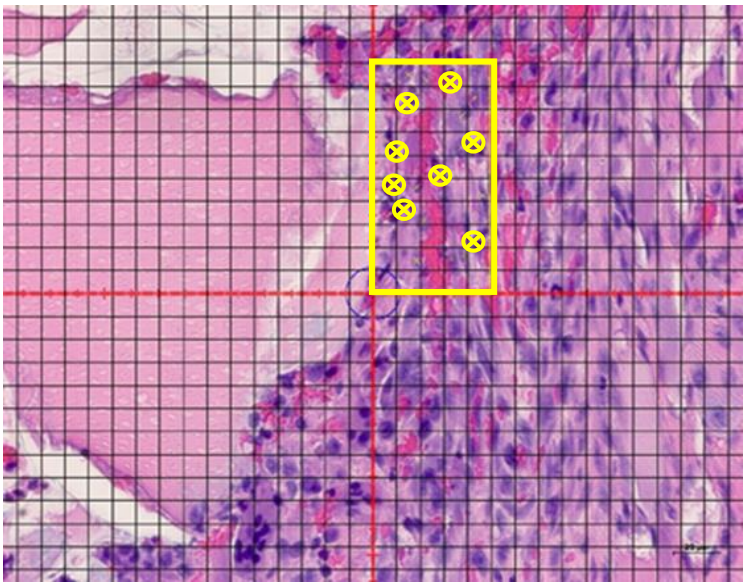


Figure 11 Example of grid pattern diagram, the yellow rectangular box represent area of 10x10 μm^2 superimposed with H&E staining for quantitative histological analysis.

Inflammatory cell count was divided into 4 grades according from Pinna *et al*.[87] including;

- Absent: No inflammatory cells were detected.
- Mild: One to ten inflammatory cells were detected.
- Moderate: Eleven to twenty inflammatory cells were detected.
- Intense: Over twenty inflammatory cells were detected

Statistical analysis:

The results were analyzed using statistical analysis software (SPSS version 16.0, USA). The significance level was set at 5% (P-value < 0.05). The clinical evaluation score was analyzed by Chi-Square test. The quantitative data of Micro-CT evaluation & Histological evaluation were test normality by Kolmogorov-Smirnov test and Shapiro-Wilk test. The parametric statistic: Repeated ANOVA was used for the normal distribution data and the Non-parametric statistic: Friedman analysis was used for the abnormal distribution data. In addition, the Descriptive analysis was used to examine the Histological evaluation.

CHAPTER 3

RESULTS

Biocompatibility evaluation

All rabbits recovered uneventfully without weight loss. Clinical observation at surgical sites were evaluated. Mild inflammation was detected at 1 week and decreased with time. Local tissue response was grading into grade 0 (normal healing, absence of infection and inflammation) at all time point. (Fig.12 A,B)

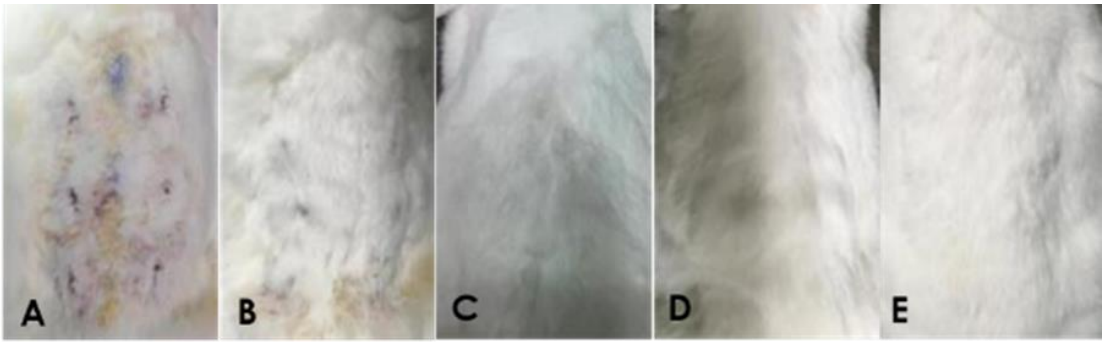


Figure 12(A) Clinical observations of rabbit's skin revealed normal tissue healing after intramuscular implantation at 1, 2, 4, 8, 12 weeks. (A = 1 week, B = 2 weeks, C = 4 weeks, D = 8 weeks, E = 12 weeks)

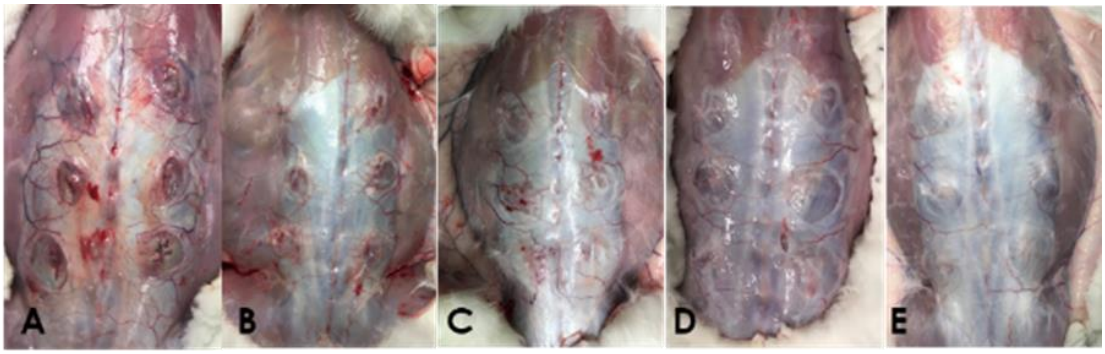


Figure 12(B) Clinical observations of rabbit's muscle revealed normal tissue healing after intramuscular implantation at 1, 2, 4, 8, 12 weeks. (A = 1 week, B = 2 weeks, C = 4 weeks, D = 8 weeks, E = 12 weeks)

Histological observation

Normal histological observation of paralumbar muscle of rabbits without inflammatory cell was showed in Fig.13

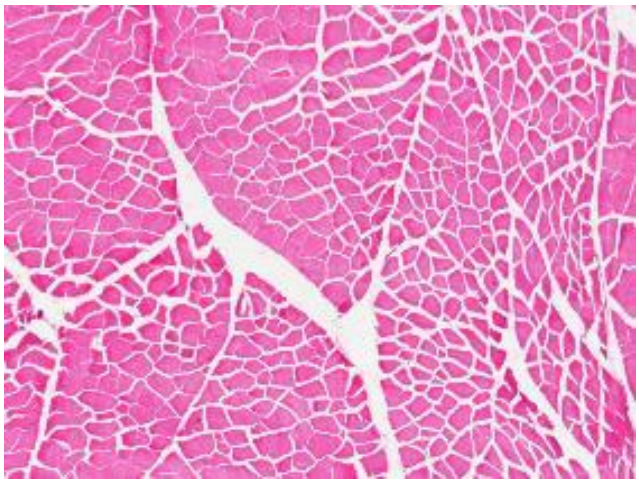


Figure 13 The histological image of normal tissue in paralumbar muscle of rabbits (H&E staining)

Histological observation of Deproteinized hDTM (dpDTM), Anorganic deproteinized bovine bone (ABB) (Cerabone®) and Poly-vinyl alcohol (PVA)

At 1 week, both dpDTM and ABB showed intense inflammatory cells response either macrophages or lymphocytes which were chronic inflammatory reaction. No muscle necrosis was observed. Numerous blood vessel was seen adjacent to the materials surface. For PVA group, the image showed the intense inflammatory cells close contact to the materials. (Fig.14A-F)

Week 1

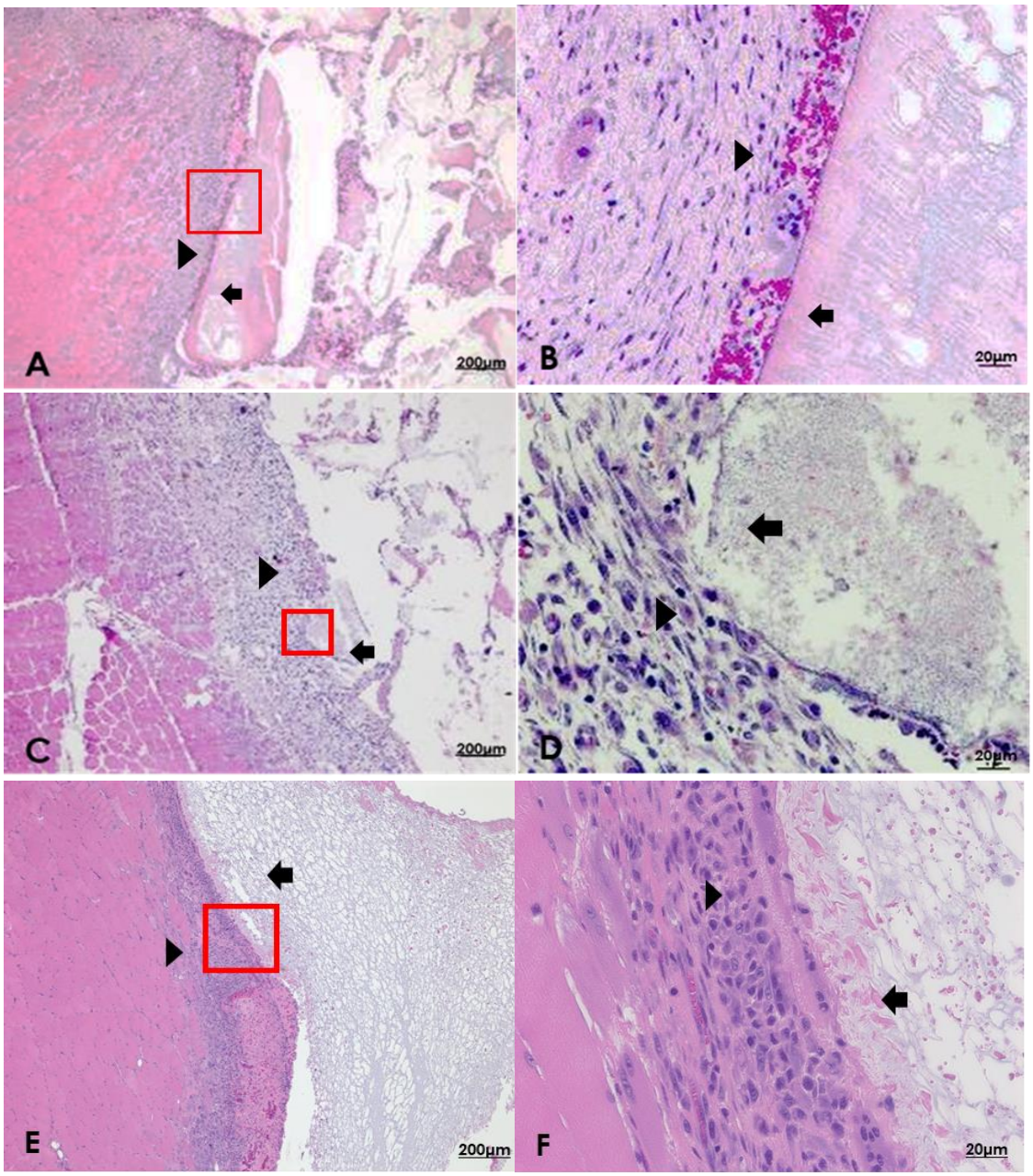


Figure 14 Histological images of (A,B) Deproteinized hDTM (dpDTM), (C,D) Anorganic deproteinized bovine bone (ABB) and (E,F) Polyvinyl-alcohol (PVA) in paralumbar muscle at 1 week with the magnification of 5X(A,C,E) and 40X(B,D,F) (H&E staining) (arrow head = inflammatory cells, black arrow = materials)

At 2 weeks, the histological images of both dpDTM and ABB showed moderate to intense inflammatory infiltration composed of macrophages and lymphocyte predominantly. In general, not only the inflammatory infiltrations were lower than 1 week but also the invasion of inflammatory cells into muscle layer. For PVA group, the image showed the intense of inflammatory cells close contact to the materials. (Fig.15A-F)

Week 2

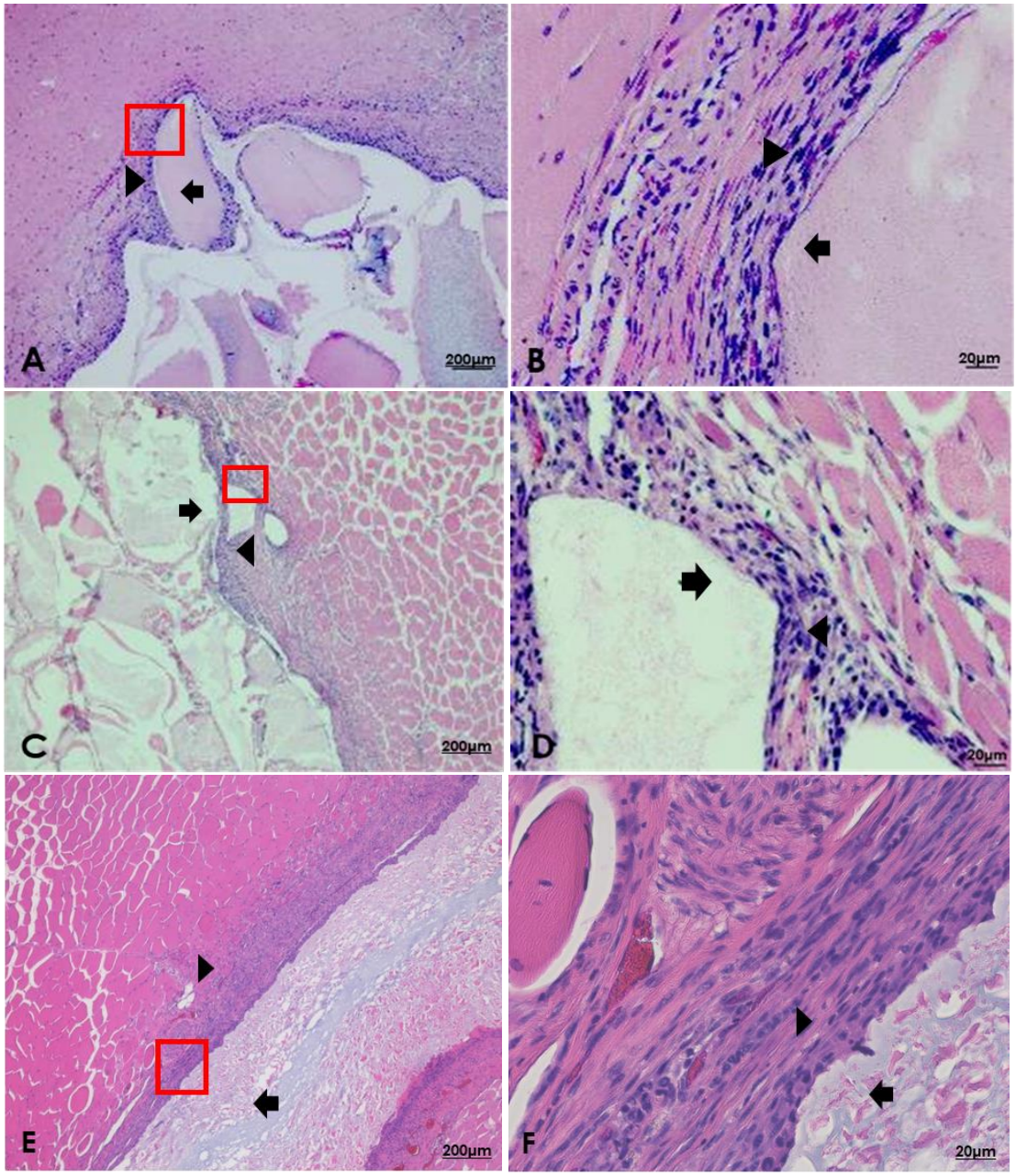


Figure 15 Histological images of (A,B) Deproteinized hDTM (dpDTM), (C,D) Anorganic deproteinized bovine bone (ABB) and (E,F) Polyvinyl-alcohol (PVA) in paralumbar muscle at 2 weeks with the magnification of 5X(A,C,E) and 40X(B,D,F) (H&E staining) (arrow head = inflammatory cells, black arrow = materials)

At 4 weeks, the specimens of both dpDTM, ABB and PVA demonstrated mild to moderate inflammatory cells infiltration. The inflammatory cells were generally decreased with time in both dpDTM, ABB and PVA groups. The predominant inflammatory cells were macrophages, lymphocyte, and plasma cells. Multinucleated giant cells were seen at the adjacent to ABB particle (Fig.16D) At this time point, there was less degree of blood vessels. (Fig.16A-F)

Week 4

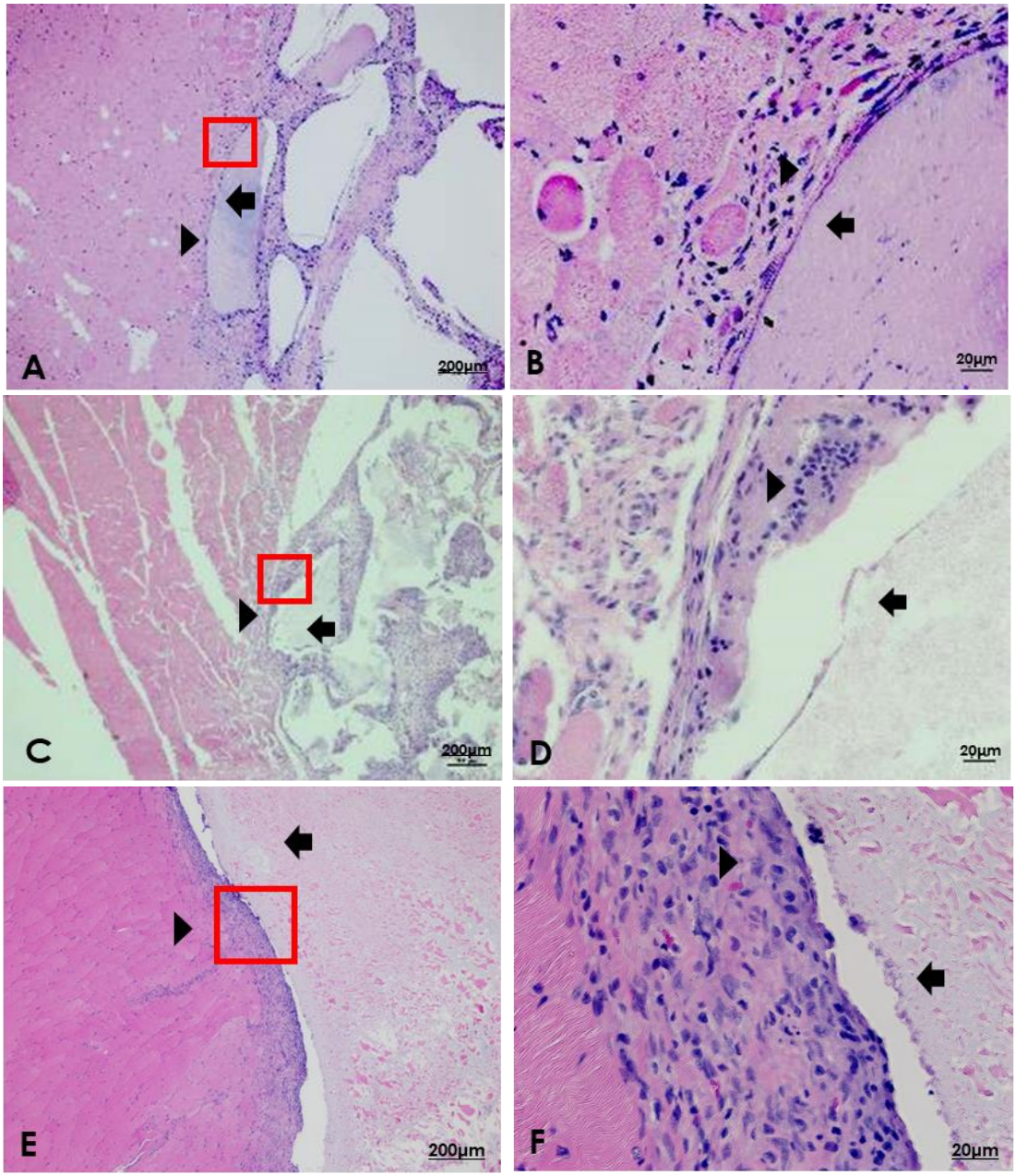


Figure 16 Histological images of (A,B) Deproteinized hDTM (dpDTM), (C,D) Anorganic deproteinized bovine bone (ABB) and (E,F) Polyvinyl-alcohol (PVA) in paralumbar muscle at 4 weeks with the magnification of 5X(A,C,E) and 40X(B,D,F) (H&E staining) (arrow head = inflammatory cells, black arrow = materials)

At 8 weeks, the histological images showed mild to moderate inflammatory infiltration composed of macrophages, lymphocyte and plasma cells predominantly. The inflammatory cells were obviously decrease but remained. There was some degree of graft resorption related to multinucleated giant cells in both dpDTM and ABB groups. For PVA group, there was only mild of inflammatory cells presented. (Fig.17A-F)

Week 8

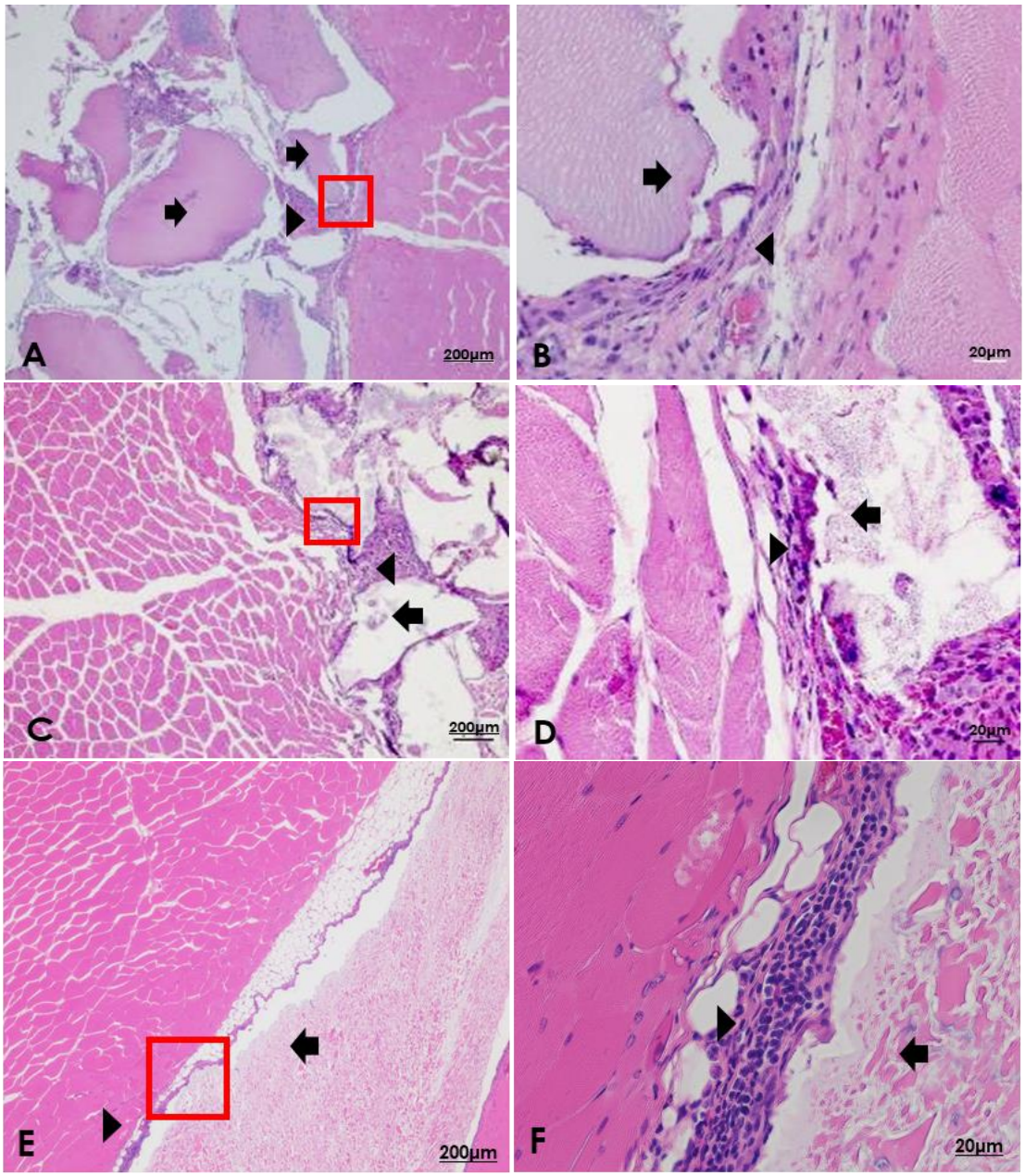


Figure 17 Histological images of (A,B) Deproteinized hDTM (dpDTM), (C,D) Anorganic deproteinized bovine bone (ABB) and (E,F) Polyvinyl-alcohol (PVA) in paralumbar muscle at 8 weeks with the magnification of 5X(A,C,E) and 40X(B,D,F) (H&E staining) (arrow head = inflammatory cells, black arrow = materials)

At 12 weeks, the histological images of both dpDTM and ABB groups showed mild inflammatory infiltration. The inflammatory cells were obviously decrease compare with the previous time. Blood vessel was not seen at this time point. Mild inflammatory cells were detected in PVA group. (Fig.18A-F)

Week 12

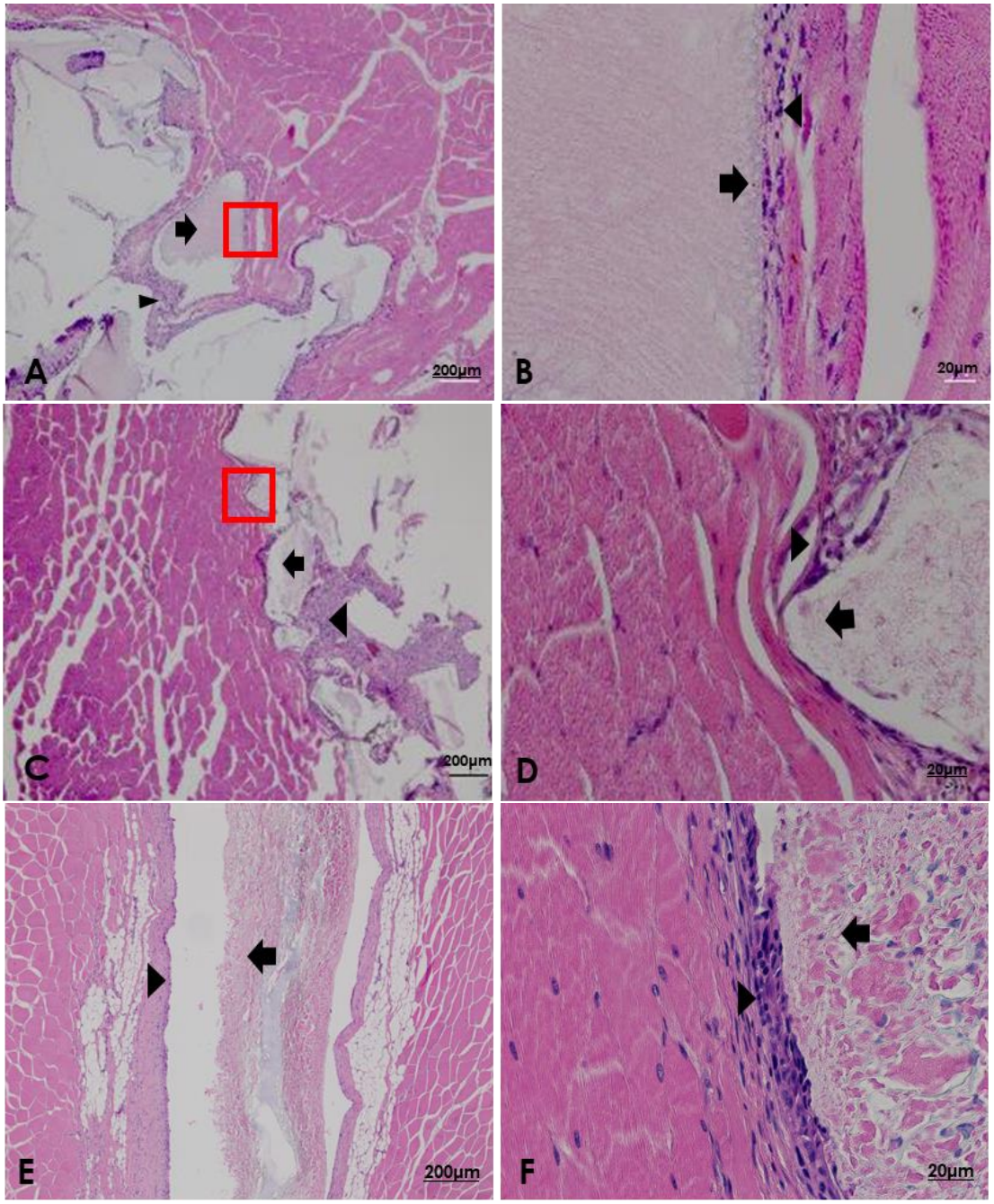


Figure 18 Histological images of (A,B) Deproteinized hDTM (dpDTM), (C,D) Anorganic deproteinized bovine bone (ABB) and (E,F) Polyvinyl-alcohol (PVA) in paralumbar muscle at 12 weeks with the magnification of 5X(A,C,E) and 40X(B,D,F) (H&E staining) (arrow head = inflammatory cells, black arrow = materials)

When compare between two groups, the histological results were not apparently different. From 1 week to 4 weeks both dpDTM and ABB showed moderate to intense inflammatory response. The local tissue reaction was decreased with time. At 8 weeks to 12 weeks, both dpDTM and ABB showed mild inflammatory response.

Only thin layer of inflammatory cells was observed at 12 weeks. However, dpDTM group presented lower inflammation response at 12 weeks. Moreover, no heterotopic bone was seen at all time point. (Fig.19)

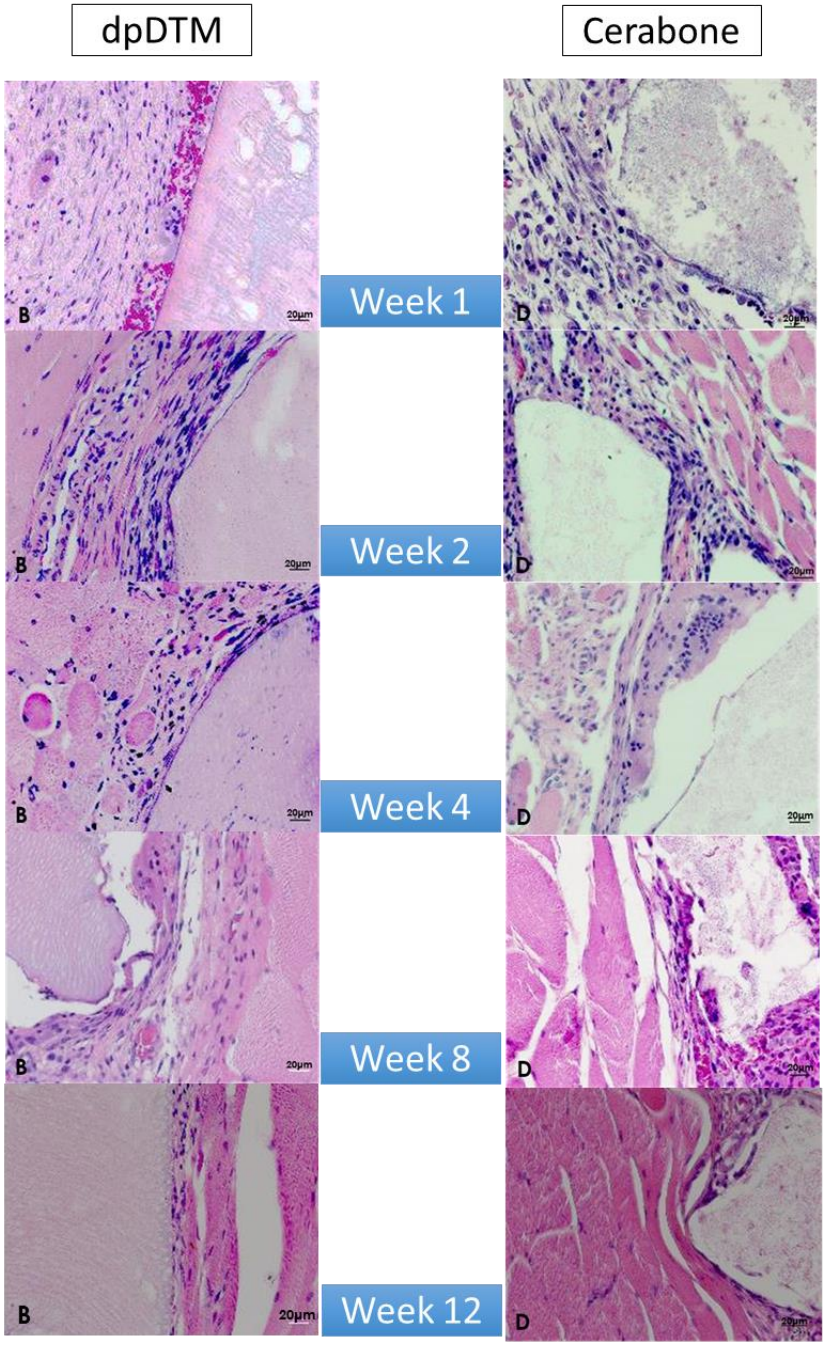


Figure 19 Histological Comparison between deproteinized hDTM (dpDTM) and anorganic deproteinized bovine bone (ABB) in paralumbar muscle at 1-12weeks, the inflammatory response was decrease with time (H&E staining), (Magnification 40X B,D)

The histological images of PVA which was negative control group showed in Fig.20(A-E). At the early period of local tissue response, 1– 4 weeks showed the multi-layer of inflammatory cells close contact to the materials. However, at 8-12

weeks there was only thin layer of inflammatory cells presented between materials and muscle tissue.

Inflammatory cells count (Grid pattern diagram)

The quantitative data of inflammatory cells count was informed as mean ± SD (Table 5). The mean of inflammatory cells count was decrease with time from 1 week to 12 weeks in both dpDTM and ABB groups. For intra-group comparison, the inflammatory cells count at 4, 8 and 12 weeks were lower than 1 week significantly (p = 0.00). On the other hand, there was no significant different between 2 weeks and 1 week. When compare between groups (dpDTM and Cerabone®), there was no significant different of inflammatory cells count between dpDTM and ABB at all time point. According from Pinna et al, 2011 [87], the mean inflammatory cells count at 1 and 2 weeks were categorized into intense (Over twenty inflammatory cells were detected) and the cells count were categorized into moderate at 4,8 and 12 weeks.

Table 5 The data showed mean ± SD of the Inflammatory cells count of dpDTM group and ABB group after materials were implanted in paralumbar muscle at 1, 2, 4, 8 and 12 weeks.

Inflammatory cells count	**Mean ± SD**				
	1 week	**2 weeks**	**4 weeks**	**8 weeks**	**12 weeks**
dpDTM	*21.83 ± 1.83 Intense	20.17 ± 1.72 Intense	17.50 ± 1.38* Moderate	15.17 ± 1.16* Moderate	13.67 ± 1.50* Moderate
ABB (Cerabone®)	*24.83 ± 1.46 Intense	24.16 ± 1.67 Intense	17.50 ± 1.38* Moderate	14.67 ± 1.11* Moderate	13.00 ± 1.29* Moderate
PVA	23.67 ± 2.42 Intense	19.17 ± 1.47 Moderate	15 ± 1.26 Moderate	10 ± 1.41 Mild	4.83 ± 0.75 Mild

(Intense = Over twenty inflammatory cells were detected.) (Moderate = Eleven to twenty inflammatory cells were detected) (n=6) (* = Significant different was found at 1 week group, intra-group comparison, P value = 0.00) (No significant different was found between groups).

Biodegradation evaluation

The three-dimensional morphology of dpDTM and ABB scaffolds were showed in Fig.20-21. The particle size of dpDTM were slightly larger than those of ABB group. In addition, there were no distinct morphological changes of dpDTM and ABB scaffolds from 1st week to 12th week.

According from MicroCT data, the results indicated that the relative bone volume/total volume (BV/TV) in dpDTM scaffolds was greater than that in ABB scaffolds at all time point. For dpDTM scaffolds, the BV/TV was 0.528 ± 0.024 at the beginning and decrease with time until 12 weeks (0.519 ± 0.016). While the same tendencies were observed in ABB scaffolds group, the BV/TV was 0.484 ± 0.020 at the beginning and at 12 weeks the BV/TV was equal to 0.475 ± 0.017. The percentage of reduction in dpDTM and ABB were 1.663% and 1.897% respectively. (Fig.22)

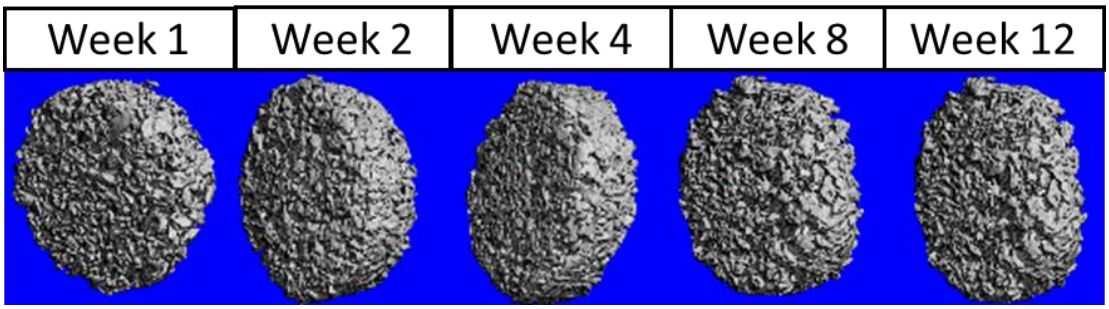


Figure 20 Three-dimensional morphology of dpDTM scaffold were scarcely degraded with time at 1, 2, 4, 8, 12 weeks. (From left to right)

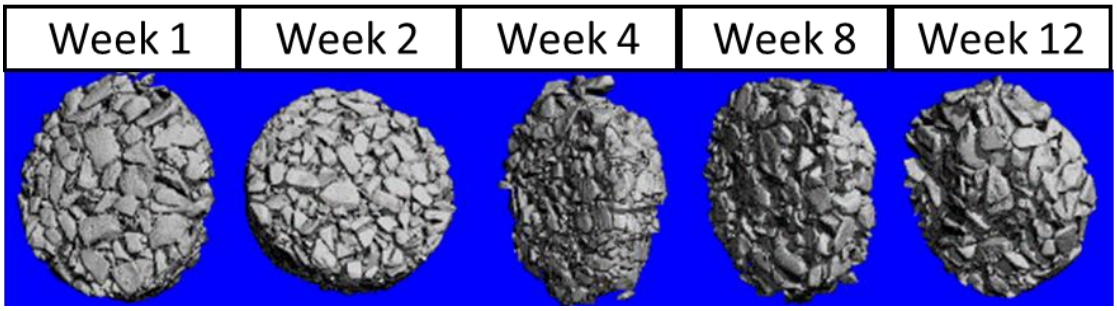


Figure 21 Three-dimensional morphology of ABB scaffold were scarcely degraded with time at 1, 2, 4, 8, 12 weeks. (From left to right)

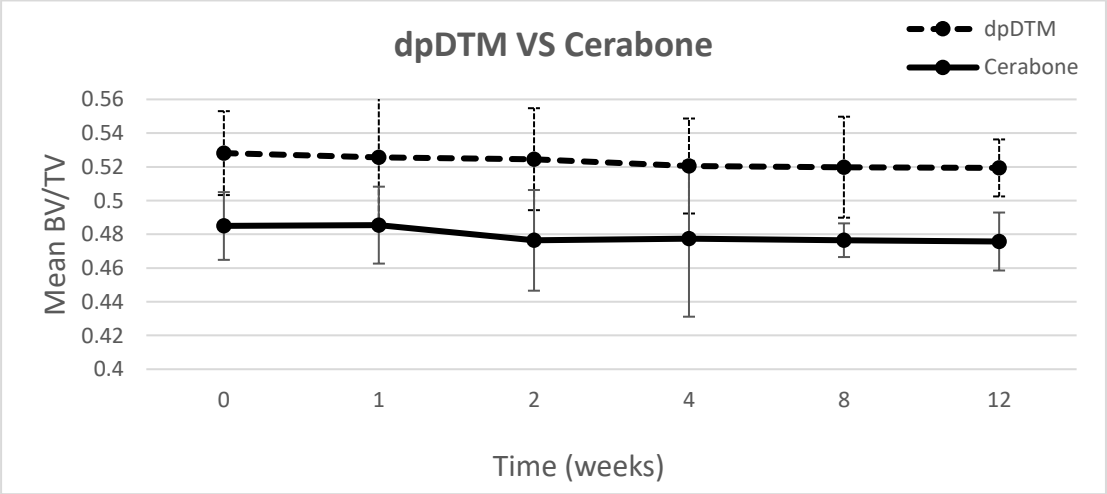


Figure 22 The association between the dpDTM (dot line) and ABB (black line) on bone volume/total volume (BV/TV) at 1, 2, 4, 8 and 12 weeks were slightly decrease in both groups with no significant different between group. (dpDTM: p value = 0.531 and ABB: p value = 0.490 respectively)

CHAPTER 4

DISCUSSIONS

Nowadays, all of new materials for human used must be test before clinically used both *In vitro* and *In vivo* testing. In our previous study, the dpDTM has been performed the physicochemical property testing, cell cytotoxicity and cell biocompatibility and the results demonstrated good physicochemical properties and non-toxicity to the cells. *(Unpublished data)* In this study, to prove its *In vivo* biocompatibility and biodegradation the materials were implant into paralumbar muscle area which given the high cellular activity and high vascularity.

According to the biocompatibility of materials, ISO-10993[41]. indicated that the muscle or subcutaneous implantation study was helpful as a screening test to assess local toxicities of the new materials. From the clinical observation of this study, there was no sign of infection in all rabbits represented the good biocompatibility which was similar to many studies[88, 89]. For histological observation, Farzad *et al*[88]. reported no evidence of any inflammatory infiltration after implantation of human dentin graft into abdominal muscle of the rabbit at 12 weeks. In contrast to our study, the inflammatory cells were remained until 12 weeks, but decrease with time. In generally, the non-specific immune response was activated when the tissue trauma has been occurred. Damaged cells release inflammatory chemical signals and evoke the initial inflammatory cells migrating to the trauma site until 7-10 days. When compare to our results, the initial inflammatory cells were sustained until 2 weeks which was comparable to general situation. The mean inflammatory cells count of dpDTM group were lower than ABB group at 1 and 2 weeks distinguishably. After 2 weeks the inflammatory cells count was comparable between those dpDTM and ABB groups.

In the present study, PVA was selected to fabricate the dpDTM scaffold as binding material. According from the *In vivo* study of PVA biocompatibility[84]. Some degree of inflammatory cells could be detected up to 4 weeks which comparable to our results. Thus, the moderate inflammatory reaction at the early period (1,2 and 4 weeks) of dpDTM probably be related by PVA.

The biodegradation of materials may be influenced by many factors including physicochemical properties, experimental models, implantation site and even animal species[90, 91]. Several methods were being used to investigate the biodegradation of biomaterials not only *In vitro* but also *In vivo* study. It depended on the purpose of the study to select the methods. In this study, the histological and micro CT evaluation were selected to test the *In vivo* degradability of materials. Histological analysis revealed the sign of graft resorption only at the region of interest but didn't showed the total graft volume change. While micro CT could analyze the BV/TV of the graft and revealed the reduction of graft with time by 3D images.

During the materials were implanted into muscular area, the cell-related biodegradability composed of mainly macrophages and multinucleated giant cells. On the other way, when the biomaterials were implanted into bony area such as calvaria

defects. The cell-related biodegradability should be mainly osteoclast. In our study, the main cell-related to biodegradation composed of macrophages, lymphocytes and plasma cells representing the chronic inflammatory response which was similar to other *In vivo* biodegradable studies[92, 93]. According from histological images of dpDTM at 8 and 12 weeks. Not only the multinucleated giant cells but also the resorption pits and some degree of material chippings were detected at dpDTM surface. This could be suggesting that our material is the bioresorbable biomaterials.

Lu *et al*[91]. studied the biodegradation rate of HA and β-TCP implanted into rabbit femoral and tibia condyle. The degradation rate of HA was about 2% and for β-TCP was about 34% at 12 weeks. Compare to our study, the degradation rate of dpDTM and ABB were 1.663% and 1.897% respectively at 12 weeks which were similar to HA but lower than β-TCP. However, in our study the materials were implanted into paralumbar muscle which was difference implantation site from the previous study. Another recent study, Zhang *et al*[86]. reported the 20% degradation rate of demineralized bone matrix (DBM) and 15% degradation rate of β-TCP after implantation of materials into calvaria defect of Spraque-Dawley rats at 8 weeks. The percentage of degradation of DBM and β-TCP were rather high when compare to our dpDTM, which may due to the different composition of tooth and bone.

In the present study, the percentage of reduction in dpDTM and ABB were comparable and relatively low (1-2%). In this situation, the evaluation time was only at 12 weeks. For better biodegradable outcomes, the longer time period up to 6 or 12 months might be concerned.

According from the previous study of our research team, the dpDTM using dual deproteinization step, chemical/thermal process has been first demonstrated *(Unpublished data).* The dpDTM prepared from the NaOH/thermal protocol demonstrated good physicochemical and suitable degradation properties. In addition, the dpDTM scaffold was also favorable for cell attachment and proliferation. In this present study, the materials demonstrated good biocompatibility and slow degradability rate in rabbit model. The slow degradability rate of dpDTM was potentially useful for maintain volume stability of the graft. Therefore, dpDTM could applied as bone graft substitute for many clinical procedures such as socket preservation, sinus augmentation and alveolar bone augmentation. The dpDTM can be fabricated from extracted teeth and prepare in stock for being used as allogeneic osteoconductive material for bone regeneration.

In terms of biocompatibility and biodegradation properties, there was no histologically and radiographically different from dpDTM and ABB. dpDTM could be suggested as bone graft substitution which could provide biocompatible, bioresorbable and osteoconductive properties as commercially anorganic deproteinized bovine bone xenograft.

This study was an animal experimental study including positive control, negative control and test groups. The measurements of inflammatory cell count were repeated to improve both validity and reliability. The number of experimental site (n) was 6/group/timepoint which sufficient to obtain reliable outcomes. Only single examiner performed the measurement of all parameter to minimize an error. Moreover,

Grid pattern diagram was used to evaluate the present of inflammation cell histologically which could support the data to become more quantitative.

For the limitation of this study, even though the evaluation time of 1,2,4,8 and 12 weeks were sufficient for biological results, however the longer period of healing should be performed for better outcome of biodegradation results. During the specimen collection process, it was difficult to locate the materials position due to the flexible of muscle tissue in rabbit model.

According from the evidence of this present study, the dpDTM was favorable for reconstructive bone graft substitution due to its good biocompatibility together with the slow degradation profiles. Therefore, dpDTM might be an alternative choices of bone graft materials. For the implication, the dpDTM could applied as bone graft substitute for many aspects of clinical procedures such as socket preservation, sinus augmentation and alveolar bone augmentation.

The identification of the inflammatory cell from histological sections were subjective and difficult to performed accurately. Many studies described the inflammatory cell response as descriptive data. In this study, the author categorized the inflammatory cell count as absent, mild, moderate and Intense to improve the outcomes.

For future research direction, to prove the potential of the material in the way of bone grafts, the *In vivo* bone regeneration is the important part at this state. For the next period, the clinically relevant implantation studies should be concerned before human used of this tooth graft material.

CHAPTER 5

CONCLUSIONS

In terms of biocompatibility, deproteinized human demineralized tooth matrix (dpDTM) and anorganic bovine bone had good biocompatibility with less degree of inflammatory cells infiltration at 12 weeks. The inflammatory response of those dpDTM and ABB at all time point were hardly different. Furthermore, both materials had potential to degrade with time with a slow degradability rate. Suggesting that dpDTM was favorable for reconstructive bone graft substitution due to its good biocompatibility together with the slow degradation profiles. However, clinically relevant implantation studies should be concerned before human used of this tooth graft material.

REFERENCES

1. Kumar P, Vinitha B, Fathima G. Bone grafts in dentistry. ***J Pharm Bioallied Sci*** 2013; 5(1): 125-7.
2. Hansson S, Halldin A. Alveolar ridge resorption after tooth extraction: A consequence of a fundamental principle of bone physiology. ***J Dent Biomech*** 2012; 3: 1758736012456543.
3. Nasr HF, Aichelmann-Reidy ME, Yukna RA. Bone and bone substitutes. ***Periodontol 2000*** 1999; 19: 74-86.
4. Laurencin C, Khan Y, El-Amin SF. Bone graft substitutes. ***Expert Rev Med Devices*** 2006; 3(1): 49-57.
5. Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. ***Clin Orthop Relat Res*** 2000; (371): 10-27.
6. LeGeros RZ. Calcium phosphate-based osteoinductive materials. ***Chem Rev*** 2008; 108(11): 4742-53.
7. Lambert F, Bacevic M, Layrolle P, Schupbach P, Drion P, Rompen E. Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites. ***Clin Oral Implants Res*** 2017; 28(10): 201-7.
8. Alves de Sousa C, Lemos C, Santiago-Jr J, Faverani L, Pellizzer E. Bone augmentation using autogenous bone versus biomaterial in the posterior region of atrophic mandibles: A systematic review and meta-analysis. ***J Dent*** 2018; 76: 1-8.
9. Troeltzsch M, Troeltzsch M, Kauffmann P, Gruber R, Brockmeyer P, Moser N, et al. Clinical efficacy of grafting materials in alveolar ridge augmentation: A systematic review. ***J Craniomaxillofac Surg*** 2016; 44(10): 1618-29.
10. Cypher TJ, Grossman JP. Biological principles of bone graft healing. ***J Foot Ankle Surg*** 1996; 35(5): 413-7.
11. Roberts TT, Rosenbaum AJ. Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing. ***Organogenesis*** 2012; 8(4): 114-24.
12. Stevenson S. Biology of bone grafts. ***Orthop Clin North Am*** 1999; 30(4): 543-52.
13. Figueiredo A S, O., & Cabrita, S. Inflammatory reaction post implantation of bone graft materials. ***Exp Pathol Health Sci*** 2012; 6(15): 8.
14. Burchardt H. The biology of bone graft repair. ***Clin Orthop Relat Res*** 1983; (174): 28-42.
15. Horowitz MC, Friedlaender, G. E. Bone and cartilage allografts. ***J Am Acad Orthop Surg*** 1991; 1: 85-101.
16. Arron AD, Wiedel, J. D. Allograft use in orthopedic surgery. ***Orthopedics*** 1994; 17: 41-8.
17. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. ***Am J Physiol*** 1987; 253(3): 699-703.
18. Swain SD, Rohn TT, Quinn MT. Neutrophil priming in host defense: role of oxidants as priming agents. ***Antioxid Redox Signal*** 2002; 4(1): 69-83.

19. Martin P, D'Souza D, Martin J, Grose R, Cooper L, Maki R, et al. Wound healing in the PU.1 null mouse--tissue repair is not dependent on inflammatory cells. ***Curr Biol*** 2003; 13(13): 1122-8.
20. Chin D, Boyle GM, Parsons PG, Coman WB. What is transforming growth factor-beta (TGF-beta)? ***Br J Plast Surg*** 2004; 57(3): 215-21.
21. Cotran RS, Kumar, V., Robbins, S. L. In Pathologic Basis of Disease. ***J. Pathol*** 1989: 163-237.
22. Khan SN, Cammisa FP, Jr., Sandhu HS, Diwan AD, Girardi FP, Lane JM. The biology of bone grafting. ***J Am Acad Orthop Surg*** 2005; 13(1): 77-86.
23. Silva TA, Lara VS, Silva JS, Garlet GP, Butler WT, Cunha FQ. Dentin sialoprotein and phosphoprotein induce neutrophil recruitment: a mechanism dependent on IL-1beta, TNF-beta, and CXC chemokines. ***Calcif Tissue Int*** 2004; 74(6): 532-41.
24. Silva TA, Lara VS, Silva JS, Oliveira SH, Butler WT, Cunha FQ. Macrophages and mast cells control the neutrophil migration induced by dentin proteins. ***J Dent Res*** 2005; 84(1): 79-83.
25. Jaha H, Husein D, Ohyama Y, Xu D, Suzuki S, Huang GT, et al. N-terminal Dentin Sialoprotein fragment induces type I collagen production and upregulates dentinogenesis marker expression in osteoblasts. ***Biochem Biophys Rep*** 2016; 6: 190-6.
26. Tanford C. Protein denaturation. ***Adv Protein Chem*** 1968; 23: 121-282.
27. Tanford C. Protein denaturation. C. Theoretical models for the mechanism of denaturation. ***Adv Protein Chem*** 1970; 24: 1-95.
28. Koizumi M, Hirai H, Onai T, Inoue K, Hirai M. Collapse of the hydration shell of a protein prior to thermal unfolding. ***J Appl Crystallogr*** 2007; 40(s1): 175-8.
29. Murugan R, Rao KP, Kumar TSS. Heat-deproteinated xenogeneic bone from slaughterhouse waste: Physico-chemical properties. ***B Mater Sci*** 2003; 26(5): 523-8.
30. Hiller JC, Thompson TJ, Evison MP, Chamberlain AT, Wess TJ. Bone mineral change during experimental heating: an X-ray scattering investigation. ***Biomaterials*** 2003; 24(28): 5091-7.
31. Uklejewski R, Winiecki M, Musielak G, Tokowicz R. Effectiveness of various deproteinization processes of bovine cancellous bone evaluated via mechano-biostructural properties of produced osteoconductive biomaterials. ***Biotechnol Bioproc e*** 2015; 20(2): 259-66.
32. Lambert F, Bacevic M, Layrolle P, Schüpbach P, Drion P, Rompen E. Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites. ***Clin Oral Implants Res*** 2016: n/a-n/a.
33. Conz MB, Granjeiro JM, Soares Gde A. Physicochemical characterization of six commercial hydroxyapatites for medical-dental applicatons as bone graft. ***J Appl Oral Sci*** 2005; 13(2): 136-40.
34. do Desterro Fde P, Sader MS, Soares GD, Vidigal GM, Jr. Can inorganic bovine bone grafts present distinct properties? ***Braz Dent J*** 2014; 25(4): 282-8.
35. Lim KT, Suh JD, Kim J, Choung PH, Chung JH. Calcium phosphate bioceramics fabricated from extracted human teeth for tooth tissue engineering. ***J Biomed Mater Res B Appl Biomater*** 2011; 99(2): 399-411.

36. Broz JJ, Simske SJ, Corley WD, Greenberg AR. Effects of deproteinization and ashing on site-specific properties of cortical bone. ***J Mater Sci Mater Med*** 1997; 8(6): 395-401.
37. Bi L, Li DC, Huang ZS, Yuan Z. Effects of sodium hydroxide, sodium hypochlorite, and gaseous hydrogen peroxide on the natural properties of cancellous bone. ***Artif Organs*** 2013; 37(7): 629-36.
38. Tadic D, Epple M. A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone. ***Biomaterials*** 2004; 25(6): 987-94.
39. GOKDUMAN K. Effects of hydrogen peroxide bleaching on human dentin and enamel microstructure and function. ***J Dent*** 2005; 86(11):1040-5.
40. ISO10993-6. Biological evaluation of medical devices part 6 : test of local effects after implantation. 2007: 1-21.
41. ISO10993. Biological evaluation of medical devices part 1:Evaluation and testing. 2013: 1-35.
42. Moran CJ, Ramesh A, Brama PA, O'Byrne JM, O'Brien FJ, Levingstone TJ. The benefits and limitations of animal models for translational research in cartilage repair. ***J Exp Orthop*** 2016; 3(1): 1.
43. Pripatnanont P, Nuntanaranont T, Vongvatcharanon S. Proportion of deproteinized bovine bone and autogenous bone affects bone formation in the treatment of calvarial defects in rabbits. ***Int J Oral Maxillofac Surg*** 2009; 38(4): 356-62.
44. Eid K, Zelicof S, Perona BP, Sledge CB, Glowacki J. Tissue reactions to particles of bone-substitute materials in intraosseous and heterotopic sites in rats: discrimination of osteoinduction, osteocompatibility, and inflammation. ***J Orthop Res*** 2001; 19(5): 962-9.
45. Scott MA, Levi B, Askarinam A, Nguyen A, Rackohn T, Ting K, et al. Brief review of models of ectopic bone formation. ***Stem Cells Dev*** 2012; 21(5): 655-67.
46. Lister LSDaL. Biocompatibility Safety Assessment of Medical Devices: FDA/ISO and Japanese Guidelines. ***Ministry of Health, Labour and Welfare*** 2003: 13-5.
47. Das D, Zhang Z, Winkler T, Mour M, Gunter C, Morlock M, et al. Bioresorption and degradation of biomaterials. ***Adv Biochem Eng Biotechnol*** 2012; 126: 317-33.
48. Lu J, Descamps M, Dejou J, Koubi G, Hardouin P, Lemaitre J, et al. The biodegradation mechanism of calcium phosphate biomaterials in bone. ***J Biomed Mater Res*** 2002; 63(4): 408-12.
49. Lu J. DM, Dejou J., Koubi G., Hardouin P., Lemaitre J., Proust J.P. The biodegradation mechanism of calcium phosphate biomaterials in bone. ***J Biomed Mater Res*** 2002; 63: 408–412.
50. Piattelli M, Favero GA, Scarano A, Orsini G, Piattelli A. Bone reactions to anorganic bovine bone (Bio-Oss) used in sinus augmentation procedures: a histologic long-term report of 20 cases in humans. ***Int J Oral Maxillofac Implants*** 1999; 14(6): 835-40.
51. Valentini P, Abensur DJ. Maxillary sinus grafting with anorganic bovine bone: a clinical report of long-term results. ***Int J Oral Maxillofac Implants*** 2003; 18(4): 556-60.

52. Isaksson H, Harjula T, Koistinen A, Iivarinen J, Seppanen K, Arokoski JP, et al. Collagen and mineral deposition in rabbit cortical bone during maturation and growth: effects on tissue properties. ***J Orthop Res*** 2010; 28(12): 1626-33.
53. Kim YK, Lee J, Um IW, Kim KW, Murata M, Akazawa T, et al. Tooth-derived bone graft material. ***J Korean Assoc Oral Maxillofac Surg*** 2013; 39(3): 103-11.
54. Kim YK, Kim SG, Byeon JH, Lee HJ, Um IU, Lim SC, et al. Development of a novel bone grafting material using autogenous teeth. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2010; 109(4): 496-503.
55. Kim YK, Kim SG, Oh JS, Jin SC, Son JS, Kim SY, et al. Analysis of the inorganic component of autogenous tooth bone graft material. ***J Nanosci Nanotechnol*** 2011; 11(8): 7442-5.
56. M. S. Mini-review article Dentin as bone graft substitution. ***Songklanakarin Dent J*** 2014; 2(1): 21-7.
57. Sivakumar GR, Girija EK, Narayana Kalkura S, Subramanian C. Crystallization and Characterization of Calcium Phosphates: Brushite and Monetite. ***Cryst Res Technol*** 1998; 33(2): 197-205.
58. Kim YK, Kim SG, Yun PY, Yeo IS, Jin SC, Oh JS, et al. Autogenous teeth used for bone grafting: a comparison with traditional grafting materials. ***Oral Surg Oral Med Oral Pathol Oral Radiol*** 2014; 117(1): 39-45.
59. Butler WT, Mikulski A, Urist MR, Bridges G, Uyeno S. Noncollagenous proteins of a rat dentin matrix possessing bone morphogenetic activity. ***J Dent Res*** 1977; 56(3): 228-32.
60. Linde A. Dentin matrix proteins: composition and possible functions in calcification. ***Anat Rec*** 1989; 224(2): 154-66.
61. Yamakoshi Y. Dentinogenesis and Dentin Sialophosphoprotein (DSPP). ***J Oral Biosci*** 2009; 51(3): 134.
62. Bessho K, Tanaka N, Matsumoto J, Tagawa T, Murata M. Human dentin-matrix-derived bone morphogenetic protein. ***J Dent Res*** 1991; 70(3): 171-5.
63. Guo S, Lim D, Dong Z, Saunders TL, Ma PX, Marcelo CL, et al. Dentin sialophosphoprotein: a regulatory protein for dental pulp stem cell identity and fate. ***Stem Cells Dev*** 2014; 23(23): 2883-94.
64. Zhang M, Jiang F, Zhang X, Wang S, Jin Y, Zhang W, et al. The Effects of Platelet-Derived Growth Factor-BB on Human Dental Pulp Stem Cells Mediated Dentin-Pulp Complex Regeneration. ***Stem cells tranal Med*** 2017.
65. Maas MC. Enamel structure and microwear: an experimental study of the response of enamel to shearing force. ***Am J Phys Anthropol*** 1991; 85(1): 31-49.
66. Michel Goldberg ABK, Marian Young, and Adele Boskey. Dentin: Structure, Composition and Mineralization: The role of dentin ECM in dentin formation and mineralization. ***Front Biosci*** 2012; 3: 711–35.
67. Montoya C, Arango-Santander S, Pelaez-Vargas A, Arola D, Ossa EA. Effect of aging on the microstructure, hardness and chemical composition of dentin. ***Arch Oral Biol*** 2015; 60(12): 1811-20.
68. Reis-Filho CR, Silva ER, Martins AB, Pessoa FF, Gomes PV, de Araujo MS, et al. Demineralised human dentine matrix stimulates the expression of VEGF and accelerates the bone repair in tooth sockets of rats. ***Arch Oral Biol*** 2012; 57(5): 469-76.

69. de Oliveira GS, Miziara MN, Silva ER, Ferreira EL, Biulchi AP, Alves JB. Enhanced bone formation during healing process of tooth sockets filled with demineralized human dentine matrix. ***Aust Dent J*** 2013; 58(3): 326-32.
70. Farzad P A-AA, Dahlin A, Andersson L, Dahlin C. . On tissue reactions to dentin as a bone substitute material. ***Department of biomaterials Institute of clinical sciences Sahlgrenska Academy at University of Gothenburg*** 2017: 46-9,58,67.
71. Moharamzadeh K, Freeman C, Blackwood K. Processed bovine dentine as a bone substitute. ***Br J Oral Maxillofac Surg*** 2008; 46(2): 110-3.
72. Murata M, Sato D, Hino J, Akazawa T, Tazaki J, Ito K, et al. Acid-insoluble human dentin as carrier material for recombinant human BMP-2. ***J Biomed Mater Res A*** 2012; 100(3): 571-7.
73. Liu G, Xu G, Gao Z, Liu Z, Xu J, Wang J, et al. Demineralized Dentin Matrix Induces Odontoblastic Differentiation of Dental Pulp Stem Cells. ***Cells Tissues Organs*** 2016; 201(1): 65-76.
74. Bormann KH, Suarez-Cunqueiro MM, Sinikovic B, Kampmann A, von See C, Tavassol F, et al. Dentin as a suitable bone substitute comparable to ss-TCP--an experimental study in mice. ***Microvasc Res*** 2012; 84(2): 116-22.
75. Gomes MF, dos Anjos MJ, Nogueira TO, Guimaraes SA. Histologic evaluation of the osteoinductive property of autogenous demineralized dentin matrix on surgical bone defects in rabbit skulls using human amniotic membrane for guided bone regeneration. ***Int J Oral Maxillofac Implants*** 2001; 16(4): 563-71.
76. Nampo T, Watahiki J, Enomoto A, Taguchi T, Ono M, Nakano H, et al. A new method for alveolar bone repair using extracted teeth for the graft material. ***J Periodontol*** 2010; 81(9): 1264-72.
77. Jeong KI, Kim S-G, Kim Y-K, Oh J-S, Jeong M-A, Park J-J. Clinical Study of Graft Materials Using Autogenous Teeth in Maxillary Sinus Augmentation2011. 471-5 p.
78. Park S-M, Um I-W, Kim Y-K, Kim K-W. Clinical application of auto-tooth bone graft material. ***J Korean Assoc Oral Maxillofac Surg*** 2012; 38(1): 2-8.
79. Koga T, Minamizato T, Kawai Y, Miura K-i, I T, Nakatani Y, et al. Bone Regeneration Using Dentin Matrix Depends on the Degree of Demineralization and Particle Size. ***PLos One*** 2016; 11(1): e0147235.
80. Paradossi G, Cavalieri F, Chiessi E, Spagnoli C, Cowman MK. Poly(vinyl alcohol) as versatile biomaterial for potential biomedical applications. ***J Mater Sci Mater Med*** 2003; 14(8): 687-91.
81. Solaro R, Corti A, Chiellini E. Biodegradation of poly(vinyl alcohol) with different molecular weights and degree of hydrolysis2000. 873-8 p.
82. DeMerlis CC, Schoneker DR. Review of the oral toxicity of polyvinyl alcohol (PVA). ***Food Chem Toxicol*** 2003; 41(3): 319-26.
83. Ozkahraman2 ETaB. Potential Evaluation of PVA-Based Hydrogels for Biomedical Applications. ***Hittite J Sci Eng*** 2015; 2(2): 165-71.
84. Moreau D, Villain A, Bachy M, Proudhon H, Ku DN, Hannouche D, et al. In vivo evaluation of the bone integration of coated poly(vinyl-alcohol) hydrogel fiber implants. ***J Mater Sci Mater Med*** 2017; 28(8): 114.
85. Gupta S, Goswami S, Sinha A. A combined effect of freeze--thaw cycles and polymer concentration on the structure and mechanical properties of transparent PVA gels. ***Biomed Mater*** 2012; 7(1): 015006.

86. Zhang H, Mao X, Du Z, Jiang W, Han X, Zhao D, et al. Three dimensional printed macroporous polylactic acid/hydroxyapatite composite scaffolds for promoting bone formation in a critical-size rat calvarial defect model. ***Sci Technol Adv Mater*** 2016; 17(1): 136-48.
87. Pinna Bde R, Stavale JN, Pontes PA, Campones do Brasil Ode O. Histological analysis of autologous fascia graft implantation into the rabbit voice muscle. ***Braz J Otorhinolaryngol*** 2011; 77(2): 185-90.
88. Al-Asfour A, Farzad P, Andersson L, Joseph B, Dahlin C. Host tissue reactions of non-demineralized autogenic and xenogenic dentin blocks implanted in a non-osteogenic environment. An experimental study in rabbits. ***Dent Traumatol*** 2014; 30(3): 198-203.
89. Rumpel E, Wolf E, Kauschke E, Bienengraber V, Bayerlein T, Gedrange T, et al. The biodegradation of hydroxyapatite bone graft substitutes in vivo. ***Folia Morphol (Warsz)*** 2006; 65(1): 43-8.
90. Daculsi G, LeGeros RZ, Heughebaert M, Barbieux I. Formation of carbonate-apatite crystals after implantation of calcium phosphate ceramics. ***Calcif Tissue Int*** 1990; 46(1): 20-7.
91. Lu JX, Gallur A, Flautre B, Anselme K, Descamps M, Thierry B, et al. Comparative study of tissue reactions to calcium phosphate ceramics among cancellous, cortical, and medullar bone sites in rabbits. ***J Biomed Mater Res*** 1998; 42(3): 357-67.
92. Klein CP, de Groot K, Driessen AA, van der Lubbe HB. Interaction of biodegradable beta-whitlockite ceramics with bone tissue: an in vivo study. ***Biomaterials*** 1985; 6(3): 189-92.
93. Malard O, Bouler JM, Guicheux J, Heymann D, Pilet P, Coquard C, et al. Influence of biphasic calcium phosphate granulometry on bone ingrowth, ceramic resorption, and inflammatory reactions: preliminary in vitro and in vivo study. ***J Biomed Mater Res*** 1999; 46(1): 103-11.

APPENDIX

ที่ ศธ 0521.11/ 1393

สำนักวิจัยและพัฒนา
เลขที่ 15 ถนนกาญจนวนิช
มหาวิทยาลัยสงขลานครินทร์
อ.หาดใหญ่ จ.สงขลา 90110

Ref.63/2017

หนังสือรับรอง

รหัสโครงการ 2560 – 03 – 063

โครงการวิจัย การศึกษาคุณสมบัติความเข้ากันได้ทางชีวภาพและการย่อยสลายทางชีวภาพของดีนิเนอราไลซ์ทูธเมทริกที่ผ่านกระบวนการสกัดโปรตีนออกในกระต่าย

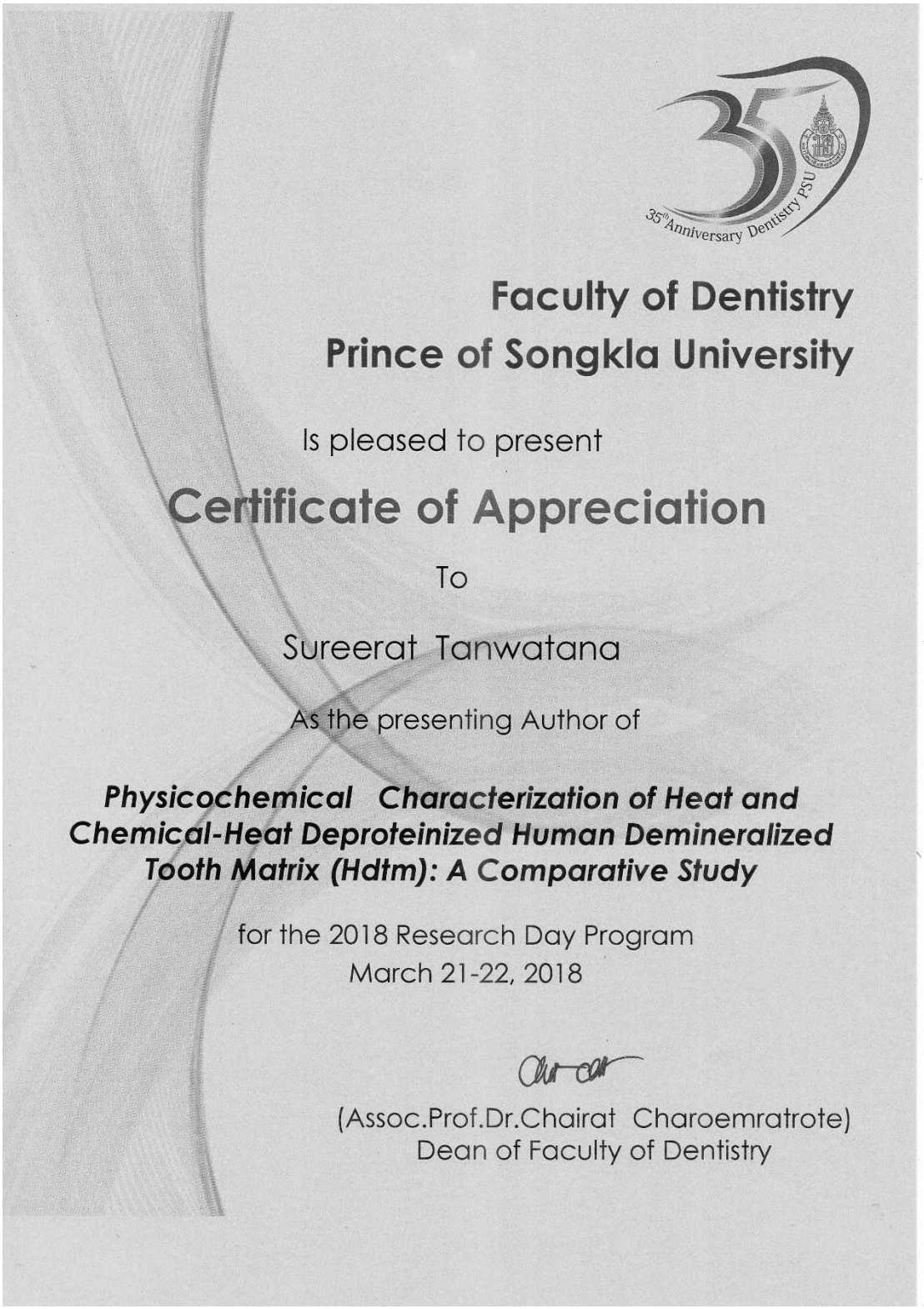
นักวิจัย รศ.ดร.ทพญ.ศรีสุรางค์ สุทธปรียาศรี
สังกัด คณะทันตแพทยศาสตร์

ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์ของสถาบัน มหาวิทยาลัยสงขลานครินทร์ โดยขอให้รายงานความก้าวหน้าของโครงการวิจัยทุก 6 เดือน

ให้ไว้ ณ วันที่ 14 ธันวาคม 2560

(รองศาสตราจารย์ ดร.วันทนา เหรียญมงคล)
รองประธานคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์ของสถาบัน
มหาวิทยาลัยสงขลานครินทร์

วันที่รับรอง: 1 มกราคม 2561
วันหมดอายุ: 30 กันยายน 2562


35th Anniversary Dentistry PSU
Faculty of Dentistry
Prince of Songkla University
Is pleased to present
Certificate of Appreciation
To
Sureerat Tanwatana
As the presenting Author of
Physicochemical Characterization of Heat and Chemical-Heat Deproteinized Human Demineralized Tooth Matrix (Hdtm): A Comparative Study
for the 2018 Research Day Program
March 21-22, 2018
(Assoc.Prof.Dr.Chairat Charoemratrote)
Dean of Faculty of Dentistry

คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ขอมอบเกียรติบัตรนี้เพื่อประกาศเกียรติคุณ

ทันตแพทย์หญิง สุรีรัตน์ ตันวัฒนะ

ในโอกาสได้รับรางวัลชนะเลิศ
การนำเสนอผลงานแบบปากเปล่า AWARD WINNER FOR ORAL PRESENTATION
การประชุมวิชาการทันตกรรมรากเทียมนานาชาติ ครั้งที่ 4 (BIS2017)
เรื่อง “Next Gen Implant Care” ระหว่างวันที่ 6-8 ธันวาคม 2560
ณ Siam Paragon at Royal Paragon Hall, Bangkok

ขอให้ประสบความสำเร็จและความเจริญยิ่งๆ ขึ้นไป
ให้ไว้ ณ วันที่ 13 เดือน ธันวาคม พุทธศักราช 2560

(รองศาสตราจารย์ ดร. ทพ. ไชยรัตน์ เฉลิมรัตนโรจน์)
คณบดีคณะทันตแพทยศาสตร์

DFCT 2019

THE 17TH International Scientific Conference of the Dental Faculty Consortium of Thailand

8 - 10 July 2019, Khon Kaen, Thailand

EMERGING TRENDS IN DENTISTRY

O_34

Biocompatibility of Deproteinized Human Demineralized Tooth Matrix (dpDTM) in Rabbit Model

Sureerat TANWATANA, Narit LEEPONG, and Srisurang SUTTAPREYASRI

Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of SongklaUniversity, Hatyai, Songkhla, Thailand

Background: Tooth is a composite structure consisting of inorganic and organic components which are very similar to those of alveolar bone. The autologous tooth matrix has been used clinically as bone substitution in various bone defect model. However, the major drawback of the autologous tooth matrix is the limited availability of the graft. Therefore, deproteinized human demineralized tooth matrix (dpDTM) was developed into allograft materials to overcome those limitations.

Objectives: To assess and compare the biocompatibility of dpDTM and anorganic deproteinized bovine bone (ABB) by intramuscular implantation at 1, 2, 4, 8, 12 weeks.

Methods: dpDTM was fabricated using dual chemical-thermal treatment (NaOH 1N, 300°C). The dpDTM scaffold construct was prepared using 5%wt/v Polyvinyl alcohol (PVA) as binding materials by freeze-thawing method. The dpDTM scaffolds (n=6/group/time point) were implanted into paralumbar muscle of the 15 adult New Zealand White rabbits. The ABB scaffold and blank PVA scaffold were used as positive and negative control. The biocompatibility analysis was performed by clinical and histological methods at 1, 2, 4, 8, 12 weeks after implantation.

Results: All rabbits recovered uneventfully without weight loss. Clinical observation revealed the absence of infection and inflammation at all time point. Histological evaluation of dpDTM and ABB in paralumbar muscle indicated the moderate to intense inflammatory response at 1-2 weeks and decreased with time.

Conclusion: In terms of biocompatibility, dpDTM and ABB demonstrated good biocompatibility with less degree of inflammatory cells infiltration at 12 weeks. Suggesting that dpDTM was favorable for reconstructive bone graft substitution due to its good biocompatibility. However, clinically relevant implantation studies should be concerned before human used of this tooth graft material.

Keywords: Biocompatibility, Bone substitution, Deproteinization, Rabbit, Tooth

VITAE

Name Miss Sureerat Tanwatana

Student ID 6010820016

Educational Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Dental Surgery	Prince of Songkla University	2015
Higher Graduate Diploma in Clinical Science (Oral and Maxillo facial Surgery)	Prince of Songkla University	2017

Work – Position and Address

Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand.

List of Proceeding and Publications

Award Winner for Oral Presentation. Tanwatana S, Suttapreyasri, Kaewjurat A, Leepong N. "Physicochemical characterization of thermal and chemical-thermal deproteinized human demineralized tooth matrix (hDTM): a comparative study". Attended to the Bangkok International Symposium of Implant Dentistry 2017 Siam Paragon, Royal Paragon Hall, Bangkok, Thailand.

Tanwatana S, Suttapreyasri, Kaewjurat A, Leepong N. "Physicochemical characterization of thermal and chemical-thermal deproteinized human demineralized tooth matrix (hDTM): a comparative study". Attended to the PSU Student Research Symposium 2018.

Tanwatana S, Suttapreyasri, Kaewjurat A, Leepong N. "Deproteinized human demineralized tooth matrix for bone tissue engineering: physicochemical characterization and cell adhesion" Attend to the International Osteology Symposium Barcelona 2019.

Tanwatana S, Suttapreyasri, Kaewjurat A, Leepong N. "Biocompatibility of Deproteinized Human Demineralized Tooth Matrix (dpDTM) in Rabbit Model" in The 17th International Scientific Conference of the Dental Faculty Consortium of Thailand July 8th –10th, 2019 at Pullman Khon Kaen Raja Orchid, Khon Kaen, Thailand.