The Influence of Demineralization Process on Physicochemical Characteristics, BMP-2 Quantification and in Vitro Biocompatibility of Human Demineralized Tooth Matrix

Anupong Jeerachaipansakul

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Master of Science in Oral and Maxillofacial Surgery

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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.

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ชื่อวิทยานิพนธ์	อิทธิพลของกระบวนการละลายแร่ธาตุ ที่มีต่อคุณสมบัติทางกายภาพเคมีปริมาณบีเอ็มพี2และการเข้ากันได้ทางชีวภาพของดีมินเนอราไลซ์ทูธเมทริกที่เตรียมจากมนุษย์
ผู้เขียน	นายอนุพงศ์ จีรชัยพันธุ์สกุล
สาขาวิชา	ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล
ปีการศึกษา	2561

บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาอิทธิพลของกระบวนการละลายแร่ธาตุออกด้วยกรดไฮโดรคลอริกที่ความเข้มข้นและระยะเวลาแตกต่างกัน ที่มีผลต่อคุณสมบัติของ ดีมินเนอราไลซ์ทูธเมทริกที่เตรียมจากฟันมนุษย์ ในแง่ของ คุณสมบัติทางกายภาพ อัตราการสลายตัว ปริมาณบีเอ็มพี2 และคุณสมบัติความเข้ากันได้ทางชีวภาพของเซลล์กระดูก

วิธีการวิจัย: นำฟันแท้ที่ได้จากการถอนฟัน ตัดแบ่งตัวฟันและรากฟัน นำเนื้อเยื่อโพรงประสาทฟันออก นำตัวฟันและรากฟันไปบดให้เป็นชิ้นเล็กขนาด 500-1,000 ไมครอน นำฟันที่ถูกบด แบ่งกลุ่มเป็น 5 กลุ่ม (0 โมล, 0 นาที (กลุ่มควบคุม), 2) 0.5 โมล 10 นาที, 3) 0.5 โมล 20 นาที, 4) 1 โมล 10 นาที และ 5) 1 โมล 20 นาที) โดยทำการทดสอบคุณสมบัติทางกายภาพเคมีในส่วนของ ลักษณะของชิ้นผงฟันในระดับจุลภาค ค่าความเป็นผลึกของชิ้นผงฟัน ธาตุองค์ประกอบ อัตราส่วนแคลเซียมฟอสเฟต การสลายตัวชิ้นผงฟันขึ้นรูป ความเข้มข้นของโปรตีนรวมและโปรตีนบีเอ็มพี2 ที่สกัดออกมาจากชิ้นผงฟัน ทำการเลือกกลุ่มที่มีลักษณะทางกายภาพเคมีที่เหมาะสม นำไปทดสอบความเข้ากันได้ทางชีวภาพของเซลล์กระดูก ในแง่ของ ลักษณะการเกาะของเซลล์บนชิ้นผงฟันขึ้นรูป จำนวนเซลล์ที่แบ่งตัว และมีชีวิตอยู่บนชิ้นผงฟันขึ้นรูป จำนวนเซลล์ที่เปลี่ยนสภาพบนชิ้นผงฟันขึ้นรูป ข้อมูลถูกนำเสนอเป็นค่าเฉลี่ยและส่วนเบี่ยงเบนมาตรฐาน โดยมีการวิเคราะห์ทางสถิติด้วยการเปรียบเทียบค่าเฉลี่ยของปริมาณ ความเข้มข้นของโปรตีน บีเอ็มพี2 ที่สกัดออกมาจากชิ้นผงฟัน, การสลายตัวชิ้นผงฟันขึ้นรูป, จำนวนเซลล์ที่แบ่งตัวและ จำนวนเซลล์ที่เปลี่ยนสภาพบนชิ้นผงฟันขึ้นรูป ที่มีนัยสำคัญทางสถิติ น้อยกว่า 0.05

ผลการศึกษา: ลักษณะชิ้นผงฟันของทุกกลุ่มมีลักษณะแข็งมีขาวและเหลือง โดยที่กลุ่มที่ผ่านกระบวนการละลายแร่ธาตุที่นานขึ้นจะมีความโปร่งใสมากขึ้น ในส่วนภาพจากกล้องจุลทรรศน์อิเลคตรอน กลุ่ม 0.5 โมล 20 นาที และ 1 โมล 20 นาที แสดงลักษณะพื้นผิวของวัสดุที่เรียบขนาดของเดนทินัลทูบูลที่กว้างขึ้น มีการเผยให้เห็นเส้นใยคอลลาเจน ร้อยละของธาตุแคลเซียมและ

ฟอสฟอรัสของกลุ่มทดลองทุกกลุ่มมีน้อยกว่า กลุ่มควบคุม (แคลเซียม ร้อยละ 15.60, ฟอสฟอรัส ร้อยละ 8.08) และพบว่ากลุ่มที่มีน้อยที่สุดคือ กลุ่ม 1 โมล 20 นาที (แคลเซียม ร้อยละ 3.43, ฟอสฟอรัส น้อยละ 1.07) อัตราส่วนแคลเซียมฟอสเฟต ของกลุ่ม 0 โมล, 0 นาที (กลุ่มควบคุม), 0.5 โมล 10 นาที, 0.5 โมล 20 นาที, 1 โมล 10 นาที และ 1 โมล 20 นาที คือ 1.49, 1.97, 2.39, 2.35 และ 2.48 ตามลำดับ ทุกกลุ่มแสดงลักษณะของไฮดรอกซีแอปาไทต์ ร้อยละค่าความเป็นผลึกของชิ้นผงฟัน ในกลุ่ม 0 โมล, 0 นาที (กลุ่มควบคุม), 0.5 โมล 10 นาที, 0.5 โมล 20 นาที, 1 โมล 10 นาที และ 1 โมล 20 นาที คือ ร้อยละ 63.09, 62.12, 56.84, 61.915 และ 54.33 การวัดการกระจายของรูพรุน มีค่าอยู่ระหว่าง 42.37-68.17A° โดยมีค่ามากที่สุดในกลุ่ม 1 โมล 20 นาที ในขณะพื้นที่ผิวมีค่า 1.77-5.43 ตร.ม./กรัม และมีค่าน้อยที่สุดในกลุ่ม 1 โมล 20 นาทีเช่นกัน ปริมาณของโปรตีนรวมและโปรตีนบีเอ็มพี2 ที่มากที่สุด พบในกลุ่ม 1 โมล 20 นาที และมีความแตกต่างอย่างมีนัยสำคัญทางสถิติกับกลุ่มควบคุม ร้อยละการสลายตัวชิ้นผงฟันขึ้นรูป ของกลุ่ม 0 โมล, 0 นาที (กลุ่มควบคุม), 0.5 โมล 10 นาที, 0.5 โมล 20 นาที, 1 โมล 10 นาที และ 1 โมล 20 นาที ในระยะเวลา 60 วัน คือ 6.41±0.30, 9.19±1.92, 8.46±0.28, 19.27±2.74 และ 22.90±10.10 ตามลำดับ (ระดับนัยสำคัญ <0.05) จากคุณสมบัติทางกายภาพเคมีที่ตรวจพบ ทำให้ กลุ่ม 0.5 โมล 10 นาที และ 0.5 โมล 20 นาที ถูกเลือกไปทดสอบความเข้ากันได้ทางชีวภาพในห้องทดลองเปรียบเทียบกับกลุ่มควบคุม การยึดเกาะของเซลล์กระดูก วันที่ 1 และ 7 ในกลุ่มควบคุมมีลักษณะรูปร่างไม่แน่นอนมีขนาดเล็กเกาะกับผิววัสดุแบบหลวมๆ ในขณะที่กลุ่มทดลอง เซลล์กระดูกมีการแผ่รูปร่างไปตามผิววัสดุและมีการเกาะเข้าไปในส่วนเดนทินัลทูบูล ต่อมาในวันที่ 14 ทุกกลุ่มมีการเกาะของเซลล์กระดูกในลักษณะเป็นแผ่นและแบ่งตัวทับกันหลายชั้น เซลล์กระดูกมีการแบ่งตัวบนพื้นผิววัสดุ เพิ่มขึ้นจากวันที่ 1 ถึงวันที่ 14 และลดลงเล็กน้อยในวันที่ 21 โดยกลุ่มควบคุมมีจำนวนเซลล์น้อยกว่ากลุ่มทดลองสองกลุ่มวันที่ 1, 14 และ 21 อย่างมีนัยสำคัญ ในขณะที่กลุ่ม 0.5 โมล 10 นาที และ 0.5 โมล 20 นาที มีจำนวนเซลล์สูงสุด ในส่วนของปริมาณอัลคาไลน์ฟอสฟาเตส ของกลุ่ม 0.5 โมล 10 นาที พบว่าเพิ่มขึ้นแตกต่างกันอย่างมีนัยสำคัญในวันที่ 14 และ 21 เมื่อเทียบกับวันที่ 1 และวันที่ 7 อย่างไรก็ตามไม่มีความแตกต่างของความเข้มข้นของ โปรตีนออสทิโอแคลซิน ระหว่างกลุ่มในทุกช่วงเวลา

บทสรุป: ระยะเวลาของปฏิกิริยาละลายแร่ธาตุด้วยกรดไฮโดรคลอริกมีผลต่อลักษณะพื้นผิว ร้อยละความเป็นผลึก ธาตุองค์ประกอบ อัตราส่วนแคลเซียมฟอสเฟต ในขณะที่ ความเข้มข้นของกรดไฮโดรคลอริกมีผลอัตราการสลายตัวของวัสดุ จากการศึกษาครั้งนี้พบว่า ดีมินเนอราไลซ์ทูธเมทริกจากฟันมนุษย์ ที่ผ่านกระบวนการเตรียมในกลุ่ม 0.5 โมล 10 นาที และ 0.5 โมล 20 นาที มีคุณสมบัติทางกายภาพเคมีและความเข้ากันได้ทางชีวภาพกับเซลล์กระดูกที่ดี เมื่อเปรียบเทียบกับฟันธรรมชาติ

Thesis Title: The Influence of Demineralization Process on Physicochemical Characteristics, BMP-2 Quantification and *In vitro* Biocompatibility of Human Demineralized Tooth Matrix
Author: Mr. Anupong Jeerachaipansakul
Major Program: Oral and Maxillofacial Surgery
Academic Year: 2018

ABSTRACT

Objectives: To examine the influence of different hydrochloric acid (HCl) demineralization processes on the physicochemical characteristic of the demineralized tooth matrix (DTM) in the aspect of physicochemical properties, degradation kinetic, the amount of bone morphogenetic protein-2 (BMP-2) and cell biocompatibility on the demineralized tooth matrix scaffold using osteoblastic cells.

Materials and methods: Caries-free permanent teeth were divided and pulp tissue were removed. The teeth were pulverized into small particles range from 500-1,000 µm. The particles were pooled and assigned to 5 groups with different protocols of HCl concentration and reaction time [0M/0min(TM), 0.5M/10min (0.5M/10DTM), 0.5M/20min (0.5M/20DTM), 1M/10min (1M/10DTM) and 1M/20min (1M/20DTM)]. The study was divided into 2 parts, physicochemical properties evaluation, and in vitro osteoblast cell biocompatibility. In physicochemical characterization, the chemical compositions were analyzed by X-ray diffraction and X-ray fluorescence spectroscopy. Structure topography and surface area were evaluated by scanning electron microscopy and BET analysis respectively. Protein extraction was performed for 24 hrs. Bradford protein assay was used to quantify total protein and ELISA was used for BMP-2 quantification. For degradation rate assessment, TM and DTM scaffolds were fabricated using 5% wt/v of polyvinyl alcohol (PVA) as a biding reagent. All groups were assessed at 60 days using 50 mM Tris-HCl solution (pH 7.4) at 37°c. Base on the physicochemical properties, DTM group from proper physicochemical properties and degradation rate was chosen for further study. For cell biocompatibility, MC3T3-E1 cells were seeded on the scaffolds. The cell-scaffold constructs of each group were divided for culturing in proliferation medium for evaluating cell viability and osteogenic induction medium for assessing cell differentiation on day 1, 3, 7, 14, and day 21. The morphology of the empty scaffolds and cell adherence were examined using SEM at 1, 7 and 14 days after cell seeding. The data was presented as mean±SD. The compared means analysis ($p<0.05$) was used for comparing total protein and BMP-2 quantification, degradation rate, cell proliferation and cell differentiation.

Results: All tooth particles were white to yellow in color with hard in consistency. DTM processed with the longer demineralization reaction times revealed less opacity. From SEM images, 0.5M/20DTM and 1M/20DTM groups exhibited a smooth surface with larger dentinal tubules and abundant of exposed collagen fibrils. Calcium and Phosphorous content in all DTM were lower compared to TM group. The lowest calcium and phosphate content were found in 1M/20DTM group (Ca=3.43%, P=1.07%) compared to TM (Ca=15.60%, P=8.08%). The Ca/P ratio for TM, 0.5M/10DTM, 0.5M/20DTM, 1M/10DTM and 1M/20DTM were 1.49, 1.97, 2.39, 2.35

and 2.48 respectively. The XRD confirmed that there was only hydroxyapatite phase. The crystallinity in TM, 0.5M/10DTM, 0.5M/20DTM, 1M/10DTM and 1M/20DTM were 63.09%, 62.12%, 56.84%, 61.915% and 54.33% respectively. From BET analysis demonstrated the average pore distributions from 42.37-68.17A° with the highest in 1M/20DTM group. In contrast to the surface areas range from 1.77-5.43 m2/g with the lowest in 1M/20m group. The highest total protein (3.53±0.24 µg/ml) and BMP-2 concentration (1,181.53±193.77 pg/ml) were found in 1M/20DTM group compared to TM (0.98±0.081 µg/ml, 2.67±5.34 pg/ml, p=0.000). The degradation at 60 days of 0M/0DTM, 0.5M/10DTM, 0.5M/20DTM, 1M/10DTM, and 1M/20DTM were 6.41±0.30%, 9.19±1.92%, 8.46±0.28%, 19.27±2.74%, 22.90±10.10% respectively ($p<0.05$).

From physicochemical characteristics, 0.5M/10DTM and 0.5M/20DTM were selected for further in vitro study compared to TM as control. SEM analysis of cell-scaffold construct in TM group demonstrated irregular shape of cells and small size on day 1 and day 7. Contrary to 0.5M/10DTM and 0.5M/20DTM groups that the cells showed larger size and propagation. At day 14, the cells proliferated to multilayers on the surface of the scaffolds in all groups. Cell proliferation of all groups increased from day 1 to day 14 but slightly decreased at day 21. There was a statistically significant difference between day 1, 14 and day 21 ($p<0.05$) in TM group. Whereas, in 0.5M/10DTM and 0.5M/20DTM group showed significant difference at day 14 compare to day 1,3 and day 7 ($p<0.05$). The cell number in 0.5M/10DTM group was significantly higher than TM group at day 1 and day 3 ($p<0.05$). The ALP activity of 0.5M/10DTM group showed significantly different increasing at day 14 and 21. However, there was no significantly different of osteocalcin (OCN) concentration between 0.5M/10DTM, 0.5M/20DTM and TM group in all time point.

Conclusions: The reaction time of HCl has effect on tooth matrix in aspect of surface morphology, crystallinity, element components, Ca/P ratio and the quantity BMP-2. The HCl acid concentration affect degradation rate of the material. The tooth matrix which demineralized using 0.5M/10min and 0.5M/20min protocol demonstrated superior physicochemical characteristic and cell biocompatibility compared with human tooth matrix.

Keywords: biocompatibility, BMP-2 protein, cell adhesion, cell proliferation, cell differentiation, degradation, demineralized, tooth matrix, physicochemical properties,

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Anupong Jeerachaipansakul

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LIST OF ABBREVIATIONS AND SYMBOLS

ACP	Amorphous calcium phosphate
ALP	Alkaline phosphatase
APDDM	Autogenous partial demineralized dentin matrix
BET	Brunauer-Emmett-Teller analysis
BMP-2	Bone morphogenetic protein-2
BSP	Bone sialoprotein
DCPD	Dicalcium phosphate dihydrate
DDM	Demineralized dentin matrix
DTM	Demineralized tooth matrix
HA	Hydroxyapatite
HCl	Hydrochloric
IGF	Insulin growth factor
NaOH	Sodium hydroxide
OCN	Osteocalcin
OCP	Octacalcium phosphate
OPN	Osteopontin
PBS	Phosphate buffered saline
rhBMP-2	Recombinant human bone morphogenetic protein-2
SBF	Simulated body fluid
SEM	Scanning electron microscope
TCP	Tricalcium phosphate
TGF-β	Transforming growth factor beta
TM	Tooth matrix
Tris	Tris (hydroxymethyl) methylamine
XRD	X-Ray Diffractometer
XRF	X-Ray Fluorescence Spectrometer

CHAPTER 1

INTRODUCTION

The autogenous bone graft is mainly used for filling in bone defect because there is no immunological reaction and provide osteoinduction, osteoconduction and osteogenesis properties but the disadvantages are donor site mobility and limited quantities.

Allograft, xenograft and allograft have been proposed as alternatives, but they have some disadvantages such as risk of transmission diseases, unpredictable degradation rate and high cost.

A variety of properties bone graft material may be required bioactivity, biocompatibility, proper porosity, crystallinity, mechanical strength and resorption. The bone graft must be biocompatible and nontoxic. It is important for the first thing of medical device investigation such as cell migration, cell adhesion, cell proliferation, cell differentiation and mineralization. Bone graft resorption and degradation properties are important in order to allow new bone to occur simultaneously with bone graft degradation[1].

The using tooth as bone graft material for bone regeneration is increase because the composition of dentin is similar to alveolar bone. The organic part composes of collagen type I, noncollagenous proteins play role in new matrix calcification. The osteoinductive property of the demineralized tooth was discovered since 1967[2], and it has been demonstrated that demineralized tooth is osteoinduction or osteoconduction properties[3].

For preparing human demineralized tooth matrix (DTM), the procedure includes long preparation time and get through multiple chemical demineralized preparation processes that affect the physicochemical as well as the degradability of the materials and biocompatibility property.

The objective of this study was to evaluate the optimum time and concentration of demineralization process for demineralizing human tooth matrix prepared for bone grafting material in the aspect of physicochemical information, degradation and *in vitro* biocompatibility of osteoblastic cells.

Review literature

The compositions of bone

Bone is a hard tissue that exhibits the mineralization matrix and cells. Bone is composed of mineral and organic part[4]. The organic matrix of bone contains mainly collagen type I and noncollagenous proteins including bone sialoprotein, bone morphogenetic proteins (BMPs), fibronectin, osteocalcin, osteonectin, osteopontin, and growth factors which play role in cell proliferation, cell differentiation, vascularization and healing process. The inorganic material of bone consists mainly of calcium and phosphorus. Calcium and phosphate components are major inorganic of bone and their compositions of human bone are 29.82±2.08% and 12.90±1.21% respectively[5]. Calcium and phosphate atomic ratio (Ca/P) between 1.5 and 1.67 are called apatites such as hydroxyapatite [6].

Bone graft material for dental implant

A bone graft is a material that promotes bone healing, whether alone or in combination with other materials[7]. Properties requirements for an ideal bone graft material as listed below[8]:

- Biocompatibility
- Osteoinduction, osteoconduction and osteoconduction
- Material porosity: macropore, micropore and nanopore
- Resorbability/degradability
- Plasticity of material
- Material Stability

The mechanism of bone graft healing at recipient site occurs following bone graft characteristics: osteoconduction, osteoinduction, and osteogenesis[9]. Osteoconduction is adherence of mesenchymal stem cells and vessels in scaffold structures and produces new bone formation at bone graft surfaces. Osteoinduction is the bone graft material supplies growth factors for osteoprogenitor cells differentiation. Osteogenesis is osteoprogenitor cells that are present in the graft, proliferate and differentiate to osteoblasts and produce new bone formation.

Type and advantages or limitations/disadvantages of bone graft materials

Other classifications of bone grafts are based on their source[10]. Autogenous bone, the gold standard of bone graft material is harvested from the same subject. There is no immunologic response to this bone graft. Autogenous bone provides all three properties; osteogenesis, osteoinduction, and osteoconduction properties. The disadvantages of this graft are the additional surgery time and the need surgery of donor site.

Allograft graft material is taken from donor with same species. Cadavers are the most common donor sources. This graft is processed to reduce immunologic response by cellular elimination but there is a chance for transmission disease or infection from the graft. This graft functions provide main osteoconduction and some osteoinduction properties.

Xenograft is the bone graft which is collected from different species. These main disadvantages of this graft are the immunological response potential and chance for transmission disease. Common sources for xenografts are bovine and porcine animals. These graft functions mainly provide osteoconduction property only.

Synthetic graft function is mainly osteoconduction. Some grafts have osteoinductive property. This property may come from free calcium phosphate ion releasing or adding synthetic proteins which enhance osteoinduction property[7].

Incorporation of the bone graft

Graft incorporation or graft remodeling has been summarized into four steps[11, 12]. First, hematoma formation in the graft site, the releasing of osteoinduction factors and cellular recruitment from platelets or neighboring cells. After inflammatory phase, the development of fibrovascular tissues connect the graft to the adjacent bone. Neovascular formation occurs inside the graft. Creeping substitution is focal resorption of the graft which is performed by recruited osteoclasts and created new bone by osteoblast.

Factors affect bone graft healing

Pore size/ interconnecting pore

A proper porosity of bone graft is between 50–80%[13]. Pore size of material can be divided into two groups: the micropore (<5 μm pore diameter) and the macropore (>100 μm pore diameter). The Macropore can enhances role in the osteoconductivity[14]. The interconnecting pore, another important factor that effects to osteoconduction and vascularization. Pore size with 75-100 μm resulted in attachment of osteoprogenitor cells and pore size with 10–75 μm is adhered by fibrous tissue[14].

Particle size of bone graft

Particle size is one of the factors that affect bone graft properties. Syftestad and Urist[15] studied bone grafts in rats and showed new bone formation was induced by demineralized bone graft particle of 125-250 μm and 500-1,000 μm, while new bone formation was not shown in demineralized bone graft particles of 44-75 μm.

Zhang et al[16] showed that particulate demineralized bone graft materials ranging from 500-710 μm showed high level of mineral deposition after the grafts were implanted in muscle of athymic mice, while particles less than 250 μm demonstrated a low level of mineral deposition.

Dozza B et al[17] found particle size of bone graft affected physicochemical of collagen structure. The particle size with 500-1,000 μm was the best condition for cytocompatibility in vitro and for osteoinduction in vivo but there was no collagen alteration found in particles range 1-2 mm and collagen degeneration found in small particles (< 500 μm).

Sampath and Reddi[18] found demineralized bone graft of rat induced new bone formation only when large particles were used (particle size 74-420 µm), while osteoinduction was not observed using a fine matrix (size 44-74 µm). They explained that the fine particles may prematurely degraded and were eliminated by a phagocytic response.

The degradation rate of bone graft

The *in vivo* biodegradation of bone graft can be performed by liquid dissolution or cell-mediated. The cells responsible for graft resorption consists of osteoclasts and multinucleated cells[14]. Ideally, bone graft resorption rate is similar to new bone formation rate but not any faster. Bartee classified the bone graft degradation into 3 groups; long term bone graft (more than 30 months), transitional bone graft (6 months-1 years) and short-term bone graft (3-6 months)[19].

Mechanical strength of bone graft

The strength and stiffness of bone material relate to mineralization, porosity, structural factors and the characteristic of collagen fibers which depends on location of a bone type and site. The demineralized bone graft material was extremely susceptible to deformation[14]. Hamed et al[20], reported the compressive strength of the demineralized bovine cortical bone matrix block (5x5x7.5 mm) was about 10-15 MPa and the deproteinized bovine cortical bone was 14-40 MPa. The mechanical strength of demineralized bone matrix is 40-60% of mineralized bones[21]. The compressive strength of porous hydroxyapatite with 50-80% porosity was found 2-10 MPa. The strength and porosity relationship that is typical of ceramic bone graft substitutions[14].

Demineralization process of bone graft

Demineralization is the process of removing minerals ions from mineral material that occure both in nature and in laboratories[22]. Senn[23] was first described the demineralized ox bone using hydrochloric acid and used these xenograft bone for repairing skeletal defects.

The acids were utilized for the demineralization such as hydrochloric acid (HCl), acetic acid, formic acid, nitric acid, and ethylenediamine tetra-acetic (EDTA). HCl is the most common use for demineralization process because of the most rapid rates of demineralization with the highest dissociation constant and solubility of calcium salts[24] and the lower risk for nearby tissue inflammation[25].

Zhang et al[26] used 0.6M of hydrochloric acid with 10-fold volume to the mass of bone for 8 hours at 4°C for producing the demineralized bone graft matrix. They found the osteoinduction property of bone graft when culturing with MC3T3-E1 osteoblasts cells in vitro.

Urist et al[27], used demineralized rat dentin matrix in pig models. The demineralization was performed by using 0.5mol/L HCl acid for 3 hours at room temp. They found de novo bone formation around the bone graft in experimental animals and this new bone came from the effect of BMPs protein.

BMPs protein

BMPs are growth factors which role in bone healing process. The osteogenic property of BMPs in the demineralized bone graft was discovered by Urist in 1965[25]. BMP-2 is a member of transforming growth factor beta (TGF-β) superfamily. BMP-2 has been demonstrated to induce mesenchymal osteoprogenitor cell differentiate in a variety of cell types[28]. Natural BMPs comprise 0.1% of total bone weight[25]. The concentration with 10 ng/ml of BMP-2 stimulates the development of adipocyte, while the concentration with 100-1000 ng/ml stimulate chondroblast and osteoblast cells. The rhBMP-2 concentrations with 100-1000 ng/ml stimulate cell differentiation. The BMP-2 can be found in bone marrow, muscles and brain[29]. Previous studies reported the average concentration of BMP-2 protein which was extracted from demineralized bone matrix range from 3.6±1.20 ng/g of DBM[30] to 26.7 ± 11.4ng/g of DBM[31].

Tooth as the bone substitute materials

The Tooth and the alveolar bone both develop from the mesenchymal origin[32]. Tooth is a hard- connective tissue organ which composes of inorganic components and organic components. The compositions of tooth and bone are similar. The enamel of tooth consists of 96% inorganic part, whereas dentin has 65% and 35% is organic part. Cementum consists of inorganic part 45-50% and organic part 50-55%. While alveolar bone has inorganic part 65% and organic part[33].

From previous study, elastic modulus measurement of human enamel molar teeth was 87.56±4.5 GPa and the hardness was 3.96±0.3 GPa. The elastic modulus measurement of human dentin molar teeth was 40–42 GPa and hardness were 0.526±0.24-1.86±0.4 GPa vary age and site of dentin[34].

The inorganic component of tooth

The assessment of the element compositions of tooth from X-ray Diffraction (XRD) that found 5 phases of inorganic components; amorphous calcium phosphate (ACP), dicalcium phosphate dihydrate (DCPD), hydroxyapatite (HA), octacalcium phosphate (OCP) and tricalcium phosphate (TCP) and. Enamel consists of hydroxyapatite, which is a high crystallinity and hardness. The crystallinity of dentin and bone are similar pattern. When using the energy dispersive X-ray spectroscopy (EDS) for calculating calcium and phosphate ratio. The Ca/P ratio of crown portion is 1.75, root portion is 1.32 and the total tooth is the range of 1.24-1.46. The ratio relates to degradable properties, more Ca/P ratio less degradation rate[35].

The organic component of tooth

Both human tooth and bone are composed mainly collagen type I (95%), non-collagenous proteins and growth factors. non-collagenous proteins which include bone sialoprotein, dentin sialophosphoprotein, dentin matrix protein 1, and osteopontin are released into the extracellular matrix in the mineralization process. Growth factors such as BMPs, transforming growth factor-beta1, insulin growth factor-I and insulin

growth factor-II (TGF-β1, IGF-I and IGF-II respectively) are found in tooth and bone and they are the chemical signaling in new bone formation[36, 37].

Demineralization process of the tooth bone graft

The demineralization process of the tooth bone graft is performed by soaking the tooth particles or blocks in a variety of strong and/or weak acids[25, 36]. The most frequently chemical using is also hydrochloric acid solution[38].

In the demineralization process, the crystallinity of tooth will decrease but the bioavailability of collagen, non-collagenous proteins and growth factors will increase[39]. From previous, they found new bone formation in rabbit muscle at 4 weeks in the demineralized dentin bone graft group, while mineralized dentin formed new bone in muscle at 8 weeks after bone graft implantation. They confirmed the osteoinductive property of demineralized dentin matrix[40].

Park et al[41], produced deciduous demineralized tooth particles by crushing tooth into powders of 800-1,000 µm. The demineralization was performed by using 0.6 mol/L HCl for 10, 15, 20, 25, 30, 60, and 90 minutes then washed by phosphate buffered saline (PBS), peracetic acid and ethanol solution. They found the particles were demineralized for 10 minutes revealed dentinal tubules with a compact mineralized surface and dense collagen matrix. While demineralization reached to 30 minutes, the tooth surface developed a crater and porous surface because of excessive loss of mineralized components. The grafts revealed in total collapse of collagen and the dentinal tubules could not be found when the time exceeding 30 minutes.

Park et al[42], reported the amount of calcium decreased when demineralization increased and this decline occurred mostly between 10 and 30 minutes.

To our knowledge, there is no previous study using different HCl acid concentrations and demineralization for preparation of the demineralized bone or tooth matrix has been reported but the proper timing may be between 10-30 minutes for tooth particle.

***in vitro, in vivo* and clinical studies using tooth as grafting materials**

There are many studies related to the human demineralized tooth and bone regeneration. Bono et al[43] studied the demineralized dentin and enamel matrices by using a demineralized agent with material: solution ratio 1:20 (g:mL). They found this process could preserve the collagen content, increasing BMP-2 releasing and exhibited biocompatibility.

Li et al[44] compared of the biocompatibility and bioactivity between EDTA demineralized dentin matrix with HA/TCP scaffold. They reported human demineralized dentin scaffolds showed better biocompatibility and has bioactivity effect when comparison to control group.

Liu et al[45] showed that the 18-140 μm particle size of the demineralized dentin matrix bone graft (DDM) induced odontoblast differentiation of dental pulp stem cells.

Kang et al[46] compared of biocompatibility and bioactivity between demineralized dentin matrix with HA/TCP scaffold. The osteoinduction potential of demineralized dentin bone graft on the human dental pulp stem cells was higher than that of HA-TCP.

Kim[47] subcutaneously implanted human demineralized dentin matrix, which prepared from Korean tooth bank into the dorsal portion of nude mice. The result showed that the demineralized tooth bone graft induced new cartilage and bone in soft tissue at 2, 4, and 8 weeks.

Lee et al[48], spent more than 120 minutes for demineralization and preparation process of demineralized tooth particles in a clinical study. They found bone formation with no immunologic reaction.

Park et al[41], used deciduous demineralized tooth particles by using 0.6 mol/L HCl for 15 minutes in cranial bone defects of Sprague-Dawley rat models. They found the exhibited new bone formation in the grafted sites.

Asfour et al[49] evaluated the healing of the human demineralized dentin onlay bone grafts in the rabbit tibia. The human demineralized dentin was fabricated as a cylindrical dentin block grafts in diameter 5 mm and 2-mm thickness. The dentin blocks were soaked in chlorhexidine for 60 minutes to reduce bacterial growth, rinsed with saline and demineralized by using 24% EDTA with pH7 for 12 hours. They found the demineralized dentin graft and bone block graft were fused to host bone, resorbed and replaced by new bone.

Pang et al[50] compared demineralized dentin bone graft with deproteinized bovine bone for alveolar bone augmentation. They found using autogenous demineralized dentin bone graft for the bone augmentation was as effective as using anorganic bovine bone.

Minamizato et al[51] reported the use of autogenous partially demineralized dentin matrix (APDDM) prepared onsite in one visit. The APDDM was used for clinical application in simultaneous implant placement with guided bone regeneration, alveolar ridge preservation, alveolar bone augmentation, and maxillary sinus augmentation.

Table 1 *in vitro*, *in vivo* and clinical studies of using demineralized tooth matrix as the bone graft material

Study	Type of study	Demineralization	Result
Li R. et al 2011	*in vitro*	• EDTA Human treated dentin matrix • Human DFCs	demineralized human dentin scaffolds showed better biocompatibility
Liu G. et al 2016	*in vitro*	• Human demineralized dentin matrix • Dental pulp stem cells (DPSCs) from pig	Demineralized dentin matrix induced odontoblast differentiation of DPSCs
Bono N. et al 2017	*in vitro*	• Demineralized enamel Particle • Demineralized dentin particle [material: solution ratio=1:20 (g: mL)] • Osteoblastic cell line	The demineralization process of dentin allowed increasing the BMP-2 releasing more than the demineralized enamel. Both groups exhibited biocompatibility
Kang et al 2017	*in vitro* *in vivo*	• Demineralized dentin matrix • Human dental pulp stem cells • Immunocompromised Nude Mice	Osteoinduction property of DDM was higher than HA-TCP group however, in vivo bone formation was similar
Kim K.W. 2014	*in vivo*	• Human demineralized dentin bone graft • Muscle of nude mice	The demineralized bone graft induced cartilage and new bone formation in soft tissues
Lee E.Y. et al 2014	clinical study	Human demineralized tooth particles • 0.6 mol/L HCl 90 mins • Peracetic + ethanol 10 mins	They found Bone formation with no immunologic reaction.

Table 1 (continued)

Study	Type of study	Demineralization	Result
Park M. et al 2015	*in vitro* *in vivo*	Human demineralized deciduous teeth • Size 800-1,000 µm • 0.6N HCl for 0, 10, 15, 20, 25, 30, 60, and 90 minutes *in vivo* • 0.6N HCl for 15 minutes • Sprague-Dawley rats	*in vitro*: increasing demineralization time, the tooth surface developed a crater and porous surface *in vivo*: exhibited new bone formation in the grafted sites.
Asfour A.A. et al 2017	*in vivo*	Human demineralized dentin onlay grafts using rabbit tibia model • Cylindrical dentin block grafts in diameter 5 mm and 2-mm • Chlorhexidine for 60 minutes • Demineralized using 24% EDTA neutral, pH7, for 12 hours.	Demineralized dentin and bone block graft were fused to host bone, resorbed, and replaced by bone and connective tissue.
Pang K. M. et al 2017	Clinical study	• Human demineralized dentin bone graft, (Korean Tooth Bank) • Anorganic bovine bone	Demineralized dentin matrix was as effective as using anorganic bovine bone.
Minamizato T. et al 2017	Clinical study	Autogenous partially demineralized dentin matrix prepared onsite in one visit • Size 400-800 µm • 2% HNO3 with pH 1.0 for 10 min	APDDM showed the efficacy and safety for • Implant placement with GBR • Socket preservation • Alveolar ridge augmentation • Maxillary sinus augmentation

Poly Vinyl Alcohol (PVA) hydrogel characteristic

Hydrogel is a hydrophilic polymer. It demonstrates good tissue compatibility. Hydrogels are composed of synthetic and natural materials. Synthetic hydrogels such as poly(hydroxyethyl methacrylate), poly(vinyl alcohol), poly(ethylene glycol dimethacrylate), poly(ethylene oxide), poly(propylene furmarate-co-ethylene glycol), polypeptides and poly(acrylic acid).Whereas natural hydrogels include agarose, alginate, chitosan, collagen, gelatin, fibrin, hyaluronic acid[52].

Poly vinyl alcohol (PVA) is water-soluble polymer. It has been used as a binding supporting material for tissue engineering applications. It shows mechanical strength and non-toxicity, biocompatibility, swelling property in aqueous with high similarity to those of human tissues and anabolic effect on bone formation[53].

Wei et al 2012[53] studied material biodegradability characteristic of PVA/collagen/hydroxyapatite (PVA/Col/HA) hydrogel scaffold by seeding MC3T cells in the scaffold. They found that this scaffold demonstrated increased cell adhesion, proliferation, and differentiation.

Biocompatibility tests for dental materials

The biomaterials which contact the cells and tissues require good physicochemical properties and must have good biocompatibility. An international standard for medical devices (ISO 10993) is used for biological assessment in the aspect of [54-56].

- Physicochemical and material compositions,
- Surface topography; macro, micro and nano
- Crystallinity
- Porosity
- Surface energy, surface electrical property
- Cytotoxicity, genotoxicity, carcinogenicity
- Hemocompatibility
- Sensitization
- Material-mediated pyrogenicity
- Acute, subacute, subchronic and chronic toxicity
- Implantation
- Reproductive/developmental toxicity
- Biodegradation

The cytotoxicity test is an important and preferred as a screen test for the evaluation of medical devices. This test uses cells to observes cell adhesion, cell growth, proliferation and differentiation by the medical materials[55].

A sequence of safety studies of new medical materials is *in vitro* studies, followed by investigations in animals, and clinical studies[57]. Li et al[44] studied the *in vitro* cytotoxicity of human demineralized tooth bone graft. They used the relative growth rate (RGR) for cytotoxicity analyzing which calculated following the ISO 10993 as %RGR = (absorbance of test group/absorbance of blank group) x100%

***In vitro* biodegradation test**

There are many buffer solutions for degradation testing. Tris-HCl solution is used for ceramics material degradation testing follow ISO 10993-14 requirement. SBF and PBS can occur crystal precipitation on surface of material[58, 59].

Tris (hydroxymethyl) methylamine (Tris) has been increasingly used as a buffering agent in biological experiments. Tris can soluble in water, inert in many enzymatic systems and no reaction with other components especially bicarbonate[60].

Wu S.C. et al[61] studied the degradability of calcium-phosphate porous microsphere materials were soaked in distilled water (pH7.4) at 37 ℃. for 3, 7, 14, and 28 days.

Kirste G. et al[62] studied Tris buffer solution affected ion release and apatite precipitation of bioactive glass, by soaking bioactive glass powder form in Tris-HCl (pH7.4) solution for 7 days.

Guarino V. et al[63] studied the degradability of a PCL scaffold with HA particles in PBS pH 7.5, NaOH and simulating body fluid solution pH 7.5 for 56 days. They reported the scaffold in NaOH solution had weight loss more than PBS pH 7.5. Moreover, they found the scaffold in simulating body fluid solution had bioactivity property by performing precipitation of appetite on material surface, which corresponds to a weight gain.

Juraski A.D. et al[64] studied in vitro degradability property of glass ceramic scaffold. They immersed Glass-ceramic material in the Tris-HCl solution (pH 7.4, 37 °C) for 21 days.

Zhong Q. et al[65] studied the degradation of porous calcium carbonate and hydroxyapatite microspheres materials. They soaked the material in Tris-HCl solution (pH 7.4, 37 °C) for 12 weeks.

From the previous studies, the tooth can be a bone graft material in alveolar bone repair and regeneration. Moreover, the difference in demineralization processes leads to question about the effects of demineralized tooth matrix (DTM) preparation on physicochemical properties, degradation kinetic, BMP-2 quantification, and biocompatibility of human demineralized tooth matrix.

Research question

How does the demineralized agent of demineralization process affect physicochemical information, BMP-2 protein quantification and osteoblastic cell biocompatibility of human demineralized tooth matrix (DTM)?

Research objectives

- To examine the influence of 5 different protocols of hydrochloric acid [1) 0M, 0min 2) 0.5M, 10min, 3) 0.5M, 20min, 4) 1M, 10min, and 5) 1M, 20min) on physicochemical properties of demineralized tooth matrix (DTM) in aspect of
    - Surface morphology and porosity
    - Crystallinity
    - Calcium and phosphate contents and ratio
    - Degradation kinetic
- To quantify the amount of the Bone morphogenetic protein-2 (BMP-2) protein in the tooth matrix after 5 different demineralization processes.
- To evaluate cell biocompatibility property on demineralized tooth matrix scaffold using osteoblastic cells in the aspect of
    - Cell adhesion
    - Cell proliferation
    - Cell differentiation

Hypothesis

A reaction time and different concentrations of hydrochloric acid in demineralization process affect physicochemical properties, BMP-2 quantification and osteoblastic cell biocompatibility of human demineralized tooth matrix (DTM).

CHAPTER 2

MATERIALS AND METHODS

Samples

This experimental research was performed at the oral surgery clinic, Faculty of Dentistry, Prince of Songkla University. Caries-free third molar or premolar permanent teeth from healthy patients undergoing surgical removal or extracted for orthodontic treatment were included in this study but the teeth were excluded if they presented any pathology/anomaly of tooth structure.

Methods

The teeth were divided into crown and root portion by high-speed rotating diamond burs, whereas pulp tissue was removed by handed endodontic files (Fig.1A, B) and washed in 0.9%NSS. The teeth were crushed into small particles by mixer ball mill machine (Mixer Mill M301, Retsch GmbH, Haan, Germany). Sievers with filter aperture 500 and 1,000 µm (Endecotts, Endecotts Ltd, London, UK) were used for selecting particle size (Fig.1C-I).

Figure 1 Demonstration of the human teeth milling process; tooth section (A-C), tooth milling (C-G) and particle size selection (H, I)

The tooth particles were pooled and devided into 5 groups (Table 1). The demineralization process was performed by using hydrochloric acid (HCl) at 25°C. and the ratio of material: demineralized solution was 1:10 (g/ml)

After demineralization process, the tooth particles were washed using distilled water with 3 folds of demineralized solution and second washing with PBS was performed respectively (3 folds of demineralized solution) for 10 minutes (Fig.2).

Table 2 The groups of tooth particles for demineralization process

Group	HCl concentration (mol/L)	Demineralization time (minute)
TM(control)	0	0
0.5M/10DTM	0.5	10
1M/10DTM	1.0	10
0.5M/20DTM	0.5	20
1M/20DTM	1.0	20

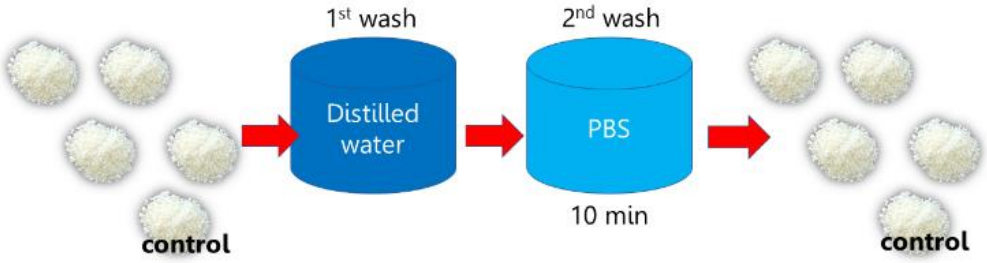


Figure 2 Demonstration of all groups after demineralization process

This experimental study was divided into 2 parts. The physicochemical properties evaluation: to evaluated and compared the effect of hydrochloric acid from demineralization on physicochemical characteristics, BMP-2 quantifications, and *in vitro* degradation rate of the materials. The most appropriate demineralization protocol which defined from physicochemical properties and degradation rate of samples was chosen for further study. Then, cell proliferation and cell differentiation assay were evaluated cell biocompatibility on selected demineralized tooth matrix scaffolds.

Part 1: Physicochemical characteristic

The surface characteristics of particles

The surface characteristics of human tooth particle in TM group and 4 experimental groups were evaluated by the scanning electron microscope (SEM; Quanta 400, FEI, Oregon, USA) with 500x, 7,000x and 20,000x magnifications (n= 1/group).

BET analysis

Brunauer-Emmett-Teller (BET) analysis is used to evaluate surface characteristics and average pore diameter. The BET Analysis (ASAP2460, Micromeritics, USA), nitrogen gas was used for surface area and average pore diameter calculation by gas adsorption in the materials (n= 1/group).

Analysis of crystallinity

To examine the crystallinity percentage of elemental component and phase of calcium phosphate compound, the samples (n= 1/per group) were assessed by X-Ray Diffractometer (XRD) (X'Pert MPD, PHILIPS, Netherlands).

Analysis of inorganic components

To determine the amount of elemental compositions especially calcium and phosphorous contents, all sample groups (n= 1/group) were assessed by X-Ray Fluorescence Spectrometer (XRF) (PW 2400, PHILIPS, Netherlands).

The Ca/P ratio

The percentages of calcium and phosphorus were calculated for Ca/P ratio. The stoichiometric Ca/P ratio was calculated by using the following formula: Ca (mol)/P (mol) = [Ca (wt%)/40.08 (g/mol)]/ [P (wt%)/ 30.97 (g/mol)]

Quantification of bone morphogenetic protein (BMP-2)

The extraction of BMP-2

The Guanidine HCl/EDTA method protein extraction was performed[66]. A 100 mg of all groups was put in 5 ml Eppendorf tubes and 1.9 ml buffer solution (4M Guanidine HCl in 1,668.2 µL of 20mM Tris-HCl solution, 190 µL of 5mM Benzamidine, 38 µL of 1mM phenylmethylsulfonyl and 3.8 µL of 0.1mM aminocaproic acid) was added to the tube. The samples were shaken at 4^{o}C for 24 hrs. The solution supernatant, collected each day, was conserved at 4^{o}C for total protein assay and ELISA test (n= 3 per group).

Total protein assay

The total protein assay [Pierce Coomassie Plus (Bradford) Assay Kit; ThermoFisher Scientific, MA, USA] was measured on the sample solutions to quantify total proteins according to manufacturer protocol.

Quantification of growth factors using ELISA

The quantification of BMP-2 of all groups were measured with human BMP-2 Quantikine ELISA Kit (R&D Systems, Minnesota, USA). Briefly, a diluent reagent was added to standard and sample solutions. The solution was incubated for 2 hours at room temperature. Then the solution was removed and the wells were washed by buffer. After the last wash, BMP-2 conjugate solution was added to each well and incubated for 2 hours at room temperature on the shaker. Then, a substrate solution was added to each well and incubated for 30 minutes at room temperature with light protection. Finally, a stop solution was added to each well. The optical density by using a microplate reader set to 540 nm or 570 nm wavelength.

Preparation of the TM/PVA and DTM/PVA scaffolds

PVA (MW 145,000 Da; hydrolysis; Merck, Germany) was used to fabricate scaffold using hydrogel solution as binding material.

PVA solutions (compositions: 5% wt/v) were modified from the method of Gupta et al[67]. Briefly, PVA powders were dissolved in distilled water, heated at 80°C and constantly stirred respectively. The PVA solutions were kept at room temperature for the cooling and air bubbles were removed.

To fabricate the TM/PVA and DTM/PVA scaffolds, a 200 mg of TM of DTM particles were added into 100 µl of 5% wt/v of PVA solutions (mg/µl) and poured into a container (diameter of 11 mm and height of 3 mm). Then the scaffolds were subjected to repeated freeze-thawing cycles at temperatures of -20±2 °C (freezing) and 25±2 °C (thawing) for 3 days. The scaffolds were stored at −20 °C and sterilized by hydrogen peroxide (H_2O_2) gas before use.

Degradation of TM and DTM scaffold

Tooth Matrix (TM)/PVA and all groups of DTM/PVA were immersed in 1 mL of 50mM Tris-HCl solution (pH 7.4) at 37°C for 60 days. The weight of all scaffolds was examined at day 0, 1, 3, 5, 7, 14, 21, 30, 45, and 60 (n= 3 per group). The percentage of degradation was calculated by the following equation:

Degradation rate (%) $=100 \times (W0 - Wt)/W0$, where W0 was the initial weight of materials and W*t* was the weight after time *t* treatment[65].

Part 2: The *in vitro* biocompatibility of TM and DTM scaffold

Preparation of cell-scaffold construct

MC3T3-E1 immortalized osteoblast lining cells were seeded onto the surface of TM/PVA and DTM/PVA scaffolds at a cell density of 5×10^4/ml in 24-well plates (Ø=10 mm). The cell-scaffolds constructs were cultured in basal medium or osteogenic medium according to the experiment.

Surface characteristic and cell morphology on scaffolds

The morphology of the empty scaffolds were detected with a scanning electron microscope by using Field Emission Scanning Electron Microscope (Apreo, ThermoFisher Scientific, Waltham, MA, USA) (n=1per group).

The scaffolds seeded with MC3T3-E1 cells were incubated in 1 ml basal medium consisted of α-modified Eagle medium (α-MEM) supplemented with 10% fetal bovine serum (Gibco, ThermoFisher Scientific, MA, USA), 0.1% of fungizone and 1% of penicillin-streptomycin solution (Gibco, ThermoFisher Scientific, MA, USA) The constructs were cultured at 37°C with 5% CO2 for 3 days and the culture medium was changed every two days.

The cell adherence was examined at 1, 7 and 14 days after culturing on the scaffolds by scanning electron microscopy. For SEM evaluation, the cells-scaffold

constructs were fixed using 10% formaldehyde with PBS for both the DTM and control scaffold materials for 2 hours, freeze dry all scaffolds were performed respectively (n=1per group).

Cell proliferation assay

A cell proliferation reagent (PrestoBlue® cell viability reagent, Invitrogen, ThermoFisher Scientific, MA, USA) was used to quantify MC3T3E1 cells viability and proliferation at day 1, 3, 7, 14 and day 21 (n=5 per group).

PrestoBlue® is a cell permeable resazurin-based solution that functions as a cell viability indicator by using the metabolic activity of living cells to measure the proliferation of cells. Resazurin is a nonfluorescent blue dye that is irreversibly reduced to a highly fluorescent pink to red dye, resorufin environment of metabolically active cells. The color change can be detected by using fluorescence or absorbance measurements with good sensitivity. Resazurin-based assays can be used without any cellular lysis or washing steps. Stained cells can be further used in a multiplexed assay format. The reagent can be removed from the cells and replaced with growth medium for further proliferation[68].

Briefly, 100 μL of PrestoBlue Reagent was added to 900 μL of culture medium directly to cells and incubated at 37 °C for 90 minutes according to the manufacturer's protocols. The optical density of the solution was measured at 570 and 600 nm using microplate reader.

Cell differentiation assay

The cell-scaffold constructs were incubated in 1 ml of osteogenic medium; basal medium α-modified Eagle medium (α-MEM) with 50 μg/ml of ascorbic acid, 10mM of beta-glycerophosphate and 10 nM dexamethasone (Sigma, St Louis, MO, USA). All cell-scaffolds were cultured in incubator with 5% CO_2 and 37°C temperature for 21 days and the medium were changed every 2 days.

For cell differentiation assay, solution samples were collected at day 1, 7, 14, and day 21. PBS was used for washing the scaffolds. Scaffolds were added with 200 μL of 1%Triton X with PBS and crushed. The cells on scaffolds were lysed by freeze-thaw technique (−20°C 15 minutes and room temperature for 15 min) for 3 times. The solutions in wells were moved to microtubes and centrifuged at 2,000g for 10 minutes. The clear supernatant in microtube was collected. The quantification of protein in solution was processed following to the manufacturer's instruction (Bio-Rad protein assay, Bio-Rad, California, USA). The light absorbance at 450 nm was evaluated using the microplate reader machine.

The alkaline phosphatase (ALP) activitiy were calculated following to instruction using Alkaline Phosphatase, AMP Buffer commercial kit (Hu-man, Germany) [(U/L)/μg protein]. The quantification of osteocalcin (OCN) protein was calculated following to the instruction using Osteocalcin ELISA commercial kit (Biomedical Technologies Inc., USA). The solutions were read at 450 nm by the microplate reader, and their concentrations were calculated with the serial diluted standard solution (ng/ml).

Table 3 Measurement tools of DTM properties

Test	No. sample	Tools	Measurement
Physicochemical properties of the graft particles	n=1	SEM	surface characteristics
	n=1	BET analysis	Percentage of surface area and average pore diameter (Aº)
	n=1	X-Ray Diffractometer	Percentage of crystallinity
	n=1	X-Ray Fluorescence Spectrometer	Percentage of inorganic component Ca/P ratio
Surface characteristic of scaffolds	n=1	SEM	Surface characteristic
Morphology of cell attachment	n=1	SEM	Morphology of cell attachment
Degradation of scaffolds	n=3	1 mL of 50mM Tris-HCl (pH 7.4)	Degradation rate
The total protein concentration	n=3	Bradford Assay Kit	The concentration of total protein
Quantification of BMP-2	n=3	BMP-2 ELISA kit	The concentration of BMP-2
in vitro biocompatibility	n=5	PrestoBlue®	Cell proliferation
	n=5	ALP AMP Buffer osteocalcin ELISA	Cell differentiation

Statistical analysis

The data analysis was calculated by using software IBM SPSS statistics version 23. The data was present as mean±SD. Compare means analysis ($p<0.05$) was used for comparing total protein and BMP-2 quantification, degradation rate of material, cell proliferation and cell differentiation.

CHAPTER 3

RESULTS

The teeth were collected and pooled together. All 125 teeth weighting 220 grams were pulverized into particle, divided into 5 groups before demineralized with HCl acid according to the assigned protocols.

Particle characterization

The particles of TM and DTM were like a sand particle with hard in consistency. The color of TM particles was opaquer than all DTM groups. 1.0/20DTM group showed the most yellowish in color. The particle sizes in all groups were between 500-1,000 µm (Fig.3).

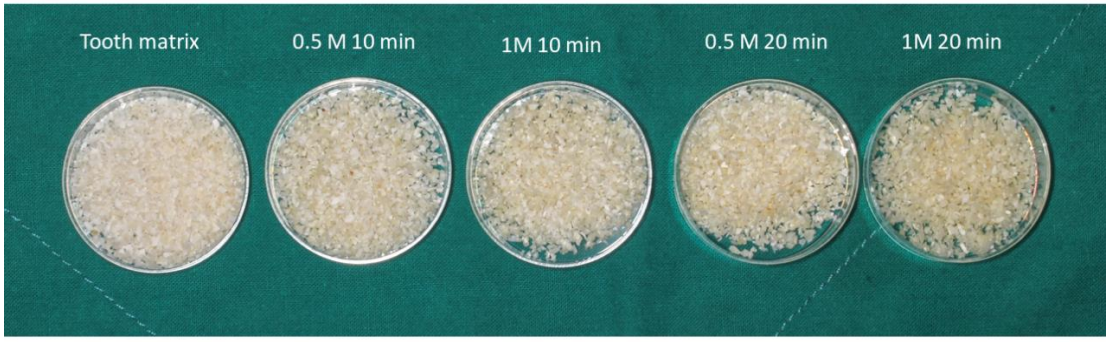


Figure 3 The characteristic of the particles of tooth matrix and demineralized tooth matrix from 5 different protocols**.**

SEM analysis

At x500 magnification, TM group showed inorganic irregular surface. When the demineralized time increased, 0.5/10DTM group showed a smooth surface with some dentinal tubule, while the other DTM groups showed a smooth surface with numerous dentinal tubules.

At x7,000 magnification, the lateral surface of TM and 0.5M/10DTM group showed inorganic irregular surface. Contrary to DTM that presented some dentinal tubule. While 1M/10DTM, 0.5/20DTM and 1M/20DTM showed collagen fiber exposure on the surface, the 1M/20DTM presented the smoothest surface.

At x20,000 magnification, the collagen fiber exposure was seen in all DTM groups but hardly to identify in TM group. The diameter of dentinal tubule in TM group was 1 µm with irregular inorganic surface. In DTM group, the dentinal tubule ranged from 0.5- 3 µm. As demineralization time increased, the size of dentinal tubule was increased. 1M/20DTM group showed the smoothest surface, collagen fiber exposed, and the diameter of dentinal tubule was 2-3 µm (Fig.4).

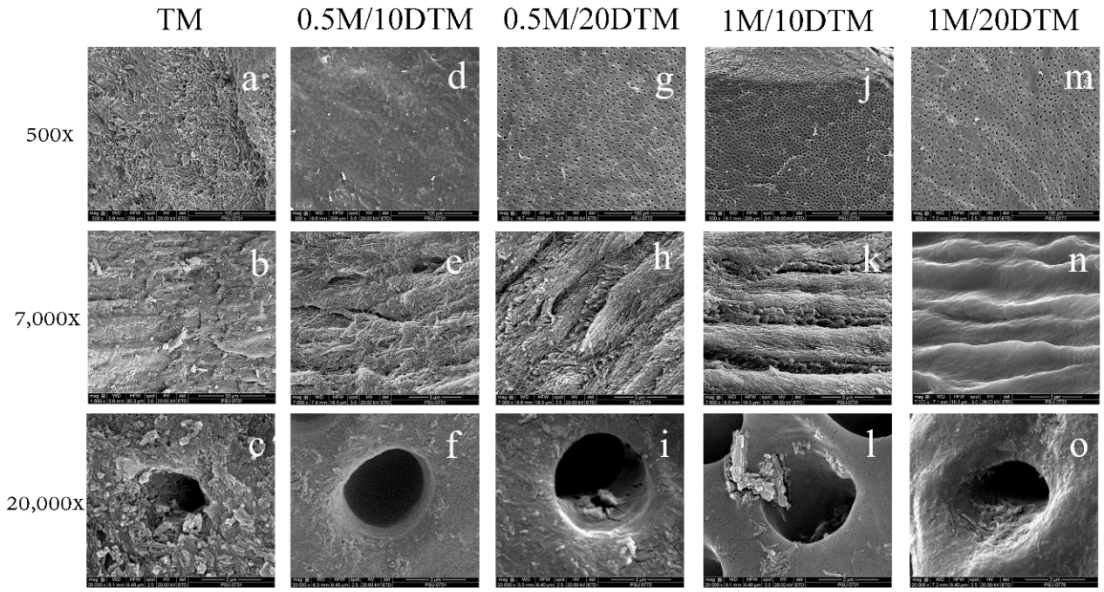


Figure 4 Showing SEM image of TM and DTM groups at 500x, 7,000x, 20,000x magnification. The DTM group demonstrated smooth surface with collagen fibrils and wider dentinal tubules

At x30,000 magnification, a crack line on the surface of 0.5M/20DTM and 1M/20DTM groups were observed (Fig.5).

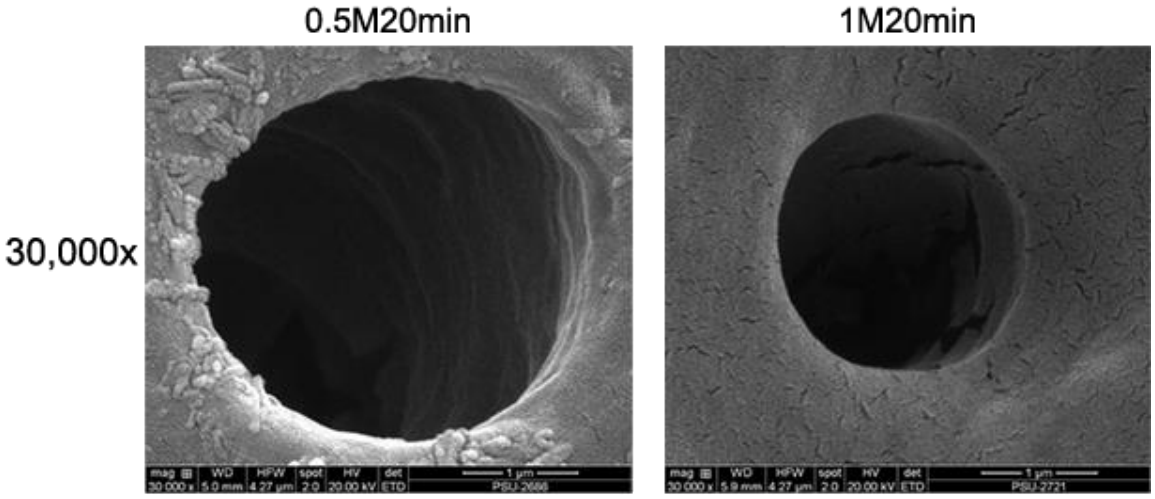


Figure 5 Showing exposed collagen fibrils and small crack lines on particle surface of 0.5M/20DTM and 1M/20DTM groups at 30,000x magnification

XRF analysis

The elemental components in TM and DTM groups were shown from XRF analysis. The amount of inorganic elemental components (calcium and phosphorus) tended to decrease while that of organic elements components (carbon, oxygen, and nitrogen) tended to increase. The DTM groups demonstrated lower

percentages of calcium and phosphorous compared to TM group. The lowest percentages of calcium and phosphorous was 1M/20DTM group.

The Ca/P ratio of TM group was 1.49 and the ratio was increase when concentration and demineralization time increasing. The highest Ca/P ratio was 1M/20DTM group (2.47). The metal elements were found in all groups with small percentage but Iron element (Fe) was found only in 1M/20DTM group (Table 4).

Table 4 The percentages of element components and Ca/P ratio in TM and DTM groups from XRF analysis

Groups	% element (% by weight)									Ca/P ratio
	Ca	P	S	Mg	Na	Cl	K	Zn	Fe	
TM	15.6	8.08	0.04	0.42	0.50	0.08	0.01	<0.01	0	1.492
0.5M/10DTM	11	4.32	0.15	0.13	0.16	0.15	<0.01	<0.01	0	1.968
1M/10DTM	8.25	2.71	0.19	0.01	0.15	0.17	<0.01	<0.01	0	2.352
0.5M/20DTM	8.17	2.64	0.21	0.07	0.12	0.01	<0.01	<0.01	0	2.391
1M/20DTM	3.43	1.07	0.25	0.05	0.08	0.2	<0.01	<0.01	<0.01	2.477

XRD analysis

According to numbers of peak and peak compatible patterns in XRD graph, Calcium and Phosphate minerals in all groups were found only in hydroxyapatite phase (Fig. 6). When concentration of HCl acid and demineralization time increased, the percentages of crystallinity decreased. The highest percentage of crystallinity was presented in TM group (63.09%) and the lowest was presented in 1M/20DTM group (54.33%) (Table 5).

Table 5 Showing percentages of crystalinity and phase of Calcium phosphate component from XRD analysis

Groups	% Crystallinity	Phase of Ca-P
TM	63.09	Hydroxyapatite
0.5M/10DTM	62.12	Hydroxyapatite
1M/10DTM	61.91	Hydroxyapatite
0.5M/20DTM	56.84	Hydroxyapatite
1M/20DTM	54.33	Hydroxyapatite

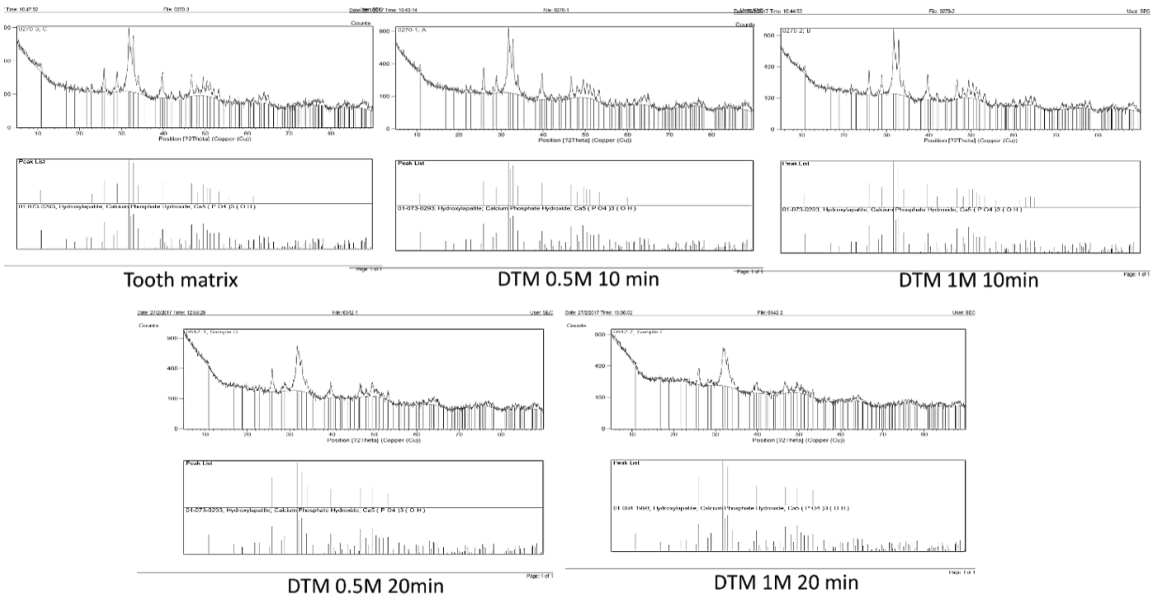


Figure 6 The numbers of peak patterns in XRD analysis graph showed calcium phosphate minerals in all groups were found only in hydroxyapatite phase

BET analysis

When the concentration of hydrochloric acid and demineralization time increased, the BET surface area decreased relatively with surface characteristic of particles but the average pore diameter was increase. The most average pore diameter was 1M/20DTM group (68.17A°) (Fig. 7).

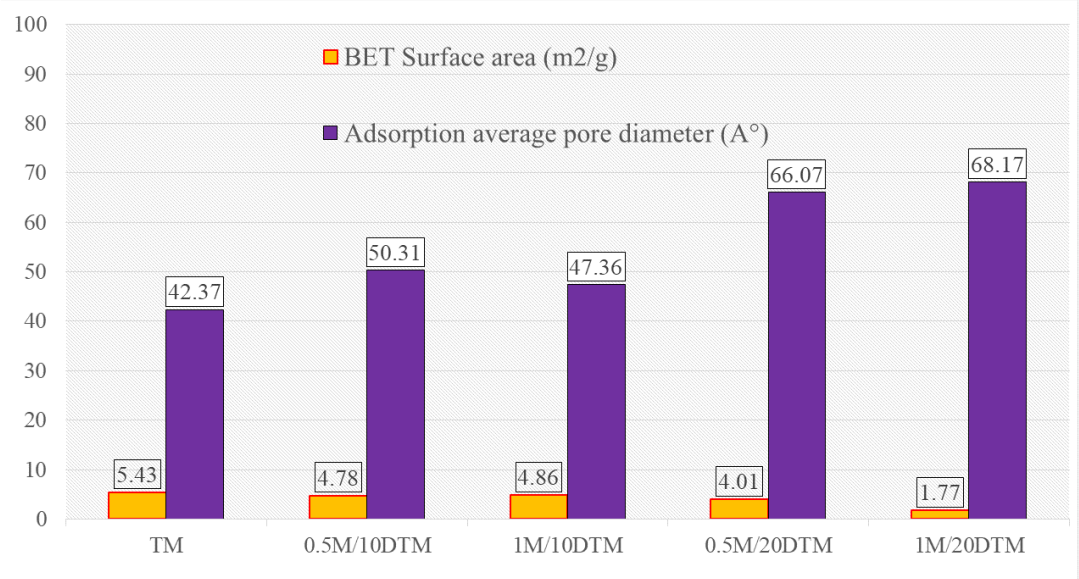


Figure 7 The bar chart of BET analysis demonstrated surface area and Adsorption average pore diameter of all groups

BMP-2 quantification

The cumulative total protein demonstrated the highest concentration in 1M/20DTM group (3,526.250±238.410 µg/ml) with a significant difference to other groups (p=0.000) (Table 6). The ELISA measurements of cumulative BMP-2 released from TM and DTM groups showed the highest concentration in 1M/20DTM group (1,181.529±193.766 pg/ml/100mgDTM) with a significant difference between the other groups (p=0.000) (Table 6). The BMP-2/total protein presented the highest ratio from 1M/20DTM group (0.336±0.060 pg/µg/100mgDTM) with a significant difference between TM group, 0.5M/10DTM group and 1M/10DTM group (p=0.000) (Table 6).

Table 6 The protein and BMP-2 concentration from TM and DTM groups. The asterisk (*) is significant different using post hoc tukey test (p<0.05)

Groups	Total protein (µg/ml)	BMP2 (pg/ml)	BMP2/total protein (pg/µg)
TM	975.00±80.85*	2.67±5.34*	0.002±0.005*
0.5/10DTM	1,166.88±307.66*	111.54±6.68*	0.101±0.029*
1M/10DTM	1,843.13±105.54*	309.72±237.10*	0.167±0.132*
0.5M/20DTM	1,876.88±105.88*	528.36±154.78*	0.278±0.070
1M/20DTM	3,526.25±238.41	1,181.53±193.77	0.336±0.060

Surface characteristic of DTM/PVA scaffolds

All scaffold-constructs in cylindical form, presented hard in consistency but brittle with interconnecting pore between particle. The color of TM group was opaquer than tooth color of DTM groups (Fig.8).

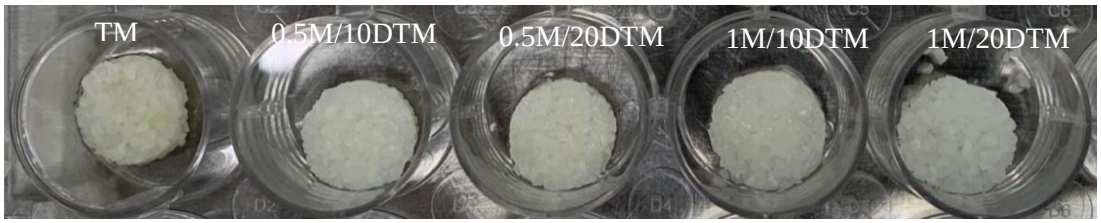


Figure 8 The characteristic of tooth matrix and Demineralized tooth matrix scaffolds

Degradation of TM and DTM scaffold

During material degradation test, the tooth particles in 1.0/10DTM and 1.0/20DTM groups dislodged from the scaffold since day 7 and the were progress with time (Fig.9).

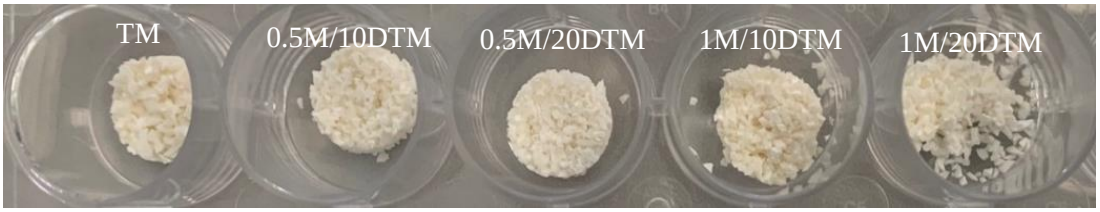


Figure 9 Showing all of TM and DTM scaffold groups after soaking in Tris-HCl solution at day 60. The Scaffolds of 1M/10DTM and 1M/20DTM groups showed some particle dislodgement

The TM group and all DTM groups degraded with time (fig.10). The degradation rate at day 60 of 1M/10DTM and 1M/20DTM groups were statistically significant difference higher than other groups (Table 7) (p=0.033, 0.006).

Table 7 The degradation rate of TM and DTM groups at day 60. The asterisk (*) is significant statistical different in using post hoc tukey test when compare to all groups (p<0.05)

Groups	N	Degradation rate day 60 (%)	Sig
TM	3	6.405±0.302	
0.5M/10DTM	3	9.194±1.916	0.966
0.5M/20DTM	3	8.462±0.279	0.991
1M/10DTM	3	19.273±2.743	0.033*
1M/20DTM	3	22.897±10.091	0.006*

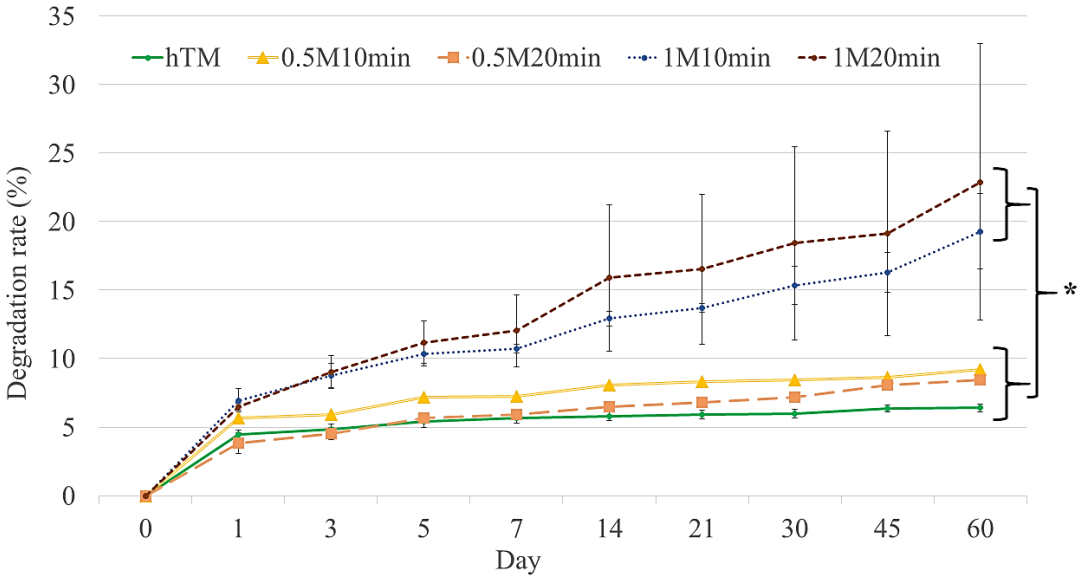


Figure 10 Line chart demonstrated degradation rate of TM and all DTM groups

From the physicochemical characteristic analysis, all demineralized groups showed wider dentinal tubule diameter, decreasing calcium and phosphate element, decreasing crystallinity and increasing BMP-2 releasing relate with demineralization time and concentration. 0.5M/10DTM, 0.5M/20DTM groups had slow degradation rate compared to human tooth. Therefore, two groups of 0.5M HCl DTM were chosen for in vitro biocompatibility test.

Cell biocompatibility Assay

Morphology of cell attachment assay

From SEM, TM, 0.5/10DTM and 0.5/20DTM scaffold groups demonstrated PVA hydrogel coating on the surface of materials at low power magnification. In high power magnification, TM groups demonstrated irregular surface of inorganic structure with small diameter of dentinal tubules. Contrariwise, 0.5M/10DTM and 0.5M/20DTM showed larger dentinal tubules and smooth surface (Fig.11).

Base on cell-scaffold construct, the morphology of cell attachment at day 1 on TM scaffold showed irregular shape with small size. The loose attachment of cells on scaffolds were observed. Contrast with 0.5M/10DTM and 0.5M/20DTM groups, the cells showed larger size and propagated on graft surface with a flat shape and extended pseudopodia periphery through the dentinal tubules.

At day 7, the cells were increased in number and size in all groups. The loose attachment of cells on TM scaffolds were still observed. The cells on 0.5/10DTM and 0.5M/20DTM groups demonstrated spreading over a material surface. At day 14, the cells proliferated to multilayers on the surface of the scaffolds in all groups. The size of cells were increase. It was difficult to see dentinal tubules or the material surface in this day. (Fig.12).

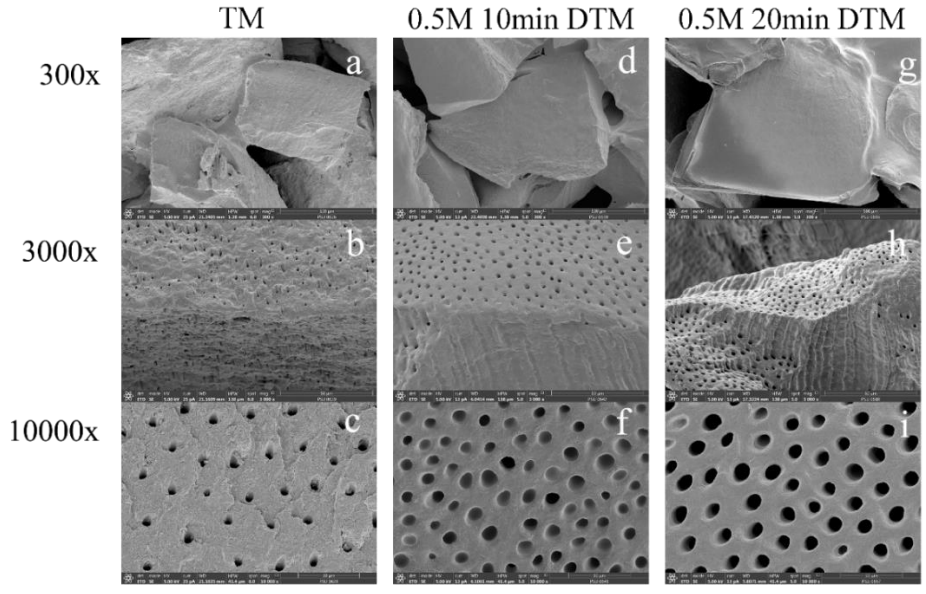


Figure 11 SEM image of empty scaffold with PVA of TM and DTM groups at 300x, 3,000x and 10,000x magnification

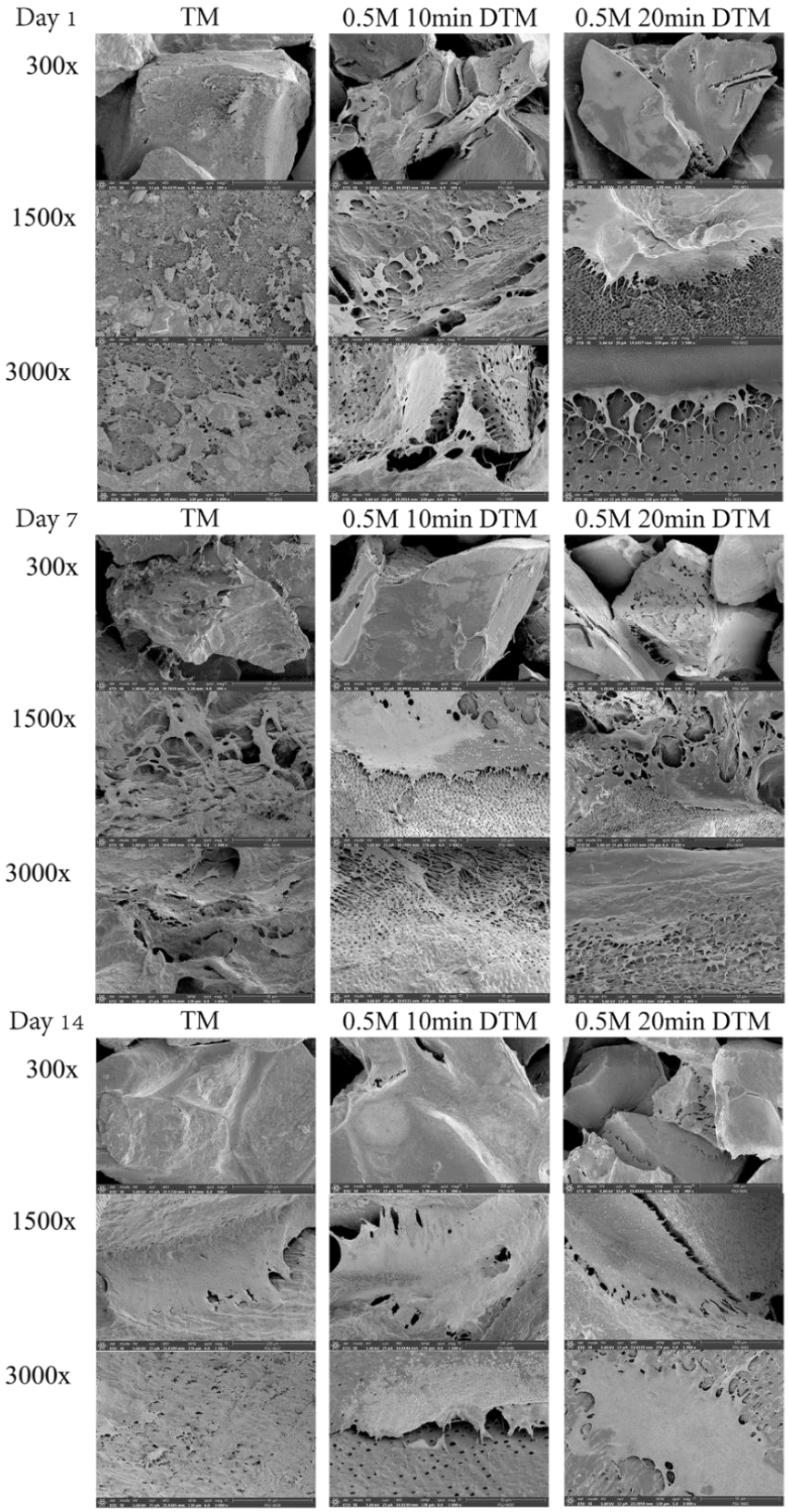


Figure 12 SEM image of cell morphology and cell attachment on TM and DTM scaffolds at day 1,7 and day 14 with 300x, 1,500x and 3,000x magnification

Cell proliferation assay

The result showed cell proliferation of all groups were increase from day 1 to day 14 but slightly decreased at day 21 (Table 8 and fig.13). In TM group, there was statistically significant difference of the cell number between day 1 and day 14 (p=0.020), and day21(p=0.013). In 0.5/10DTM and 0.5/20DTM group, the number were increased significantly on day 14 compared to day 1 (p=0.000, p=0.000), day 3 (p=0.004, p=0.003) and day7 (p=0.028, p=0.015).

When compared between TM and DTM groups, the cell number was higher in 0.5/10DTM group compared to TM group at day 1(p=0.039), and day3 (p=0.033)

Table 8 The cell proliferation of TM and experimental groups. The "a" is significant different when compare with 0.5M/10DTM group ($p<0.05$). The "b" is significant different when compare to day 1 ($p<0.05$). The "c" is significant different when compare to day 14.

Cell number (mean±SD)					
	Day1	**Day3**	**Day7**	**Day14**	**Day21**
TM	8,960± 1,089.85(a)	19,346.67± 3,526.42(a)	23,333.33± 5,878.99	33,300± 9,246.09(b)	36,950± 20,935.70(b)
0.5M/ 10DTM	13,200± 2,343.81(c)	27,026.67± 3,896.40(c)	30,173.33± 2,140.86(c)	41,290± 1,608.60	38,840± 6,822.23
0.5M/ 20DTM	10,280± 846.36(c)	25,666.67± 3,708.33(c)	28,813.33± 2,746.05(c)	44,250± 4,648.28	41,120± 9,426.70

Cell differentiation assay

The ALP activity was shown in Figure 14 and Table 19. The TM group presented the lowest level of ALP over the observation period. In 0.5/10DTM group, the ALP increased to the hightest level on day 21. In 0.5M/20DTM, the highest level of ALP was detected on day 1, the slightly decreassed over time.

Table 9 Alkaline phosphatase activity of three groups. "(a)" was a significant difference when compare with 0.5M/20DTM group ($p<0.05$)."(b)" was a significant difference when compared with day 1 ($p<0.05$).

ALP/Total protein (U/L/µg protein)				
	Day1	**Day7**	**Day14**	**Day21**
TM	0.12±0.1 (a)	0.19±0.10	0.6±0.01	0.2±0.08
0.5M/10DTM	0.18±0.14	0.20±0.13	1.5±0.83	2.0±1.7
0.5M/20DTM	0.61±0.17	0.15±0.06 (b)	0.7±0.03 (b)	0.1±0.05 (b)

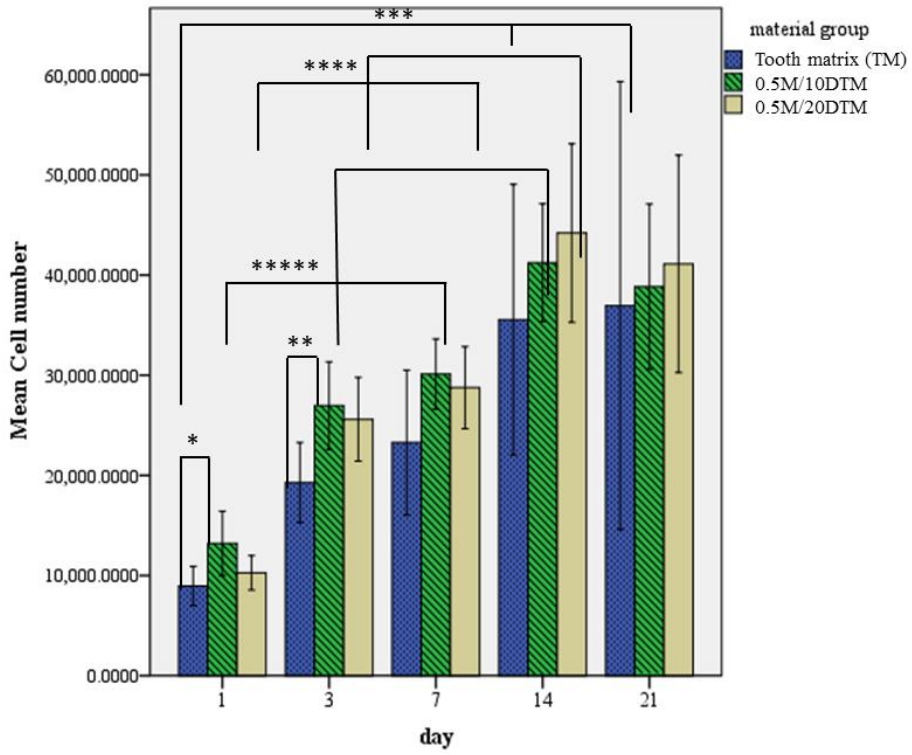


Figure 13 Bar chart of cell proliferation at day 1, 3, 7, 14 and 21. *, ** significant different in when compare between TM and 0.5M/10DTM group. ***, ****, ***** significant different when compare between days ($p<0.05$).

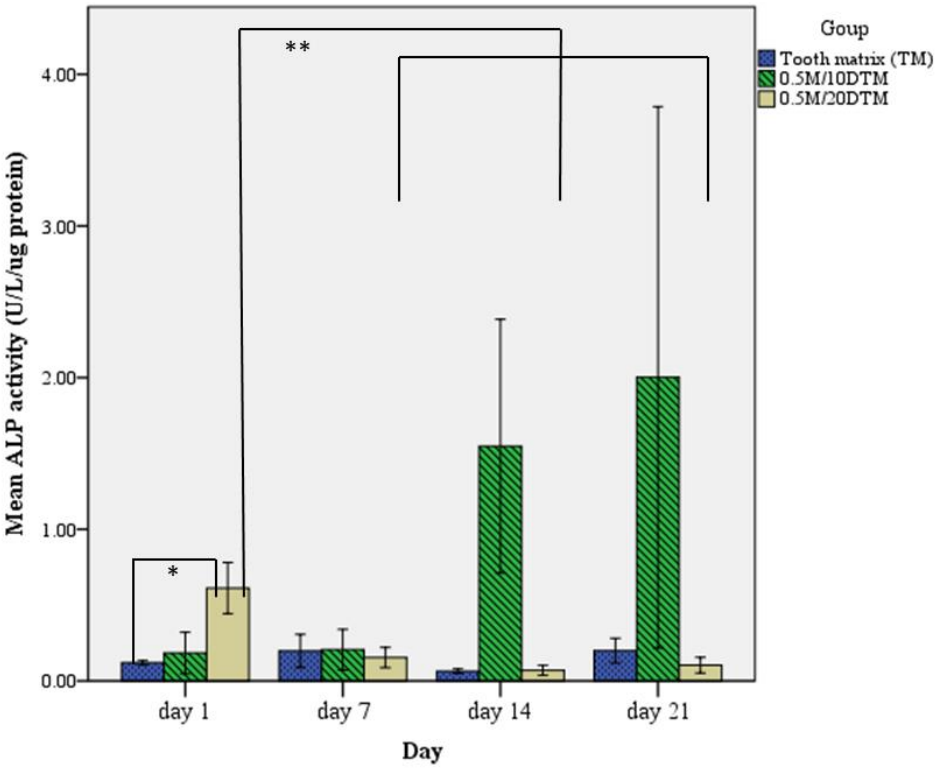


Figure 14 Bar chart of Alkaline phosphatase activity at day 1, 7, 14 and 21. *,** were significant different when compare with 0.5M/20DTM group ($p<0.05$).

The concentration of OCN protein was demonstrated on Figure 15 and Table 10. In TM group, the OCN level was stable during the observation period. The level of OCN in 0.5M/10DTM and 0.5M/20DTM were increase on day 14 and decreased to the lowest on day 21. However, the OCN level of all group was not statistically different.

Table 10 Osteocalcin concentration of TM and DTM groups at day 1, 7, 14 and day 21.

OCN (ng/ml)				
	Day1	**Day7**	**Day14**	**Day21**
TM	7.50±1.58	8.00±4.80	7.00±3.26	6.88±1.25
0.5M/10DTM	8.00±0.94	6.50±2.85	11.00±8.02	6.00±4.20
0.5M/20DTM	8.50±0.61	6.00±4.18	10.50±5.42	6.00±2.85

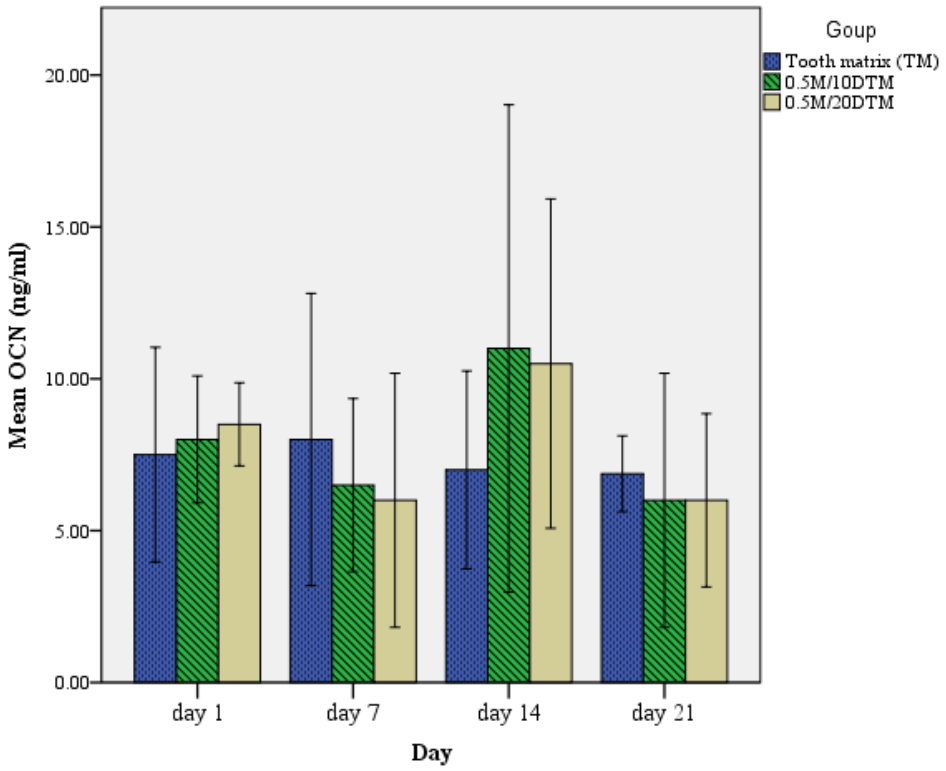


Figure 15 Bar chart of Osteocalcin concentration (OCN) of TM and DTM groups at day 1, 7, 14 and 21.

CHAPTER 4

DISCUSSIONS

The demineralization process is the most important factor affecting the physicochemical property of the graft material. Various protocols have been performed to fabricate tooth-derived bone graft material and the results showed new bone formation and different degradation rate of the graft. In the present study, the different demineralization time and HCl concentration for preparing tooth-derived bone graft were examined and compared with an attempt to preserve the structure of the tooth as the osteoconduction, while facilitate the osteoinduction property by removing some inorganic content to expose organic components which are collagen fibril and BMP-2. The result demonstrated that the demineralized process decreases inorganic content and change the surface morphology of the materials. In particular, increasing the reaction time of hydrochloric acid resulted in lowering the crystallinity, and the surface area. While increasing the HCl concentration result in increasing degradation rate and the quantity of BMP-2.

The demineralization process affects physicochemical and BMP-2 releasing of tooth and bone. In the demineralization process, acid dissolves the inorganic of bone (hydroxyapatite) so the crystallinity of tooth decreases but the bioavailability of collagen, non-collagenous proteins and growth factors increase[39]. In the present study, SEM analysis at high magnification (20,000x), showed wider diameter of dentinal tubules with collagen fibers orientation. The exposed fiber pattern was also reported on the surface of the dentin block graft from previous study[69]that showed increasing the demineralization time could increase the diameter of dentinal tubules, as well as exposed collagen fiber and crack lines on dentin block with ultrasonic demineralization. The dentinal tubules and crack lines can increase the effective porous volume because of increasing in the body fluid contact surface area and growth factor releasing[70].

In the present study, demineralization time increased but the percentages of crystallinity decreased. Similar to previous study[69],they demonstrated tooth would lose their crystallinity of hydroxyapatite structure and became amorphous after 10 and 30 minutes of ultrasonic demineralization process. XRD graph pattern also suggested that the main phase in all group was HA.

XRF analysis demonstrated lower amount of calcium and phosphorous in DTM group than in TM group that like previous studies[71]. Park et al[42], reported that increasing demineralization time, inorganic materials (calcium and phosphate) were decreased, while organic materials (carbon, oxygen, nitrogen) were increased. When inorganic materials decrease, this can imply the mechanical strength of graft will decrease[6, 14].

From the literature, the Ca/P molar ratios for HA phase of calcium phosphate were 1.67[72], in this study the Ca/P ratio of TM group is 1.49 which was slightly decreased from the depletion of calcium and phosphate from demineralization process[72].The Ca/P ratio has been both lower or close to stoichiometric HA (1.67).

However, Ca/P ratio which more than 1.67 has been reported.[73] The strength of HA increases as Ca/P ratio increases to the ratio is 1.67 and decrease suddenly when the ratio is more than 1.67[74].

The higher calculated Ca/P molar ratio in this study suggests that in addition to apatite calcium phosphate. DTM may consist of either amorphous calcium phosphate or unstable calcium phosphate ions such as Ca^{2+}, HPO_4^{2-}, CO_3^{2-}, octacalcium phosphate (OCP) or dicalcium phosphate dihydrate (DCPD) which can release free ions in liquid environment and may enhance their osteoinduction[6]. So, calcium ion and phosphate free ion should be investigated in the further study.

The metal elements in this study which are found in TM and DTM groups are basic ions which form the tooth tissue structures. The elements such as Cu, Fe, Mg, Mn, Pb, Sr and Zn can present in enamel layer[75].

From BET analysis, when concentration of hydrochloric acid and reaction time increased, the BET surface area decreased. Contrast with previous study[41] that they reported the increasing of BET surface area in deciduous teeth relate demineralization time in ultrasonic demineralization process. This evidence confirmed that demineralization process affects the surface area of the material. The average pore diameter in this study was increased relate to demineralization time that may be affected from increasing size of dentinal tubule and crack line in the surface of materials. Previous report suggested that more porous results may increase protein and liquid opportunity in the materials which enhance for protein adhesion as well as ion exchange in apatite formation[14].

Tris-HCl was used in the material degradation assay follow ISO 10993 recommendation. Xin and Chu[58] found the OH^- from material and H_20 reaction could bond hydrogen ion in Tris–HCl and the precipitation of the materials were inhibited. Contrast with SBF solution, hydrocarbonate and hydrophosphate in SBF could react with OH^- and degradation of material was interfered by phosphate and carbonate precipitate on the surface.

The degradation rate of the bioceramic materials depends on cation and anion in calcium phosphate component, particle size, porosity of material. Increasing porosity may affect mechanical strength and surface area which contact environment fluids and leads to faster degradation rate. Moreover, degradation is dependent on acidity and temperature of environment.[6]

Bartee[19] classified bone graft material degradation into 3 groups: long term bone graft, transitional bone graft and short term bone graft. The degradation rate of 0.5M/10DTM and 0.5M/20DTM over 60 days were 9.19±1.92% and 8.46±0.28% respectively which were sorted as transitional resorption rate.This degradation rate was similar to previous clinical studies[50, 51]. Minamizato et al,[51] found the autogenous demineralized dentin matrix remaining in the specimens after 4-6 months socket preservation. Pang et al,[50] there was 8.95±6.15% of residual autogenous demineralized tooth bone graft in the specimen after 6 months socket preservation. Base on the degradation rate, 0.5M/10DTM and 0.5M/20DTM may suitable to be used as the bone material for socket preservation in early or delay implant placement and for guide bone regeneration in small peri-implant defect. However, *in vivo* study should be evaluated

degradation of material which effect from extracellular liquid, enzymes and cell-mediated resorption.[76]

Cell adhesion on biomaterial is a process that can be divided into 4 phases. First, a cell can physically light contact with the substrate. Then, the cell adheres passively and starts sensing the substrate. Third, cell reshape and initial cell propagation. Forth, focal adhesion is formed by connecting between the extracellular matrix macromolecular complexes and the cytoskeleton[77].

From this study, the cell biocompatibility on day 1 demonstrated lower amount of cell proliferation in all groups especially TM group. Similar previous study, Li et al[44], reported cell morphology and cell proliferation of dental follicle cells on human tooth dentin matrix treated with EDTA and untreated human tooth were low on day 1. However, the polygonal shape of cells which was characteristic of cell viability adhesion. They discussed that demineralization protocol must be optimized. They found insufficient demineralization revealed a less bone graft scaffold property, while excessive demineralization process could destroy the structure of dentin. Same with previous study, Liu et al[78] showed that demineralized dentin matrix (DDM) slightly inhibited proliferation of dental pulp stem cells in the DDM group only on day 3 but cell proliferation result was increase by time to day 7. They concluded that a number of porosity surface, wettability, surface charge from solubilities of Ca/P ions and binding proteins releasing after demineralization were effect on cell adhesion process.[77]

Liu et al[78] explained that cell adhesion, cell expansion and cell proliferation were key factors of cell biocompatibility. Cell-biomaterial interactions were crucial for cell differentiation and tissue formation such as such as BMP-2 and TGF-β1.

Base on the present study, it is suggested that the slightly reduction of cell proliferation at day 21 was not attributed from the toxicity of the DTM material but might be due to the insufficient space of the scaffold, because the control group (TM) also exhibited the same pattern.

BMP-2, an osteoinductive growth factor which is one of transforming growth factor beta (TGF-b) superfamily. BMP-2 has been demonstrated to induce osteoblast cell differentiation into a various of cell types.[28] BMP-2 is a highly soluble protein and degraded *in vivo* study with 7-16 minutes of half-life[29] so the scaffolds are used to keep BMPs for staying at the grafted site. In this situation, scaffold should act as a carrier and should slow release growth factors[39]. Therefore, the organic matrix of DTM in this study may functions as a container for BMP-2.

BMP-2 was also quantified in other biological materials. The content varies from tissue to tissue. Demineralized bone matrix (DBM), the BMP-2 concentration were range from 3.6±1.20[30] to 26.7 ± 11.4 ng/g DBM[31]. The BMP-2 in DTM was comparable to those in DBM. BMP-2 in dental pulp tissue was 77.4±8.8 pg/mg of dental pulp tissue[79]. BMP-2 content in deciduous tooth was 0.42 ± 0.3 ng of BMP-2 per gram of tooth particles and demineralized deciduous tooth matrix contained was 1.2 ± 0.3 ng of BMP-2 per gram of particles[80]. BMP-2 with 100-1,000 ng/ml can induce the differentiation of cells and the development of mineral matrix. From this present study, 24 hours extracted BMP-2 of DTM groups were

111.538±6.679 to 1,181.529±193.766 pg/ml of 100 mg of DTM. It can imply DTM has a sustained release carrier property and BMP-2 of one gram human tooth may be extracted 1.11-11.8 ng/ml for 24 hours.

The demineralization process may affect to BMP-2 quantification if excessive demineralization. Pietrzak et al[81] reported that extensive demineralization with 0.5M hydrochloric acid more than 90 minutes may induce BMPs depletion in demineralized bone matrix.

From cell differentiation analysis, alkaline phosphatase (ALP), bone morphogenetic protein-2, osteonectin (ON), Runx-2 and transforming growth factor-beta 1 are early markers of osteogenic cell differentiation, whereas osteocalcin and osteopontin (OPN) are expressed later in cell differentiation process[82]. Casagrande et al[83], reported BMP-2 are necessary to induce cell differentiation from human exfolicle of deciduous teeth into odontoblast cells. Bono et al[80], reported the BMP-2 content found in demineralized tooth graft could enhanced ALP activity.

The exposure of collagen may affect to BMP-2 releasing and enable to cell differentiation. 0.5M/20DTM group showed higher ALP level on day 1 and decreased over time. This finding may come from higher level of BMP-2 which ready to release from the material. While in 0.5M/10DTM, the BMP-2 may be entrapped inside the inorganic matrix and released later after period of time. From this study, the decreasing of late cell differentiation on day 21 may affect from pH condition of the graft and BMP-2 quantification[84].

Further study should be performed to investigate the surface wettability, calcium and phosphate free ion, as well as the in vivo degradation profile.

CHAPTER 5

CONCLUSION

The reaction time and the concentration of HCl affected differently on the demineralized materials. While the reaction time of HCl has effect on tooth matrix in aspect of surface morphology, crystallinity, element components, Ca/P ratio and the quantity BMP-2. HCl concentration has effect on degradation rate of the material. The demineralized process enhanced physicochemical properties of the tooth matrix by exposing the collagen fibrils, growth factors releasing, increasing the porosity and surface area without disturbing the inorganic component.

The demineralized process which using 0.5M HCl acid for 10 minutes demonstrated the most suitable protocol for fabricating DTM as the evidence from the degradation rate, cell proliferation and cell differentiation and may have the potential for further clinical application.

Further study, surface wettability, material porosity, calcium and phosphate free ion, pH of the material, enzyme related degradation and BMP-2 releasing profile should be investigated.

REFERENCES

1. Roseti L, Parisi V, Petretta M, Cavallo C, Desando G, Bartolotti I, et al. Scaffolds for Bone Tissue Engineering: State of the art and new perspectives. ***Mater Sci Eng C Mater Biol Appl*** 2017; 78: 1246-1262.
2. Yeomans JD, Urist MR. Bone induction by decalcified dentine implanted into oral, osseous and muscle tissues. ***Arch Oral Biol*** 1967; 12(8): 999-1008.
3. 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): 228-232.
4. Florencio-Silva R, Sasso GRD, Sasso-Cerri E, Simoes MJ, Cerri PS. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. ***Biomed Res Int*** 2015.
5. Buddhachat K, Klinhom S, Siengdee P, Brown JL, Nomsiri R, Kaewmong P, et al. Elemental Analysis of Bone, Teeth, Horn and Antler in Different Animal Species Using Non-Invasive Handheld X-Ray Fluorescence. ***PLoS One*** 2016; 11(5): e0155458.
6. Eliaz N, Metoki N. Calcium Phosphate Bioceramics: A Review of Their History, Structure, Properties, Coating Technologies and Biomedical Applications. ***Materials (Basel)*** 2017; 10(4): 1-104.
7. Elsalanty ME, Genecov DG. Bone grafts in craniofacial surgery. ***Craniomaxillofac Trauma Reconstr*** 2009; 2(3): 125-134.
8. Kolk A, Handschel J, Drescher W, Rothamel D, Kloss F, Blessmann M, et al. Current trends and future perspectives of bone substitute materials - from space holders to innovative biomaterials. ***J Cranio Maxill Surg*** 2012; 40(8): 706-718.
9. Kao ST, Scott DD. A review of bone substitutes. ***Oral Maxillofac Surg Clin North Am*** 2007; 19(4): 513-521, vi.
10. Roden RD, Jr. Principles of bone grafting. ***Oral Maxillofac Surg Clin North Am*** 2010; 22(3): 295-300, v.
11. Burchardt H. The biology of bone graft repair. ***Clin Orthop Relat Res*** 1983; (174): 28-42.
12. Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. ***Clin Orthop Relat Res*** 2000; (371): 10-27.
13. Hing KA. Bioceramic bone graft substitutes: Influence of porosity and chemistry. ***Int J Appl Ceram Tec*** 2005; 2(3): 184-199.
14. Hannink G, Arts JJC. Bioresorbability, porosity and mechanical strength of bone substitutes: What is optimal for bone regeneration? ***Injury*** 2011; 42: S22-S25.
15. Syftestad G UM. Degradation of bone matrix morphogenetic activity by pulverization. ***Clin Orthop Relat Res*** 1979; 141: 281-285.
16. Zhang M PR, Wolfinbarger L. Effect(s) of the demineralization process on the osteoinductivity of demineralized bone matrix. ***J Periodontol*** 1997; 68: 1085-1092.
17. Dozza B LI, Duchi S, Della Bella E, Martini L, Salamanna F, Falconi M, Cinotti S, Fini M, Lucarelli E, Donati D. When size matters: differences in demineralized bone matrix particles affect collagen structure, mesenchymal stem cell behaviour, and osteogenic potential. ***J Biomed Mater Res*** 2016: 1-37.

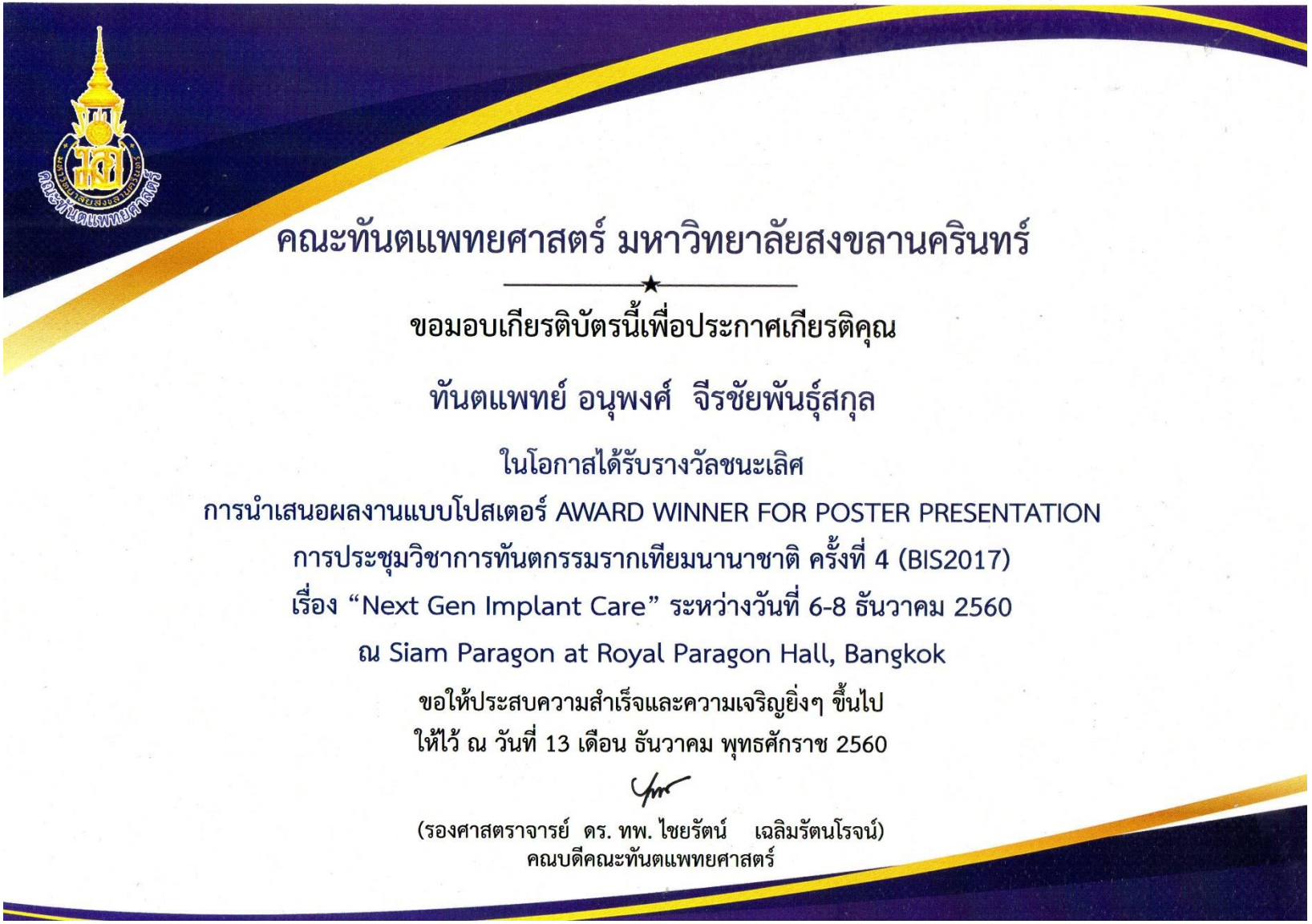
18. Sampath TK RA. Importance of geometry of the extracellular matrix in endochondral bone differentiation. ***J Cell Biol*** 1984; 98: 2192-2197.
19. Bartee BK. Extraction site reconstruction for alveolar ridge preservation. Part 1: rationale and materials selection. ***J Oral Implantol*** 2001; 27(4): 187-193.
20. Hamed E, Novitskaya E, Li J, Chen PY, Jasiuk I, McKittrick J. Elastic moduli of untreated, demineralized and deproteinized cortical bone: validation of a theoretical model of bone as an interpenetrating composite material. ***Acta Biomater*** 2012; 8(3): 1080-1092.
21. Liu MY, Lv YG. Reconstructing Bone with Natural Bone Graft: A Review of In Vivo Studies in Bone Defect Animal Model. ***Nanomaterials (Basel)*** 2018; 8(12).
22. Hermann Ehrlich PGK, Konstantinos D. Demadis, Oleg S. Pokrovsky. Principles of demineralization: Modern strategies for the isolation of organic frameworks Part I. Common definitions and history. ***Micron*** 2008; 39: 062–1091.
23. Senn N. On the Healing of Aseptic Bone Cavities by Implantation of Antiseptic Decalcified Bone. ***Ann Surg*** 1889; 10(5): 352-368.
24. Morris RE, Jr., Benton RS. Studies on demineralization of bone. I. The basic factors of demineralization. ***Am J Clin Pathol*** 1956; 26(6): 579-595.
25. Urist MR. Bone: formation by autoinduction. ***Science*** 1965; 150(3698): 893-899.
26. Zhang Y, Wang J, Ma Y, Niu X, Liu J, Gao L, et al. Preparation and biocompatibility of demineralized bone matrix/sodium alginate putty. ***Cell Tissue Bank*** 2017; 18(2): 205-216.
27. Marshall R. Urist BSS. Bone Morphogenetic Protein. ***J Dent Res*** 1971; 50: 1392.
28. Glowacki J. Demineralized Bone and BMPs: Basic Science and Clinical Utility. ***J Oral Maxillofac Surg*** 2015; 73(12): S126-131.
29. Begam H, Nandi SK, Kundu B, Chanda A. Strategies for delivering bone morphogenetic protein for bone healing. ***Mat Sci Eng C-Mater*** 2017; 70: 856-869.
30. Wildemann B, Kadow-Romacker A, Haas NP, Schmidmaier G. Quantification of various growth factors in different demineralized bone matrix preparations. ***J Biomed Mater Res*** 2006: 437-442.
31. Pietrzak WS, Woodell May J, McDonald N. Assay of bone morphogenetic protein-2, -4, and -7 in human demineralized bone matrix. ***J Craniofac Surg*** 2006; 17(1): 84-90.
32. 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): 1264-1272.
33. Kim Y-K. Bone graft material using teeth. ***J Korean Assoc Oral Maxillofac Surg*** 2012; 38: 134-138.
34. Zhang YR, Du W, Zhou XD, Yu HY. Review of research on the mechanical properties of the human tooth. ***Int J Oral Sci*** 2014; 6(2): 61-69.
35. 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): 7442-7445.
36. Masaru Murata TA, Masaharu Mitsugi, Md Arafat Kabir, In-Woong Um, Yasuhito Minamida, Kyung-Wook Kim, Young-Kyun Kim, Yao Sun, Chunlin Qin. Autograft of Dentin Materials for Bone Regeneration. Pignatello R, editor2013.

37. Aubeux D, Beck L, Weiss P, Guicheux J, Enkel B, Perez F, et al. Assessment and Quantification of Noncollagenic Matrix Proteins Released from Human Dentin Powder Incorporated into a Silated Hydroxypropylmethylcellulose Biomedical Hydrogel. ***J Endod*** 2016; 42(9): 1371-1376.
38. Margarida Figueiredoa SC, Gabriela Martinsa, João Freitas, Fernando Judas, Helena Figueiredo. Influence of hydrochloric acid concentration on the demineralization of cortical bone. ***Chem Eng Res des*** 2011; 89: 116-124.
39. 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.
40. Bang G UM. Bone induction in excavation chambers in matrix of decalcified dentin. ***Arch Surg*** 1967; 94(9): 781-789.
41. Park M, Mah YJ, Kim DH, Kim ES, Park EJ. Demineralized deciduous tooth as a source of bone graft material: its biological and physicochemical characteristics. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2015; 120(3): 307-314.
42. Park SM, Kim DH, Pang EK. Bone formation of demineralized human dentin block graft with different demineralization time: In vitro and in vivo study. ***J Craniomaxillofac Surg*** 2017; 45(6): 903-912.
43. Bono N, Tarsini P, Candiani G. Demineralized dentin and enamel matrices as suitable substrates for bone regeneration. ***J Appl Biomater Funct Mater*** 2017; 15(3): e236-e243.
44. Li R, Guo WH, Yang B, Guo LJ, Sheng L, Chen G, et al. Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration. ***Biomaterials*** 2011; 32(20): 4525-4538.
45. 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.
46. Kang KJ, Lee MS, Moon CW, Lee JH, Yang HS, Jang YJ. In Vitro and In Vivo Dentinogenic Efficacy of Human Dental Pulp-Derived Cells Induced by Demineralized Dentin Matrix and HA-TCP. ***Stem Cells Int*** 2017; 2017: 2416254.
47. Kim KW. Bone Induction by Demineralized Dentin Matrix in Nude Mouse Muscles. ***Maxillofac Plast Reconstr Surg*** 2014; 36(2): 50-56.
48. Eun-Young Lee E-SK, Kyung-Won Kim. Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy Studies on Processed Tooth Graft Material by Vacuum-ultrasonic Acceleration. ***Maxillofac Plast Reconstr Surg*** 2014; 36(3): 103-110.
49. Al-Asfour A, Farzad P, Al-Musawi A, Dahlin C, Andersson L. Demineralized Xenogenic Dentin and Autogenous Bone as Onlay Grafts to Rabbit Tibia. ***Implant Dent*** 2017; 26(2): 232-237.
50. Pang KM, Um IW, Kim YK, Woo JM, Kim SM, Lee JH. Autogenous demineralized dentin matrix from extracted tooth for the augmentation of alveolar bone defect: a prospective randomized clinical trial in comparison with anorganic bovine bone. ***Clin Oral Implants Res*** 2017; 28(7): 809-815.
51. Minamizato T, Koga T, I T, Nakatani Y, Umebayashi M, Sumita Y, et al. Clinical application of autogenous partially demineralized dentin matrix prepared immediately after extraction for alveolar bone regeneration in implant dentistry: a pilot study. ***Int J Oral Max Surg*** 2017.

52. Yang JM, Yang JH, Tsou SC, Ding CH, Hsu CC, Yang KC, et al. Cell proliferation on PVA/sodium alginate and PVA/poly(gamma-glutamic acid) electrospun fiber. ***Mater Sci Eng C Mater Biol Appl*** 2016; 66: 170-177.
53. Song W, Markel DC, Jin X, Shi T, Ren W. Poly(vinyl alcohol) /collagen/hydroxyapatite hydrogel: properties and in vitro cellular response. ***J Biomed Mater Res A*** 2012; 100(11): 3071-3079.
54. Williams DF. On the mechanisms of biocompatibility. ***Biomaterials*** 2008; 29(20): 2941-2953.
55. Li W, Zhou J, Xu Y. Study of the in vitro cytotoxicity testing of medical devices. ***Biomed Rep*** 2015; 3(5): 617-620.
56. Anderson JM. Future challenges in the in vitro and in vivo evaluation of biomaterial biocompatibility. ***Regen Biomater*** 2016; 3(2): 73-77.
57. de Souza Costa CA, Hebling J, Scheffel DL, Soares DG, Basso FG, Ribeiro AP. Methods to evaluate and strategies to improve the biocompatibility of dental materials and operative techniques. ***Dent Mater*** 2014; 30(7): 769-784.
58. Yunchang Xin PKC. Influence of Tris in simulated body fluid on degradation behavior of pure magnesium. ***Mater Chem Phys*** 2010; 124: 33-35.
59. Cheng HC, Chu KT, Teng NC, Tsai HL, Ou KL, Ou SF. The Effect of pH Value on Phase Transformation of Calcium Phosphate Cement. ***Int J Appl Ceram Tec*** 2014; 11(2): 364-370.
60. J. S. Gillespie A.T. McKnight. Adverse effects of tris hydrochloride, a commonly used buffer in physiological media. ***J Physiol*** 1976; 259: 561-573.
61. Wu SC, Hsu HC, Hsu SK, Lin FW, Ho WF. Preparation and characterization of porous calcium-phosphate microspheres. ***Ceram Int*** 2015; 41(6): 7596-7604.
62. Kirste G, Brandt-Slowik J, Bocker C, Steinert M, Geiss R, Brauer DS. Effect of chloride ions in Tris buffer solution on bioactive glass apatite mineralization. ***Int J Appl Glass Sci*** 2017; 8(4): 438-449.
63. Guarino V, Taddei P, Di Foggia M, Fagnano C, Ciapetti G, Ambrosio L. The Influence of Hydroxyapatite Particles on In Vitro Degradation Behavior of Poly epsilon-Caprolactone-Based Composite Scaffolds. ***Tissue Eng Pt A*** 2009; 15(11): 3655-3668.
64. Juraski AD, Rodas ACD, Elsayed H, Bernardo E, Soares VO, Daguano J. The In Vitro Bioactivity, Degradation, and Cytotoxicity of Polymer-Derived Wollastonite-Diopside Glass-Ceramics. ***Materials*** 2017; 10(4).
65. Zhong QW, Li WH, Su XP, Li G, Zhou Y, Kundu SC, et al. Degradation pattern of porous CaCO3 and hydroxyapatite microspheres in vitro and in vivo for potential application in bone tissue engineering. ***Colloid Surface B*** 2016; 143: 56-63.
66. Wildemann B, Kadow-Romacker A, Pruss A, Haas NP, Schmidmaier G. Quantification of growth factors in allogenic bone grafts extracted with three different methods. ***Cell Tissue Bank*** 2007; 8(2): 107-114.
67. Gupta S, Webster TJ, Sinha A. Evolution of PVA gels prepared without crosslinking agents as a cell adhesive surface. ***J Mater Sci Mater Med*** 2011; 22(7): 1763-1772.
68. Rodriguez-Corrales JA, Josan JS. Resazurin Live Cell Assay: Setup and Fine-Tuning for Reliable Cytotoxicity Results. ***Methods Mol Biol*** 2017; 1647: 207-219.

69. Park SM, Kim DH, Pang EK. Bone formation of demineralized human dentin block graft with different demineralization time: In vitro and in vivo study. ***J Cranio-Maxill Surg*** 2017; 45(6): 903-912.
70. Kabir MA, Murata M, Akazawa T, Kusano K, Yamada K, Ito M. Evaluation of perforated demineralized dentin scaffold on bone regeneration in critical-size sheep iliac defects. ***Clin Oral Implants Res*** 2017: 1-9.
71. Saebe M, Suttapreyasri S. Dentin as bone graft substitution. ***Songklanakarin J Sci Technol*** 2014; 2(1): 40-47.
72. Wang L, Nancollas GH. Calcium orthophosphates: crystallization and dissolution. ***Chem Rev*** 2008; 108(11): 4628-4669.
73. Barakat NAM, Khalil KA, Sheikh FA, Omran AM, Gaihre B, Khil SM, et al. Physiochemical characterizations of hydroxyapatite extracted from bovine bones by three different methods: Extraction of biologically desirable HAp. ***Mat Sci Eng C-Bio S*** 2008; 28(8): 1381-1387.
74. Suchanek W, Yashima M, Kakihana M, Yoshimura M. Processing and mechanical properties of hydroxyapatite reinforced with hydroxyapatite whiskers. ***Biomaterials*** 1996; 17(17): 1715-1723.
75. Teruel Jde D, Alcolea A, Hernandez A, Ruiz AJ. Comparison of chemical composition of enamel and dentine in human, bovine, porcine and ovine teeth. ***Arch Oral Biol*** 2015; 60(5): 768-775.
76. Sheikh Z, Abdallah MN, Hanafi AA, Misbahuddin S, Rashid H, Glogauer M. Mechanisms of in Vivo Degradation and Resorption of Calcium Phosphate Based Biomaterials. ***Materials*** 2015; 8: 7913–7925.
77. Janice Kal Van Tam KU, Mitsuhiro Ebara, Stefania Pagliari, Giancarlo Forte, Takao Aoyagi. Mesenchymal stem cell adhesion but not plasticity is affected by high substrate stiffness. ***Sci Technol Adv Mater*** 2012; 13: 8.
78. Liu GL, Xu GQ, Gao ZH, Liu ZH, Xu JJ, Wang JS, et al. Demineralized Dentin Matrix Induces Odontoblastic Differentiation of Dental Pulp Stem Cells. ***Cells Tissues Organs*** 2015; 201(1): 65-76.
79. Ilic J, Radovic K, Roganovic J, Brkovic B, Stojic D. The levels of vascular endothelial growth factor and bone morphogenetic protein 2 in dental pulp tissue of healthy and diabetic patients. ***J Endod*** 2012; 38(6): 764-768.
80. Bono N, Tarsini P, Candiani G. BMP-2 and type I collagen preservation in human deciduous teeth after demineralization. ***J Appl Biomater Funct Mater*** 2018: 1-8.
81. Pietrzak WS, Ali SN, Chitturi D, Jacob M, Woodell-May JE. BMP depletion occurs during prolonged acid demineralization of bone: characterization and implications for graft preparation. ***Cell Tissue Bank*** 2011; 12(2): 81-88.
82. Vater C, Kasten P, Stiehler M. Culture media for the differentiation of mesenchymal stromal cells. ***Acta Biomater*** 2011; 7(2): 463-477.
83. Casagrande L, Demarco FF, Zhang Z, Araujo FB, Shi S, Nor JE. Dentin-derived BMP-2 and Odontoblast Differentiation. ***J Dental Res*** 2010; 89(6): 603-608.
84. Jang WG, Kim EJ, Kim DK, Ryoo HM, Lee KB, Kim SH, et al. BMP2 protein regulates osteocalcin expression via Runx2-mediated Atf6 gene transcription. ***J Biol Chem*** 2012; 287(2): 905-915.

APPENDIX



คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ขอมอบเกียรติบัตรนี้เพื่อประกาศเกียรติคุณ

ทันตแพทย์ อนุพงศ์ จีรชัยพันธุ์สกุล

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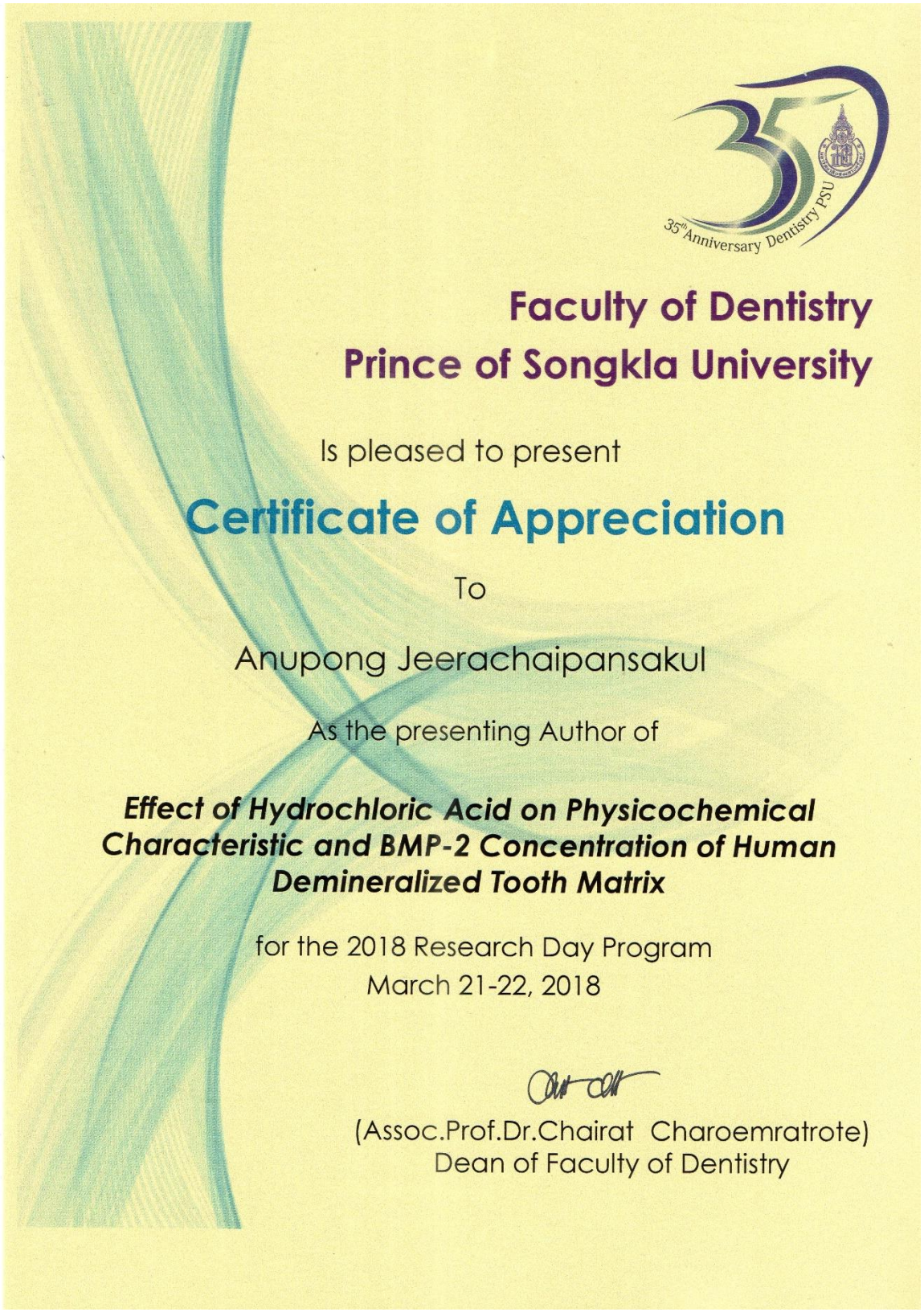
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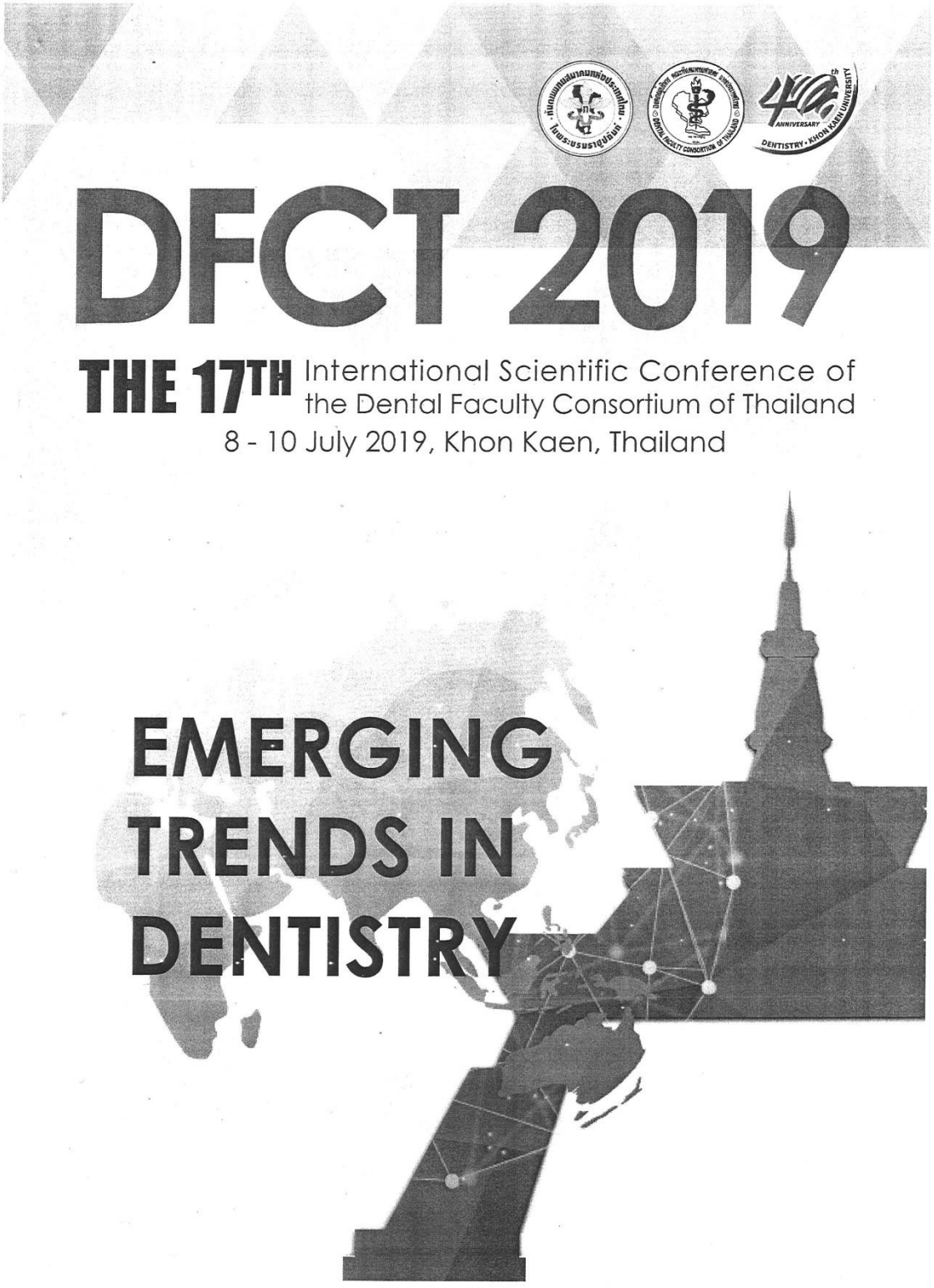
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The Effect of Demineralization Process on Physicochemical Properties, BMP-2 Releasing of Human Demineralized Tooth Matrix

Anupong JEERACHAIPANSAKUL and Srisurang SUTTAPREYASRI

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Background: Demineralized tooth matrix has been considered a grafting material. However, various demineralization procedures may result in products with different properties.

Objectives: To examine the influence of the demineralization process of human tooth using 4 different preparation protocols of hydrochloric acid on the physicochemical characteristic, degradation rate and BMP-2 protein quantification.

Methods: Caries-free permanent teeth were pulverized into particles range from 500-1,000 μm. Tooth particles were assigned to 5 groups with different protocols of demineralization (mineralized tooth;TM, 0.5M/10DTM, 0.5M/20DTM, 1M/10DTM and 1M/20DTM). Chemical compositions were analyzed by x-ray diffraction and x-ray fluorescence spectroscopy. Surface morphology was evaluated by scanning electron microscopy and Brunauer-Emmett-Teller analysis. Bradford protein assay was used to quantify total protein and human ELISA was used for BMP-2 quantification. Degradation behaviors were assessed over 60 days using 50 mM Tris-HCl solution (pH 7.4) at 37°c.

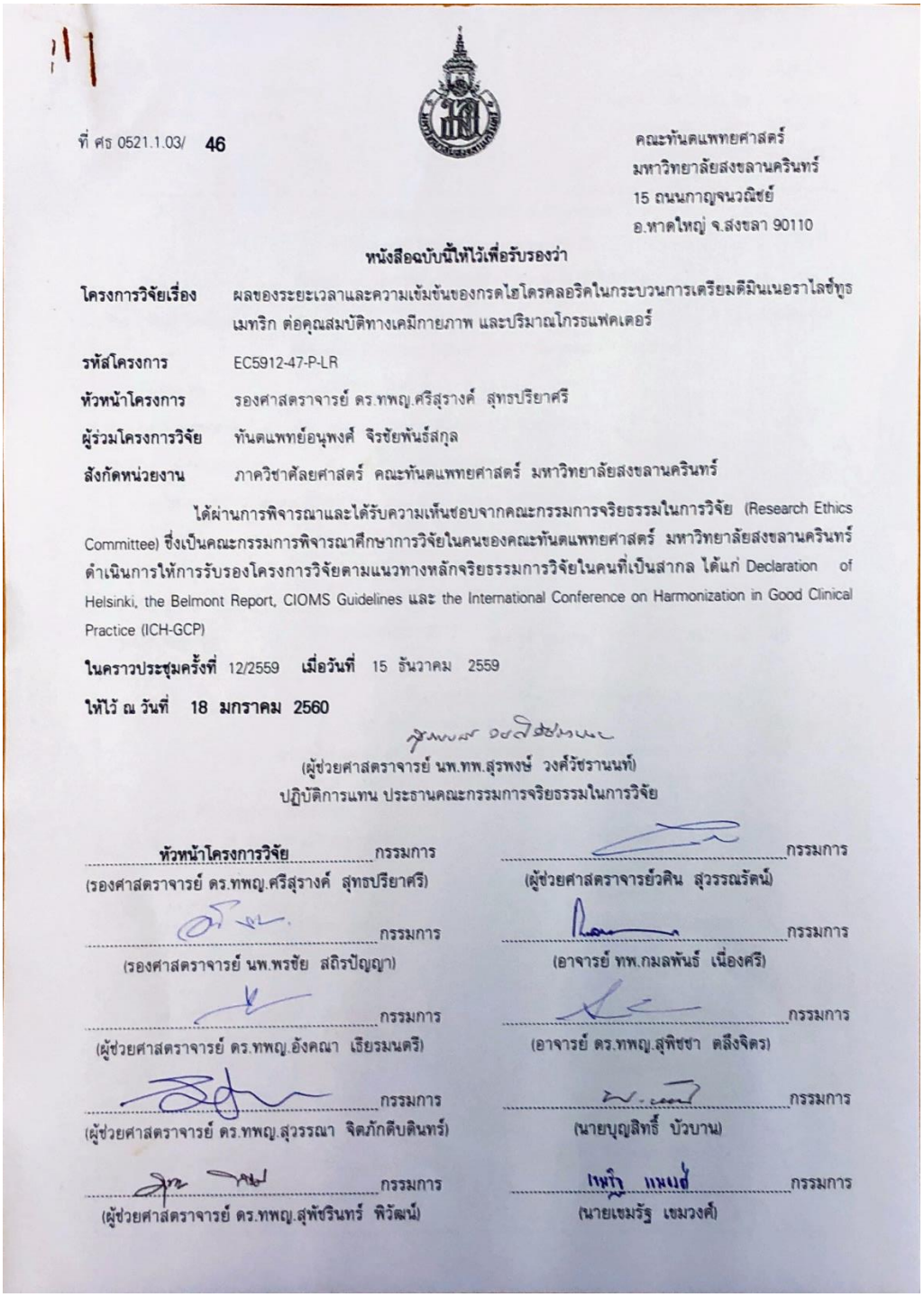
Results: Increasing reaction time led to more collagen exposed on smooth dentin surface, larger size of dentinal tubule and average pore diameter, less crystallinity, and less Ca/P ratio. The highest total protein (3.53±0.24 μg/ml) and BMP-2 concentration (1,181.53±193.77 pg/ml) were found in 1M/20DTM group compared to TM group (0.98±0.081 μg/ml, 2.67±5.34 pg/ml, p=0.000). The degradation at 60 days of TM, 0.5M/10DTM, 0.5M/20DTM, 1M/10DTM, and 1M/20DTM were 6.41±0.30%, 9.19±1.92%, 8.46±0.28%, 19.27±2.74%, 22.90±10.10% respectively (p<0.05).

Conclusion: The reaction time of HCl has greater effect on human tooth particles in aspect of surface morphology, crystallinity, element components, Ca/P ratio, the quantity of total protein and BMP-2 releasing. While increasing the concentration leads to more degradation of the human tooth matrix.

Keywords: BMP-2, Bone graft, Ca/P, Degradation, Demineralization, Tooth

FUNDING AND ETHICAL APPROVAL

All subjects were informed consent. The study protocol was approved by the Ethic Committee of the Faculty of Dentistry and this funding was supported by Graduate School Research Support Funding, Prince of Songkla University, Songkhla, Thailand.



ที่ ศธ 0521.1.03/ 46

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
อ.หาดใหญ่ จ.สงขลา 90110

หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง ผลของระยะเวลาและความเข้มข้นของกรดไฮโดรคลอริคในกระบวนการเตรียมดีมินเนอราไลซ์ทูธ เมทริก ต่อคุณสมบัติทางเคมีกายภาพ และปริมาณโกรธแฟคเตอร์

รหัสโครงการ EC5912-47-P-LR

หัวหน้าโครงการ รองศาสตราจารย์ ดร.ทพญ.ศรีสุรางค์ สุทธปริยาศรี

ผู้ร่วมโครงการวิจัย ทันตแพทย์อนุพงศ์ จิรชัยพันธ์สกุล

สังกัดหน่วยงาน ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

ในคราวประชุมครั้งที่ 12/2559 **เมื่อวันที่** 15 ธันวาคม 2559

ให้ไว้ ณ วันที่ 18 มกราคม 2560

(ผู้ช่วยศาสตราจารย์ นพ.ทพ.สุรพงษ์ วงศ์วัชรานนท์)
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Name Mr. Anupong Jeerachaipansakul

Student ID 6010820017

Educational Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Dental Surgery	Chiangmai University	2010
Higher Graduate Diploma in Clinical Science (Oral and Maxillofacial Surgery)	Prince of Songkla University	2017

Work – Position and Address

General dentist at Wiangkaen hospital public health, Wiangkaen district, Chiang Rai, Thailand.

Oral presentation

Award winner for poster presentation. Jeerachaipansakul A, Suttapreyasri S, Kaewjurat A, Leepong N. "Effect of Hydrochloric acid on physicochemical characteristic and BMP-2 concentration of human demineralized tooth matrix". Attended to the Bangkok International Symposium of Implant Dentistry 2017, Siam Paragon, Royal Paragon Hall, Bangkok, Thailand.

Oral presentation

Jeerachaipansakul A, Suttapreyasri S, Kaewjurat A, Leepong N. "Effect of hydrochloric acid on physicochemical characteristic and BMP-2 concentration of human demineralized tooth matrix". Attended to the PSU Student Research Symposium 2018.

Accepted poster presentation

Jeerachaipansakul A, Suttapreyasri S, Kaewjurat A, Leepong N. "The influence of demineralization process on physicochemical characteristics, bmp-2 quantification of human demineralized tooth matrix" Attend to the International Osteology Symposium Barcelona 2019.

Proceeding oral presentation

Jeerachaipansakul A, Suttapreyasri S "The effect of demineralization process on physicochemical properties, BMP-2 releasing of human demineralized tooth matrix" Attend to the 17th International Scientific Conference of the Dental Faculty Consortium of Thailand (DFCT), 8-10 July 2019, Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand.