RELEASE OF TRANSFORMING GROWTH FACTOR BETA-1 FROM HUMAN TOOTH DENTIN AFTER APPLICATION OF EITHER BIODENTINE™ OR PROROOT® MTA AS A CORONAL BARRIER

KUNLADA WATTANAPAKKAVONG

MASTER OF SCIENCE

IN DENTISTRY

GRADUATE SCHOOL

CHIANG MAI UNIVERSITY

APRIL 2019

RELEASE OF TRANSFORMING GROWTH FACTOR BETA-1 FROM HUMAN TOOTH DENTIN AFTER APPLICATION OF EITHER BIODENTINE™ OR PROROOT® MTA AS A CORONAL BARRIER

KUNLADA WATTANAPAKKAVONG

A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN DENTISTRY

GRADUATE SCHOOL, CHIANG MAI UNIVERSITY

APRIL 2019

RELEASE OF TRANSFORMING GROWTH FACTOR BETA-1 FROM HUMAN TOOTH DENTIN AFTER APPLICATION OF EITHER BIODENTINE™ OR PROROOT® MTA AS A CORONAL BARRIER

KUNLADA WATTANAPAKKAVONG

THIS THESIS HAS BEEN APPROVED TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN DENTISTRY

Examination Committee:

..... Chairman
(Prof. Dr. Siriporn Chattipakorn)

..... Member
(Asst. Prof. Dr. Pairoj Linsuwanont)

..... Member
(Asst. Prof. Dr. Phumisak Louwakul)

..... Member
(Asst. Prof. Dr. Tanida Srisuwan)

Advisor:

.....
(Asst. Prof. Dr. Tanida Srisuwan)

3 April 2019

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Asst. Prof. Dr. Tanida Srisuwan for her invaluable help and great support for this study. My success would not have been possible without her expert advice. Also, I would like to thank the Department of Endodontics, Chiang Mai University Faculty of Dentistry for giving me the opportunity for advanced study.

I thank the Dental Research Center, the Faculty of Dentistry, Chiang Mai University for the provision of equipment and work space. This study was supported by a grant from the Faculty of Dentistry of Chiang Mai University.

In addition, I would like to thank my research advisory committee members, Asst. Prof. Dr. Pairoj Linsuwanont, Prof. Dr. Siriporn Chattipakorn, Asst. Prof. Dr. Phumisak Laowakul, and Asst. Prof. Dr. Tanida Srisuwan, for their guidance in the study. I also thank Dr. M. Kevin O Carroll, Professor Emeritus of the University of Mississippi School of Dentistry, USA and Faculty Consultant at Chiang Mai University Faculty of Dentistry, for his assistance in the preparation of my thesis.

Kunlada Wattanapakkavong

หัวข้อวิทยานิพนธ์ การปลดปล่อยทรานส์ฟอร์มิงโกรทแฟกเตอร์บีตา-1 จากเนื้อฟันมนุษย์ภายหลังการใช้ไบโอเดนทีนหรือโปรรูทเอ็มทีเอเป็นวัสดุกั้นบริเวณคอฟัน

ผู้เขียน นางสาวกุนฑ์ลดา วัฒนภัควงศ์

ปริญญา วิทยาศาสตรมหาบัณฑิต (ทันตแพทยศาสตร์)

อาจารย์ที่ปรึกษา ผศ.ทพญ.ดร.ธนิดา ศรีสุวรรณ

บทคัดย่อ

บทนำ ในทางคลินิกพบว่าฟันที่ได้รับการรักษาด้วยวิธีรีเจนเนอเรทีฟ เอ็นโดดอนติกส์มักเกิดการสะสมของแคลเซียมกระจายอยู่ภายในคลองรากฟัน การเกิดลักษณะดังกล่าวอาจสัมพันธ์กับหลายปัจจัย โดยปัจจัยหนึ่งอาจเกิดจากวัสดุกลุ่มไบโอแอคทีฟ ได้แก่ โปรรูทเอ็มทีเอ และ ไบโอเดนทีน ที่ใช้เป็นวัสดุกั้นบริเวณคอฟันอย่างแพร่หลายในขั้นตอนสุดท้ายของรีเจนเนอเรทีฟ เอ็นโดดอนติกส์ อย่างไรก็ตามยังไม่มีการศึกษาผลของวัสดุเหล่านี้ต่อการสะสมแร่ธาตุ วัตถุประสงค์ของการศึกษานี้คือ เพื่อศึกษาผลของวัสดุทั้งสองต่อการกระตุ้นเนื้อฟันให้มีการปลดปล่อยทรานส์ฟอร์มิงโกรทแฟกเตอร์บีตา-1 และผลของวัสดุทั้งสองต่อการสะสมแร่ธาตุบนเซลล์ปลายรากฟัน

วิธีการทดลอง วัสดุโปรรูทเอ็มทีเอหรือไบโอเดนทีนถูกนำมาใช้เป็นวัสดุกั้นบริเวณคอฟันในรากฟันถอนมนุษย์ที่คลองรากฟันได้ถูกขยายให้มีขนาดใหญ่ และนำไปแช่ในสารละลายฟอสเฟตบัฟเฟอร์ซาไลน์เป็นระยะเวลา 14 วัน หลังจากนั้นจะวัดปริมาณของทรานส์ฟอร์มิงโกรทแฟกเตอร์บีตา-๑ ที่ปลดปล่อยมาจากเนื้อฟันด้วยเอนไซม์-ลิงค์ อิมมูโนแอสเซย์ หรือ อีไลซา ในส่วนของการศึกษาผลของวัสดุต่อต่อการสะสมแร่ธาตุบนเซลล์ปลายรากฟัน เซลล์ปลายรากฟันระยะที่สองไปสามจะถูกนำมาเลี้ยงร่วมกับวัสดุเพียงอย่างเดียว หรือร่วมกับฟันที่มีวัสดุกั้นบริเวณคอฟันในดิฟเฟอ-เรนทิเอชั่นมีเดีย การสะสมแร่ธาตุภายในเซลล์จะถูกประเมินที่ 14 และ 21 วันด้วยอะลิซารินเรด และวัดปริมาณการสะสมแคลเซียมภายในเซลล์ โดยทดสอบสถิติด้วยสถิติครัสคาล-วัลลิส และแมน-วิทนีย์ยู ที่ระดับนัยสำคัญ 0.05

ผลการทดลอง การปลดปล่อยทรานส์ฟอร์มิงโกรทแฟกเตอร์บีตา-1 มากที่สุดในฟันที่มีไบ-โอเดนทีนเมื่อเทียบกับฟันที่มีโปรรูทเอ็มทีเอและฟันที่ไม่มีวัสดุ ไบโอเดนทีนเพียงอย่างเดียวหรือฟันที่

มีไบโอเดนทีนเป็นวัสดุกั้นบริเวณคอฟัน เหนี่ยวนำให้เซลล์ปลายรากฟันมีการสะสมแร่ธาตุได้ดีกว่าโปรรูทเอ็มทีเอทั้งในระยะเวลา 14 และ 21วัน เป็นที่น่าสนใจว่าเมื่อไบโอเดนทีนถูกใช้เป็นวัสดุกั้นบริเวณคอฟัน พบว่าผลของการสะสมแร่ธาตุลดลงอย่างมีนัยสำคัญเมื่อเทียบกับการใช้ไบโอเดนทีนเพียงอย่างเดียว ($P < .05$)

สรุป ไบโอเดนทีนเมื่อใช้เป็นวัสดุกั้นบริเวณคอฟันสามารถกระตุ้นเนื้อฟันให้มีการปลดปล่อยทรานส์ฟอร์มิงโกรทแฟกเตอร์บีตา-1ได้ และผลของการสะสมแร่ธาตุในไบโอเดนทีนมากกว่าโปรรูทเอ็มทีเอ

Thesis Title	Release of Transforming Growth Factor Beta-1 from Human Tooth Dentin After Application of Either Biodentine™ or ProRoot® MTA as a Coronal Barrier
Author	Ms. Kunlada Wattanapakkavong
Degree	Master of Science (Dentistry)
Advisor	Asst. Prof. Dr. Tanida Srisuwan

Abstract

Introduction: The constant observation of intracanal calcification in teeth treated with regenerative endodontic procedures (REPs) has been an issue which various factors could possibly influence. Bioactive materials, including ProRoot® MTA and Biodentine™, have been widely used as a coronal barrier in the final step of REPs. However, the mineralization effect of these materials, when used as such, has not been investigated. The purposes of this study were to evaluate the effect of either ProRoot® MTA or Biodentine™, after application as the coronal barrier, on transforming growth factor beta-one (TGF-ß1) release from root canal dentin, and to observe the impact of these materials on human apical papilla cell (APC) mineralization.

Methods: Either ProRoot® MTA or Biodentine™, was applied as a coronal barrier in the enlarged root canals of human root segments. The segments were stored in phosphate buffer saline (PBS) for 14 days. Then the amount of TGF-ß1 was evaluated using enzyme-linked immunosorbent assay (ELISA). To investigate the effect of the materials on APC mineralization, APCs from passages 2 and 3 were grown in the presence of either the materials alone or material-filled root segments using differentiation medium. Cell mineralization was quantified at 14 and 21 days by alizarin red S staining and then the amount of calcium deposit was quantitatively analyzed. Statistical analysis was performed using the Kruskal-Wallis and Mann-Whitney U tests with the significance level at 0.05.

Results: The greatest amount of TGF-ß1 release was observed in the root segments treated with Biodentine™ compared to those treated with ProRoot® MTA and the untreated segments. Biodentine™, used either alone or as a coronal barrier, promoted greater APC mineralization than did ProRoot® MTA, both on days 14 and 21. Interestingly, when Biodentine™ was applied as a coronal barrier, the mineralization effect was significantly reduced compared to the use of the materials alone ($P < .05$).

Conclusions: Biodentine™, when used as a coronal barrier, promoted the release of TGF-ß1 from the root canal dentin. A higher mineralization effect was observed with BD than with ProRoot® MTA.

CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

α-MEM	Alpha-minimum essential medium
AAE	American association of Endodontists
ANGPT-1	Angiopoietin-1
APCs	Apical papilla cells
BD	Biodentine™
BMP	Bone morphogenetic protein
$Ca(OH)_2$	Calcium hydroxide
CEJ	Cemento-enamel juction
DPSCs	Dental pulp stem cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblastic growth factor
GIC	Glass inomer cement
GMTA	Grey Mineral trioxide aggregate
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
IGF	Insulin-like growth factor
IRM	Intermediate restorative material
MTA	Mineral trioxide aggregate
NaOCl	Sodium hypochloride
REPs	Regenerative endodontic procedures
RMGI	Resin glass ionomer cement
SCAPs	Stem cells from apical papilla
SEM	Scanning electron microscopy
STF	Simulated body fluids
TGF-ß	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor
WMTA	White Mineral trioxide aggregate

STATEMENT OF ORIGINALITY

This study demonstrated the effect of Biodentine™ and ProRoot® MTA on TGF-ß1 release, when each is applied as a coronal barrier in the root canals of human teeth and the effect of these materials on the mineralization potential of isolated human apical papilla cells, when each is applied as a coronal barrier in the root canals of human teeth.

CHAPTER 1

Introduction

1.1 Background and rationales

Regenerative endodontic procedures (REPs) are a new alternative treatment technique, applying the tissue engineering concept, for treating necrotic immature permanent teeth with apical periodontitis. The ultimate goal of these treatment approaches is to regenerate/generate the fully functional pulp-dentin complex and encourage the continuation of root development (1-5). To date, many studies have demonstrated successful clinical and radiographic outcomes after REPs (3, 6-8). Most of the studies revealed that teeth that had undergone this treatment approach showed root canal thickening and tooth elongation (9-15). However, the histologic results from both animal studies and human case reports have shown that the generated tissues inside the root canal were not dental pulp tissues, but likely to be periodontal tissues (16, 17). Moreover, in clinical, the presence of intracanal calcification has been stated as a common finding after REPs (12, 18, 19). Even though intracanal calcification does not impede healing, progressive intracanal calcification can affect tooth vitality, and complicate endodontic treatment and subsequent restoration of the tooth (19, 20). Various factors may be associated with the causes of intracanal calcification, for example the induction of bleeding through the root canal and the differentiation of recruited stem cells (5, 19). For this reason, several fields of exploration have been explored, for example modification of treatment techniques and selection of materials, in order to reach the ideal treatment outcome.

Basically, the treatment procedure for REPs includes disinfection steps followed by regenerative steps. The overall treatment aims to create sterile space for cells/tissues to regenerate inside the root canal. Recently, the American association of Endodontists (AAE) has proposed updated guidelines for REPs, which are regularly adjusted following to the latest evidence. Most of the techniques have been modified in order to create a

better environment for recruiting stem cells and generating tissues. In the past, efforts have been made focusing on the use of materials of low cytotoxicity, such as low concentration antibiotics and irrigating solution. The present AAE protocol has already been adjusted toaccount for changes in the concentrations of both of these agents (21-23). Currently, one of the clinical steps of interest is a dentin preconditioning step. Several studies have examined the effect of medicaments and irrigants on dentin, including changes in dentin morphology, promotion of cell attachment, and expression of growth factors (24-27), and have reported that the use of calcium hydroxide and ethylenediaminetetraacetic acid (EDTA) can promote cell attachment and solubilize growth factors (25, 26, 28), which play a role in cell migration, proliferation and differentiation of dental stem cells (4, 29).

Growth factors constitute an important component of the tissue engineering concept. Various types of growth factors are sequestered in dentin matrix during dentinogenesis and can be released after dental injuries, such as caries or trauma and after placement of restorations (26, 28, 30-32). Transforming growth factors beta-1 (TGF-ß1) is one of the key factors that plays a role in dental cell differentiation (33-35). Recent studies have revealed that TGF-ß1 acts as a chemo-attractant and can stimulate differentiation of dental pulp stem cells (DPSCs) into odontoblasts (26, 27). Other studies also have reported that endodontic disinfectants and irrigating techniques, such as calcium hydroxide, EDTA and the use of ultrasonic activation, could promote TGF-ß1 release from dentin (26, 28, 36). Additionally, some studies have investigated the effects of bioactive materials, such as MTA and Biodentine™ on the release of TGF-ß1 (30, 35, 37). Tomson et al. (30) have demonstrated that MTA can increase the level of TGF-ß1 released from dentin. Moreover, both MTA and Biodentine™ can stimulate TGF-ß1 secretion from pulp cells (35). These materials can activate the differentiation of mesenchymal stem cells into odontoblast-like cells and promote proliferation of mesenchymal stem cells (37).

Conventionally, these bioactive materials are used in the final step of REPs as coronal barriers to prevent bacterial reinfection, because they can create the perfect seal (37, 38). However, no studies have investigated the potential of these bioactive materials from the regenerative aspect for REPs, including the potential to stimulate the release of growth factors and their consequences. Therefore, the objectives of this study are to 1)

investigate the effect of Biodentine™ and ProRoot® MTA on TGF-ß1 release, when each is applied as a coronal barrier in the root canals of human teeth, and 2) investigate the effect of Biodentine™ and ProRoot® MTA on mineralization potential of isolated human apical papilla cells, when each is applied as a coronal barrier in the root canals of human teeth.

1.2 Research questions

- Do Biodentine™ and ProRoot® MTA effect on TGF-ß1 release, when each is applied as a coronal barrier in the root canals of human teeth?

- Do Biodentine™ and ProRoot® MTA effect on the mineralization potential of isolated human apical papilla cells, when each is applied as a coronal barrier in the root canals of human teeth?

1.3 Objectives of the study

- To investigate the effect of ProRoot® MTA and Biodentine™ on TGF-ß1 release, when each is applied as a coronal barrier in the root canals of human teeth

- To investigate the effect of ProRoot® MTA and Biodentine™ on the mineralization potential of isolated human apical papilla cells, when each is applied as a coronal barrier in the root canals of human teeth

1.4 Research hypotheses

H0: TGF-ß1 release on the root canal of human teeth and mineralization of isolated human apical papilla cells are not different when each is applied with either ProRoot® MTA or Biodentine™ as a coronal barrier.

H1: TGF-ß1 release on the root canal of human teeth and mineralization of isolated human apical papilla cells are different when each is applied with either ProRoot® MTA or Biodentine™ as a coronal barrier.

CHAPTER 2

Literature review

2.1 Regenerative Endodontics

2.1.1 Background

Regenerative endodontic procedures (REPs) are defined as biologically-based procedures created to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex (39). Currently, it is one of the alternative treatment approaches for treatment immature teeth with apical periodontitis.

Traditionally, the treatment of necrotic immature teeth is apexification by either long term calcium hydroxide medicament (40) or inducing artificial barriers with materials such as mineral trioxide aggregate (MTA) (41). However, a major concern has been raised when using those techniques because the promotion of root development is limited, making the tooth prone to fracture (3). Thus, REPs have become a treatment of interest since the continuation of root development has been constantly reported after treatment with this new approach (18).

REPs, originally termed as "revascularization techniques," was popularized when two studies, by Iwaya et al (42) and Banchs and Trope (43), reported case studies of immature necrotic teeth treated with the particular technique and showing continued root development. The background of this treatment protocol is to create a sterile environment in the tooth, an environment which can promote the repair process of the destroyed tissue. Therefore, the first clinical procedure is to eliminate bacteria by effective canal disinfection, using both irrigants and medicaments. Then, a blood clot is stimulated to act as a scaffold for regeneration by penetrating the instruments through the apical end of the immature tooth. Then, to prevent the ingrowth of bacterial reinfection, a bacteria-tight seal filling is created. To date, many case studies demonstrating successful clinical and radiographic outcome using REPs have been

published (3, 6-8).

However, some drawbacks have also been proposed, since unexpected treatment results, for example root canal obliteration, have been observed, both in clinical and histological studies (12, 16, 44). Therefore, improvement of both protocols and techniques is required to allow more predictable treatment outcome after REPs.

2.1.2 Current treatment protocols

The American Association of Endodontists (AAE) has deliberated and summarized the protocol for REPs. The protocol refers to many steps in REPs from case selection criteria, informed consent, treatment procedures, suggested follow-up periods and, lastly, the goals of treatment. In general, REPs have two critical steps. Firstly, disinfection of the root canal by irrigation and intracanal medicaments. After two to four weeks, the next step in regeneration is engaged if signs/symptoms are resolved. The second step is creating a blood clot by intentionally stimulating the apical tissues, allowing stem cells from the periapical tissues to reside and generate (23).

Regenerative Endodontic Procedures Recommended by AAE 2016

- Case selection
    - Tooth with necrotic pulp and an immature apex.
    - Pulp space not needed for post/core, final restoration.
    - Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).
- First Appointment
    - Local anesthesia, dental dam isolation and access.
    - Copious, gentle irrigation with 20 mL of 1.5% NaOCl for five minutes, using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vent, or EndoVacTM). Dry canals with paper points
    - Place calcium hydroxide or low concentration of triple antibiotic paste via syringe. Then seal with 3–4 mm of a temporary filling material. Dismiss patient for one to four weeks.

- Second Appointment (one to four weeks after first appointment)
    - Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with disinfection, or alternative disinfection
    - Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation
    - Copious, gentle irrigation with 20 ml of 17% EDTA. Dry with paper points
    - Create bleeding into canal system by over-instrumenting. Stop bleeding at a level that allows for 3-4 mm of restorative material
    - Place a resorbable matrix such as CollaPlug™ over the blood clot, if necessary, followed by capping materials, such as MTA or Biodentine™
    - Seal with 3–4 mm layer of glass ionomer and then place permanent restoration.

Various issues, for example the techniques to recruit stem cells and different medicaments used, have been continuously reported revealing their influences after REPs (5, 24, 37, 45). However, additional scientific evidence is still required to support the use of newer materials in order to improve the clinical outcome.

2.1.3 Treatment outcome

A recent study has described the outcome after REPs into three different levels: patients, clinicians and scientists (Figure 2.1) (46). The patient-based outcomes constitute the base of a pyramid, and represent the resolution of disease, tooth esthetics, tooth survival and function. The clinician-based outcomes constitute the middle of the pyramid, and represent the radiographic signs of healing and pulp sensitivity. Finally, the scientist-based outcomes constitute the top of the pyramid, and represent the successful generation of the dental-pulp tissue (46).

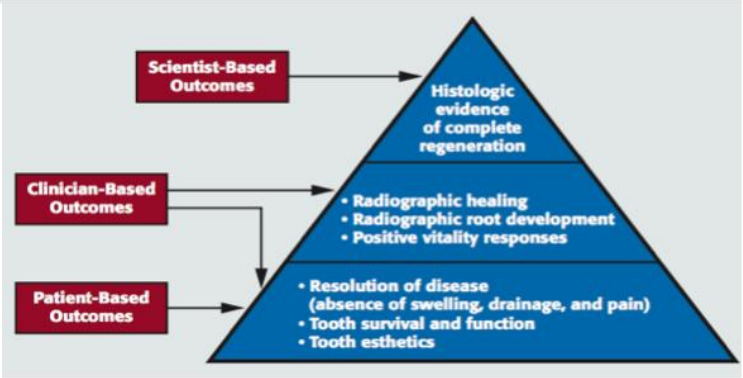


Figure 2.1 REPs outcome assessment pyramid

Most of the outcome studies in REPs have focused on the patient- and clinician-based outcomes and have reported very high survival rates ranging from 76.47%-100% (11, 14, 15, 47). Moreover, the significant clinical findings which are truly expected are the thickening of the root canal walls and the continuation of root development (12, 42, 43). However, a variety of findings have been reported after REPs were commenced, finding which can be categorized into five types as follow (Figure 2.2) (12):

- Type 1: increased thickening of the canal walls and continued root maturation (Figure 2.2*a*)
- Type 2: blunt root apex with no significant continued root development (Figure 2.2*b*)
- Type 3: continued root development, while the apical foramen remains open (Figure 2.2*c*)
- Type 4: severe calcification of the canal space (Figure 2.2*d*)
- Types 5: forming of hard tissue barrier in the canal between the coronal MTA plug and the root apex (Figure 2.2*e*)

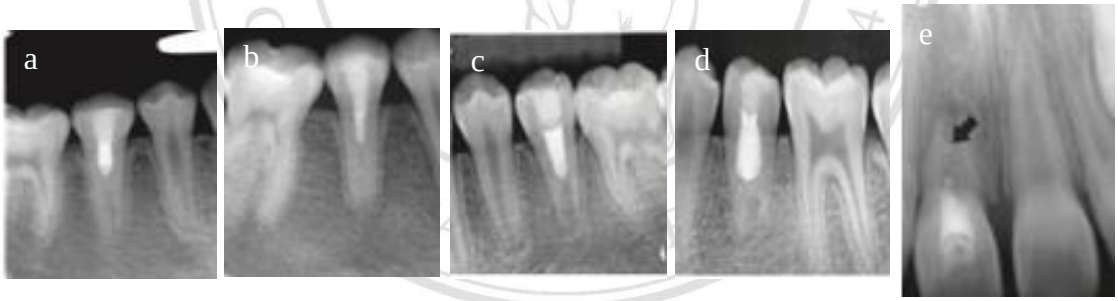


Figure 2.2 Clinical outcome of revascularization

- Tooth 45, increased thickening of the canal walls and continued root maturation.
- Tooth 45, no significant continued root development with the root becoming blunt and closed.
- Tooth 35, continued root development with the apical foramen remaining open.
- Tooth 35, severe calcification of the canal space

Several recent published outcome studies have drawn similar conclusions, showing that teeth that had undergone REPs had significant increase in root length compared to conventional MTA apexification (9, 48). In contrast, the latest study by Silujai and Linsuwanont reported that there was no significant difference between

revascularization and apexification in terms of increased root length, but there was significant increase in mesiodistal width of the root at the cemento-enamel junction (15). The changes in root development, in terms of root canal thickness, root elongation and apical closure, are summarized in the following table (Table 2.1).

Table 2.1 Summary of root development

Radiographic finding of root development after treated with REPs	Authors	Outcome (%)	Follow-up period
1. Increased root length	Jeeraphun et al. 2012 (11)	14.9 %	at least 6 months
	Kahler et al. 2014 (12)	-2.7-25.3 %	at least 18 months
	Souad et al. 2014 (14)	5 %	12 months
	Silujjai et al. 2017 (16)	-4-58 %	at least 12 months
	Li et al. 2017 (13)	23.37 %	12 months
	Edwin et al. 2017 (15)	8.1 %	30 months
2. Increased root dentin thickness	Shah et al. 2008 (48)	57 %	0.5-3.5 years
	Jeeraphun et al. 2012(11)	28.2 %	at least 6 months
	Kahler et al. 2014 (12)	-1.9-72.6 %	at least 18 months
	Souad et al. 2014 (14)	21 %	12 months
	Edwin et al. 2017 (15)	11.6 %	30 months
3. Completed apical closure	Kahler et al. 2014 (12)	19.4 %	at least 18 months
	Souad et al. 2014 (14)	55 %	12 months
	Li et al. 2017 (13)	40 %	12 months
	Edwin et al. 2017 (15)	30.8 %	30 months

2.1.4 REPs and the tissue engineering concept

The basis for regenerative endodontics is the application of the tissue engineering concept. Tissue engineering aims to repair and restore the damaged tissue/organ, returning it to function. The three main components of tissue engineering are stem cells,

scaffolds and signaling molecules (Figure 2.3) (49). The suitable combination of these factors should promote tissue regeneration (49).

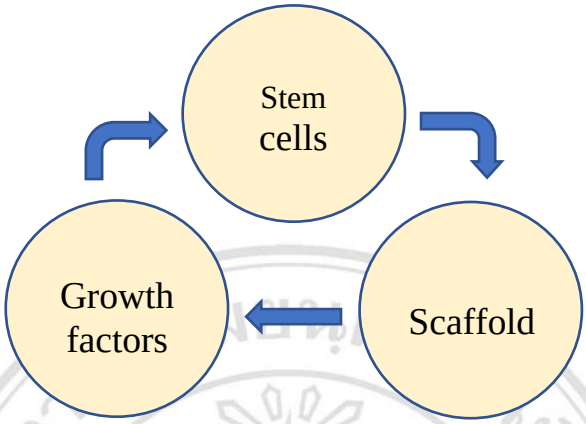


Figure 2.3 The three main components of tissue engineering

Stem cells

Focusing on regenerative endodontics, the stem cells/progenitor cells which have been generally referred to are stem cells from the apical papilla (SCAPs). These cells have been hypothesized to survive at the apical part of the root even in the presence of infection (50).

In general, SCAPs have been discovered in the apical papilla tissue at the root ends of developing teeth. They exhibit the profiles of stem cells, such as STRO-1, CD73 and CD90, which are exactly similar to those of typical mesenchymal cells. SCAPs have high proliferation rates, self-renewal properties, and can differentiate in to odontoblast-like cells (51, 52). SCAPs have been proposed to play roles in tissue regeneration after REPs (52). Therefore, it is important to effectively recruit and suitably modulate these cells in order to achieve the ideal dental tissue generation.

Previous study focusing on the role of stem cells in REPs reported interesting results after blood was collected from the root canal after the apical part of the root was intentionally penetrated (5, 53). In that study, blood collected from the canal system showed significantly higher levels of stem cell markers CD73 and CD105 (up to 600-fold) than did the systemic blood (Figure 2.4), confirming the possibility of stem cell recruitment. The findings demonstrate that undifferentiated stem cells can be induced

into the canal space, and may be associated with the possible regeneration of tissues inside root canals that have been treated with REPs (5, 53)

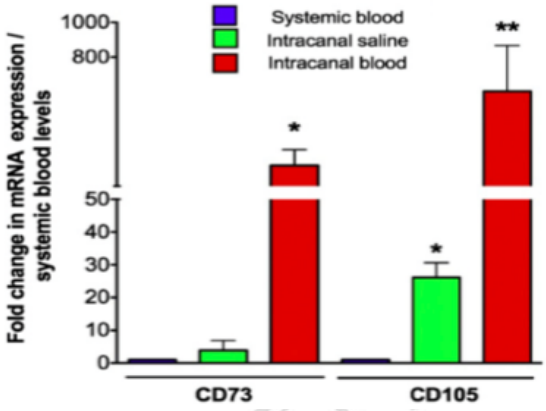


Figure 2.4 Expression of mesenchymal stem cell markers CD73 and CD105 after the stimulated-bleeding step in regenerative procedures (53)

Scaffolds and signaling molecules

Scaffolds and signaling molecules are required for the tissue generation. The scaffolds act as a three-dimensional structure, allowing cells to migrate, adhere and differentiate (29). In respect to REPs, appropriate scaffolds may provide an environment for cells, including SCAPs and other stem cells, to differentiate and, ideally, generate new dental pulp tissue. Currently, blood clots, created by intentional over-instrumentation through the apical tissue of the root, have been assumed to generate autologous scaffolding, providing a lattice for cell growth, while also recruiting stem cells from the apical tissues (4). The components of a blood clot constitute a network of fibrin, platelets, red blood cells and white blood cells, all of which are necessary for wound healing. Various methods have been applied in order to produce scaffolds from other sources, such as collagen, glycosaminoglycan, hydrogels, polylactic acid, and polyglycolic acid (5, 49). The novel construction of these scaffolds may offer clinicians the ability to improve treatment quality. Moreover, several techniques have also been investigated to conjugate the important signaling molecules into the scaffolds that potentially improve specific cell differentiation.

Growth factors are proteins which play roles in cell fate, including cell differentiation (4, 29). Various types of growth factors have been mentioned as playing parts in REPs, for example bone morphogenetic protein (BMP), transforming growth factor-beta (TGF-ß), fibroblastic growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and vascular endothelial growth factor

(VEGF) (4, 29, 54). These growth factors can be found in blood and also sequestrated in the dentin matrix (4, 29). Earlier studies have reported that growth factors and cytokines are sequestrated in dentin matrix during dentinogenesis, and released in responded to some dental injuries, such as dental caries or restorative procedures (31, 55). These growth factors are solubilized and released to the pulp tissue, and can stimulate the dentin regenerative processes (32). One of the most interesting growth factors, which has been frequently raised in REPs, is transforming growth factor-beta (TGF-ß).

The super-family of TGF-ßs can be classified in to three groups: TGF-ßs, BMP and activins/inhibins. TGF-ßs consist of TGF-ß1, TGF-ß2, TGF-ß3 and TGF-ß5, which are multifunctional regulators that influence cell proliferation, cell differentiation, matrix secretion and apoptosis (56). They are important modulators controlling odontoblast activity. They are also released from odontoblasts, and sequestrated in dentin during dentinogenesis. Therefore, the dentin has been assumed to be a reservoir of growth factors for reparative and regenerative process of the dental pulp after injuries.

Previous *in vivo* studies have reported that when pulpal cells were exposed to endogenous TGF-ß, formation of reactionary dentin, reparative dentin or dentin bridges were observed (56-58). Moreover, it has also been confirmed that TGF-ß1 can modulate odontoblastic differentiation and extracellular matrix secretion (33, 34). Melin et al. (33) used cultured tooth slice to investigate the effect of TGF-ß1 on dental pulp and observed the diffusions of TGF-ß1 through the dentinal tubules to the coronal pulp portion. The penetration of the TGF-ß1 induced cell proliferation, cell migration and collagen synthesis inside the dental pulp. Besides, TGF-ß1, with the addition of FGF-2, improves DPSC differentiation to odontoblast-like cells (34). The stimulation of the release an endogenous cocktail of growth factors may offer the appropriate signals and also the environment for recruited cells to differentiate into the desired tissue. Therefore, various methods have been invented and investigated in order to increase the release of growth factors.

- ***Effect dentin conditioning to the release of growth factor; TGF-ß***

Several studies have examined factors, for example irrigating solutions, intracanal medicaments and a variety of materials that can induce dental pulp cells or dentin to

release growth factors (26, 27, 30, 35). Irrigating solutions and intracanal medicaments not only disinfect the root canal, but also solubilizing the growth factors from dentin (Figure 2.5) (37).

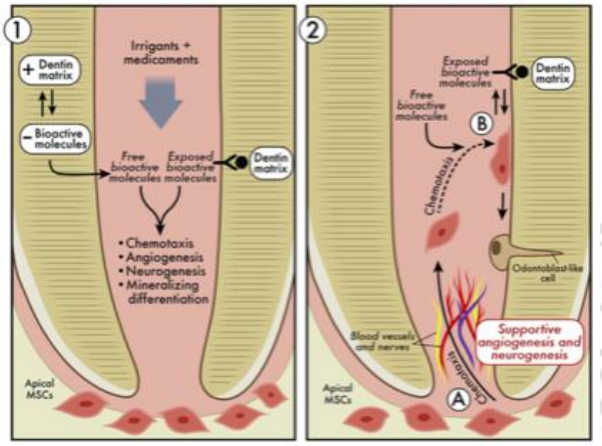


Figure 2.5 Actions of irrigants and medicaments in the release of bioactive molecules sequestered in dentin and their affect on cell regeneration

A recent study by Galler et al. (26) reported that the amount of TGF-ß1 was increased from dentin after dentin was irrigated with chlorhexidine for five minutes, followed by EDTA for 20 minutes. The study also remarked that the use of NaOCl significantly reduced the release of TGF-ß1. Furthermore, another study by the same group have found the expression of TGF-ß1 on the dentin surfaces after treatment with various irrigants using gold-labelling immunohistochemistry and scanning electron microscopy (SEM) (Figure 2.6).

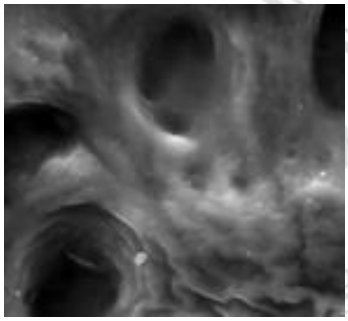


Figure 2.6 TGF-ß1 expression on dentin surfaces after EDTA conditioning. Gold nanoparticles conjugated to TGF-ß1 antibody was used for immunohistochemistry.

On the other hand, a study by Zeng et al. (27) found that when dentin was irrigated with 1.5% NaOCl or 2.5% NaOCl, followed by 17% EDTA, a greater release of TGF-ß1 was observed than from dentin irrigated with 17% EDTA alone. Interestingly, the addition of ultrasonic activation during root canal irrigation with EDTA promoted TGF-ß1 released from dentin (36). Thus, there are still some controversies at various steps of irrigation which require further evaluation.

The influence of intracanal medicaments on TGF-ß1 release has also been a topic of interest. A recent study revealed that water-base calcium hydroxide can promote slight increases of TGF-ß release from dentin compared to dentin without pre-medicament or medicated with other medicaments, including corticoid-antibiotic paste, oil-based calcium hydroxide, triple antibiotic paste and chlorhexidine gel (26). The results implied that water-based calcium hydroxide, when used as root canal medicament, can dissolve and, then, stimulate the release of growth factors from human dentin matrix (28).

Another interesting factor, can affects the release of growth factors, is the application of materials into the root canal. Since the final step in REPs is the placement of a coronal barrier, the selection of materials having active roles is attractive. Initially, filling materials, covering the blood clot were used in order to create an appropriate seal after REPs. The newer bioactive materials, when used as a coronal seal, can offer some advantages to the generating tissue. To date, a variety of materials have been applied, such as resin glass ionomer cement (RMGI), resin-bonded composite, MTA and Biodentine™. However, scientific evidence is lacking to support the use of these materials, especially in REPs. Thus, the benefits of using the newly developed bioactive materials in REPs should be evaluated since the materials may play some roles in regenerative processes, for example, promoting proliferation and differentiation of recruited mesenchymal stem cells (37).

Previous studies focusing on these bioceramic materials have reported that MTA and Biodentine™ can promote TGF-ß release from dental pulp cells which no significant differences, in terms of the TGF-ß level released, observed between the two materials (35). Other signaling molecules, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (ANGPT-1) and fibroblast growth factor-2 (FGF-2), were also evaluated in another study (59). That study revealed that the application of either MTA or Biodentine™ on dental pulp promoted VEGF release, but not that of ANGPT-1 or of FGF-2 (59). Interestingly, different formulations of the materials (white or grey MTA) affected the release of growth factor. A greater release of TGF-ß1 was detected when white MTA solution was used to dissolve the dentin powder, than when grey MTA was used (30).

Currently, MTA and Biodentine™ are the material of choice for vital pulp therapy (30, 60). Nevertheless, the evidence supporting the use of these bioactive materials is still limited. Further studies focusing on molecular aspects of regenerative potential are required.

2.2 Bioactive materials in REPs

2.2.1 Mineral trioxide aggregate (MTA)

MTA, introduced by Torabinejad in 1993 (61), is a calcium-silicate-based cement, usually used in endodontic treatment, for pulp capping and pulpotomy, and as an endodontic reparative material, apical barrier and root-end filling material (62). MTA has two types: white (WMTA) and grey (GMTA). The differences between WMTA and GMTA are color and the concentrations of Al_2O_3, MgO and FeO (63). WMTA was developed to replace GMTA, since tooth discoloration by GMTA has been consistently reported (64). Various commercial MTA products are currently available, including ProRoot® Gray MTA and White MTA (Dentsply Tulsa Dental Specialties, Memphis, TN, USA), RetroMTA® and OrthoMTA (BioMTA®, Seoul, Korea), Endocem MTA (Maruchi, Wonju-si, Korea) and MTA Angelus® (Angelus, www.angelus.ind.br) (65).

2.2.1.1 Composition

The components of MTA are powder and liquid (Table 2.2) (66). The powder-to-liquid ratio is 3:1. The components can be mixed on glass slab or paper pad using a plastic or metal spatula to a paste consistency (41, 67, 68). After mixing the pH of MTA is 10.2 (69). After three hours, the pH will increase to 12.5.

2.2.1.2 Setting reaction

MTA becomes hydrated on contact with water (70). Two main reactions are observed; 1) tricalcium silicate and dicalcium silicate react with water to form calcium silicate hydrate gel and calcium hydroxide, and 2) tricalcium aluminate and calcium sulfate produce ettringite, which is a by-product of the hydration of calcium sulfate, tricalcium aluminate and water during hydration (70). The calcium ions diffuse through the dentinal tubules and become progressively concentrated (71, 72). MTA requires moisture for setting; so, it is recommended that a wet cotton pellet be placed over the MTA (41). The setting time of MTA differs with each brand, for example, two hours 20

minutes for ProRoot® WMTA (69, 73) and less than 24 minutes for Angelus MTA® (74).

2.2.1.3 Physical and mechanical properties

The initial compressive strength of MTA is 40 MPa, which is significantly lower than that of amalgam, Intermediate Restorative Material (IRM; L.D. Caulk, Milford, DE, USA), and SuperEBA™ cement (Harry J. Bosworth, Skokie, IL, USA). However, the compressive strength is increased to 67 MPa after three weeks of setting, still lower than that of amalgam and IRM, but similar to that of SuperEBA™ (69). The surface hardness of ProRoot® MTA is 39.99 HV, which is 2.5-fold higher than that of Portland cement type I (CEM I) (16.32 HV) and Portland cement type II (CEM II) (13.51 HV) (75). Five-millimeter-thick ProRoot® MTA has been recommended as an apical barrier, since it provide better sealing ability than a lesser thickness (76). Several studies have revealed many factors affecting the compressive strength and microhardness of this material, including the MTA types, the water-to-powder ratio, the condensation pressure on the material and the conditions of MTA storage (73, 77-80).

Table 2.2 The components of MTA

Powder	Liquid
Purified Portland cement • Dicalcium silicate (Ca_2SiO_4) • Tricalcium silicate (Ca_3SiO_5): main component • Tricalcium Aluminate ($Ca_3Al_2O_6$) • Calcium sulfate ($CaSO_4$, gypsum) • Tetracalcium aluminoferrite ($4CaO.Al_2O_3Fe_2O_3$)	Distilled water
Bismuth oxide: radiopacifying agent	

Moreover, moisture is important to the strength of MTA because the hydration rates of dicalcium and tricalcium silicate are different; so, keeping MTA moist, once applied strengthens it over time (81). Moreover, a study discovered that the precipitation of apatite-like crystal between MTA and dentin plays a major role in improving the bond strength and sealing ability of this material, since it creates chemical and mechanical bonds to the dentin (82). Moreover, the ability of precipitation to form an interfacial layer and tag-like structures may influence the sealing ability and push-out bond strength of MTA (83).

2.2.1.4 Biological Properties

a) Biocompatibility

MTA is a non-toxic, non-carcinogenic, and non-genotoxic material. Several studies, both *in vitro* and *in vivo*, have evaluated the biocompatibility and cytotoxicity of MTA (62, 84) and have confirmed that it is safe to be used in clinical treatment. Various cell types, including mouse fibroblasts (85), mouse L929 (86, 87), human gingival fibroblasts (88, 89), human osteoblast cells (90) and human periodontal ligament fibroblasts (91), have been used to assess the biocompatibility of MTA. For example, Keiser et al. (91) studied the cytotoxicity of MTA, amalgam and SuperEBA™ on human periodontal ligament fibroblasts and demonstrated that MTA had the least toxicity. When MTA was cytologically tested after complete setting, no sign of cytotoxicity has been observed (90, 91). However, another *in vitro* study reported that MTA, at the initial setting up to 12 hours, was cytotoxic (92).

b) Bioactivity

The main components of MTA are calcium and silicate (63), which are able to induce hard tissue formation (93). Remineralization of dentin has also been observed when MTA was applied into carious lesions. To date, MTA has been known as a bioactive material generally used in endodontics (94).

- ***Interaction between MTA & dentin***

MTA can form an apatite-like layer, a characteristic of calcium silicate material, on the surface when contacted with physiologic fluids or simulated body fluids (STF) (83, 95). Sarkar et al. (96) reported that when MTA is dissolved in simulated body fluids, calcium is the most dominant ion released, controlling the precipitation of a hydroxyapatite layer on the MTA surface. The precipitation of hydroxyapatite continuously progresses in the porous MTA and later starts to change its composition at the dentin wall. Initially, hydroxyapatite crystals create a mechanical bond between the MTA and dentin surface. Then, the interaction of an apatite layer and dentin, creates a chemical bond between them. Both actions generate a good seal of MTA to dentin (96). Also, scanning electron microscope (SEM) pictures at the interfacial layer revealed dentin bridge, osteodentin and reparative dentin formation, indicating that this material has mineralization potential (Figure 2.7) (96-98).

Moreover, tag-like structures and lateral branch formations of apatite crystals into the dentin have been observed when using dentin discs (Figure 2.8) (83). The study commented that the initial precipitation of amorphous calcium phosphate was a precursor for apatite crystal formation and deposition. Therefore, the ability of precipitation to form interfacial layers and tag-like structures may influence the sealing ability and push-out bond strength of MTA (83).

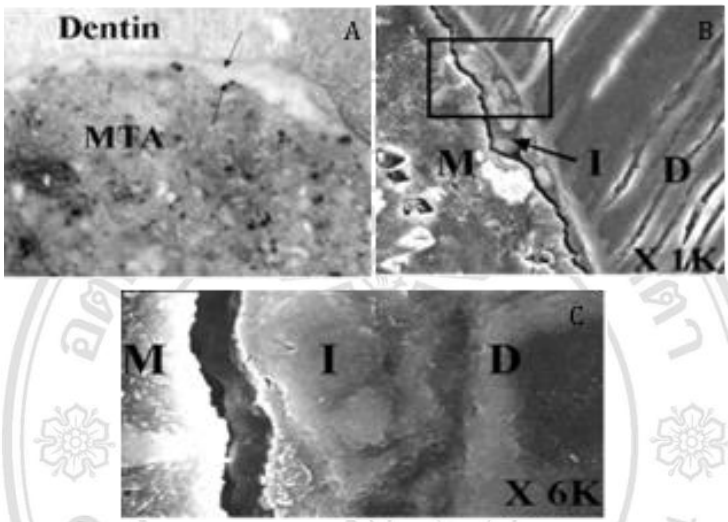


Figure 2.7 Interaction between MTA and dentin. MTA (M), Interface (I), dentin (D) (97)

A) Optical micrograph of MTA-dentin cross-section (x200) showing white interfacial layer sandwiched between MTA and the dentin wall.

B) Scanning electron micrograph of MTA-dentin cross-section (x1000)

C) Area identified by box (x6000)

Figure7B and 7C: Interfacial layer firmly attach to the dentin wall.

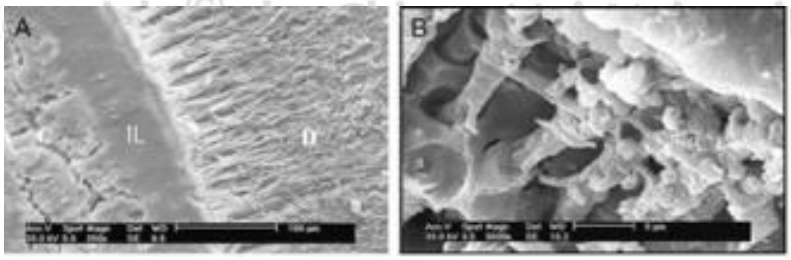


Figure 2.8 Tag-like structures and lateral branch formations into the dentin after treated with ProRoot® MTA

A) Photomicrograph of ProRoot® MTA–dentin interface showing cement (C), interlayer (IL) and dentin (D)

B) Higher magnification showing tag-like structures and lateral branches

- ***Interaction between MTA & vital tissue***

Currently, MTA is recommended as a pulp-capping material because of its biocompatibility and reliability in dentin bridge formation (95, 99, 100). Nair et al. (100) demonstrated a compact dentin-like hard tissue bridge covering the dental pulp of intact teeth after the teeth were capped with MTA for one week. The length and thickness of the hard tissue barrier increased continuously during their three-month experiment (Figure 2.9). Moreover, when compared to calcium hydroxide, a traditional medicament in vital pulp therapy, it has been regularly reported that MTA showed increased reliability and quality of the dentin bridge formation (101).

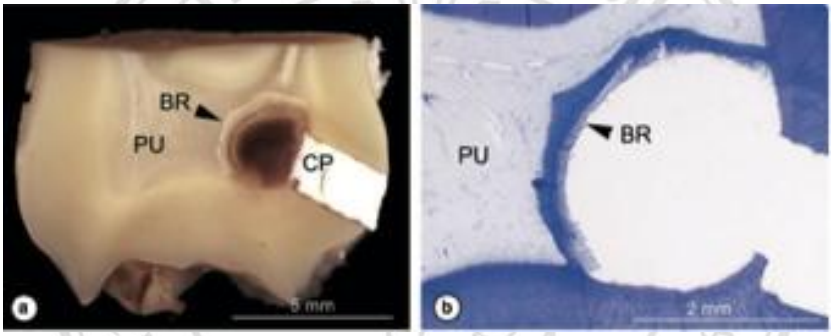


Figure 2.9 Pulpal response to MTA capping after three month's observation.

a) The mesial half of a maxillary left third molar (tooth 28) shows capping material (CP) and hard tissue bridge (BR) opposite the exposed pulp (PU)
b) Histological section of the specimen

Many studies have examined the actions of MTA after direct contact with human tissues, such as dental pulp. The actions can be sequenced as follows (102):

- Calcium hydroxide formation and Ca ion release, which induce cell attachment and proliferation
- Alkalinity of the material, creating antibacterial environment
- Cytokines in the tissue are regulated, activating the differentiation and migration of hard-tissue-producing cells
- Hydroxyapatite formation, creating a biological seal

- ***Interaction of MTA to dentin and vital tissue for releasing TGF-ß1***

MTA can stimulate either dentinal matrix or pulp cells to release TGF-ß1 (30, 35).

Tomson et al. (30) demonstrated significantly greater TGF-ß1 release in dentin matrix when WMTA solution was used to dissolve dentin powder than when GMTA solution was used. TGF-ß1 release in human gingival fibroblast cells is greater after 72 hours of contact with ProRoot® MTA than when MTA is not used (103). Even though MTA has shown to have many advantages, several limitations have also been observed, including tooth discoloration resulting from the interaction between MTA and collagen (104), and long setting time. Therefore, other innovation bioactive materials have been continuously developed.

2.2.2 Biodentine™

2.2.2.1 Composition

Biodentine™, a tricalcium-silicate-based material, was developed as a "dentin replacement" material (Septodont, http://www.septodontusa.com/). This material was established because of some disadvantages of MTA, for example tooth discoloration and difficulty in handling. Currently, Biodentine™ is used in various clinical applications such as repairing root perforations, creating an apical barrier for apexification, treating root resorption, retrograde filling, pulp capping, and also dentin replacement (105).

Biodentine™ is presented as a powder and a liquid. The components of the powder are tricalcium silicate (main component), dicalcium silicate, calcium carbonate, calcium oxide, iron oxide and zirconium oxide. The liquid is an aqueous solution of a hydrosoluble polymer (water reducing agent) with calcium chloride (setting accelerator) (Table 2.3). The powder is enclosed in a capsule and is mixed with five drops of liquid and vibrated for 30 seconds at 4000 rpm leading to the formation of paste-liked material (106).

2.2.2.2 Setting reaction

The setting reaction of the material is a hydrating reaction, which produces a hydrated calcium silicate gel (CSH gel) and calcium hydroxide ($Ca(OH)_2$). This precipitates on the surface of remaining silicate particles and in the pores of the powder, leading to a significant decrease in the material's porosity which would increase its compressive strength over time (106, 107).

$$2(3CaO.SiO_2) + 6H_2O \longrightarrow 3CaO.2SiO_2.3H_2O + 3Ca(OH)_2$$

The initial setting time of Biodentine™, according to manufacturer, is 9–12 minutes (106), whereas the final setting time is 45 minutes (108). Biodentine™ has the shortest setting kinetics among tricalcium silicate cements (ProRoot® MTA, MTA Angelus® etc.) because of the addition of calcium chloride to the liquid component (108-110).

Table 2.3 The components of Biodentine™

Powder	
Tri-calcium silicate (C3S)	Main core material
Di-calcium silicate (C2S)	Second core material
Calcium carbonate and calcium oxide	Filler
Iron oxide	Shade
Zirconium oxide	Radiopacifier
Liquid	
Calcium chloride	Accelerator
Hydrosoluble polymer	Water reducing agent

2.2.2.3 Physical and mechanical properties

The manufacturer claims that Biodentine™ has similar compressive strength and surface hardness to natural dentin (297 MPa, and 60-90 HVN respectively) (106). Therefore, it has been recommended as a dentin substitute (111). Several studies have shown that Biodentine™ has superior mechanical properties, in terms of compressive strength, push-out bond strength and microhardness, to other tricalcium silicate cements, such as mineral trioxide aggregate (MTA) and Bioaggregate™ (108, 109, 112). Moreover, blood contamination, and also various endodontic irrigants have no negative effects on the strength or setting time of Biodentine™ (109, 113). The studies suggested that the small particle size and uniformity of Biodentine™ components can reduce the porosity and solubility of the setting materials (114, 115).

2.2.2.4 Biological Properties

a) Biocompatibility

Biodentine™ is known as a biocompatible material. Several studies have

compared its cytotoxicity with that of MTA, using a variety of cell types, for example, human pulp fibroblasts, human gingival fibroblasts, osteoblast-like cells, periodontal ligament cells, DPSCs and SCAPs (59, 113, 116, 117). Both materials seemed to have slight cytoxicity (59, 113, 116, 117). However, some studies reported greater cell viability after contact with Biodentine™ than with MTA, especially at the early stages of contact (approximately four to seven days) (113, 117). An interesting study by Peters et al. (59), evaluated the effects of Biodentine™ and ProRoot® MTA on the viability of SCAPs, observing that the viability of SCAPs, either grown in direct or indirect contact with both materials, was significantly greater on the first day of their experiment than when SCAPs were grown with no contact with either material. However, when measured on the third and seventh days, there was no significant difference in the viability of SCAPs, regardless of contact with the materials.

The concentration of Biodentine™ has also been evaluated, in term of whether it has some impacts on proliferation, migration or adhesion of human dental pulp stem cells (60). The result showed that Biodentine™, when used at 0.2 and 2 mg/ml can promote stem cell proliferation. The 0.2 mg/ml concentration showed the greater cell migration and adhesion abilities. In contrast, a high concentration, Biodentine™ at 20 mg/ml, decreased cell proliferation (60).

b) Bioactivity

When Biodentine™ is mixed with water, calcium silicate hydrate gel and calcium hydroxide are produced, releasing the Ca ions. Then, after contact with physiological solutions, a layer of hydroxyapatite forms (118). Ca and Si have observed being incorporated into human root dentin (119).

• Interaction between Biodentine™ & dentin

Biodentine™ is rich in calcium compounds and calcium ion concentration, having roles in hard-tissue formation (118). Atmeh et al. (120) found tag-like structures in the dentinal tubules, just beneath the interface between dentin and Biodentine™, called the "mineral infiltration zone (MIZ)". The forming of this layer is suspected to result from the accumulation of by-products after the chemical setting reaction of Biodentine™, followed by mineral diffusion. Han and Okiji (119) also demonstrated that the

precipitates are composed mainly of calcium, phosphorus, carbon, oxygen, and silicon. In that study, various materials, including WMTA and BC sealer, were compared with Biodentine™. The results showed that the concentration of Ca ions, incorporated in root canal dentin, progressively increased when Biodentine™ was used. Moreover, the depth of incorporation of the Ca and Si in Biodentine™ into human root canal dentin was also significantly higher than that in WMTA and BC sealer, confirming the bioactivity of these materials (119). Furthermore, the high alkalinity of calcium hydroxide, produced during the chemical reaction, can degrade the organic collagen component of the interfacial dentin. This degradation increases the permeation of high concentrations of calcium, hydroxyl and carbonate ions into the interfacial dentin, leading to its increased mineralization (120).

- ***Interaction between Biodentine™ & vital tissue***

Biodentine™ has been used, as an alternative material to MTA, in vital pulp therapy because various studies have demonstrated that it causes little inflammation to the pulp tissue and is able to induce homogenous reparative dentin, similar to the effects of MTA (38, 121-123). Moreover, when Biodentine™ and WMTA were tested in pulpotomized primary pig teeth, similar results were observed with both materials in terms of inflammatory response and hard tissue formation after 90 days of treatment (121). Later, biomineralization properties of Biodentine™ have been evaluated in various cell types. Laurent et al. (35) have revealed that Biodentine™ can induce the formation of mineralized foci and odontoblast-like cell differentiation in human dental pulp cells. These effects may be associated with an increase in TGF-ß1 secretion by human pulp cells following contact with the material.

- ***Interaction of Biodentine™ to dentin and vital tissue for releasing TGF-ß1***

Various types of pulp capping materials, for example, MTA and calcium hydroxide, have been shown to solubilize TGF-ß1 from the dentin (28, 30). To date, no studies have investigated this finding in relation to Biodentine™. Most current studies have reported results when these materials were in contact with different cell types (35, 116, 124). Laurent et al. (35) have reported that Biodentine™ and MTA have the same

potential in stimulating TGF- ß1 secretion from dental pulp cells. Moreover, Nikfarjam et al. (124) have shown that, when 0.42 mg and 0.70 mg of Biodentine™ suspensions were used to culture pulp fibroblasts for four days, the release of TGF-ß1 from the dental pulp fibroblasts was reduced on the first experimental day, whereas on the second day, the quantity of TGF-ß1 returned to normal. That study suggested that Biodentine™ suspensions had no negative influences on cell morphology, proliferation or cell integrity, such suspensions may improve the healing of pulp.

Mullaguri et al. (125) have shown that TGF- ß1 release in platelet-rich fibrin (PRF) is 1.6 times greater when Biodentine™ is used to cover the PRF than when glass inomer cement (GIC) or intermediate restorative material (IRM) is used. The fibrin structure is organized in parallel strands when Biodentine™ is used to cover the PRF. In contrast, when GIC or IRM are used, the fibrin collapses and becomes disorganized (125).

To date, MTA has been the material of choice for regenerative endodontic procedures because of its properties, such as biocompatibility and bioactivity. However, some limitations have been observed, such as tooth discoloration and difficulty in material manipulation. Therefore, an innovation bioactive material, Biodentine™, has been popularized in endodontics, because it has impressive properties similar to those of MTA, while several drawbacks have been eliminated. Even though both MTA and Biodentine™ are bioactive materials, most of the published scientific evidence is mainly focused on their mechanical, rather than their biological, properties. Therefore, more research supporting this biological field, especially in regeneration, is required.

In REPs, these materials are currently used as coronal barriers in the final step of the treatment procedure. However, the advantages of their use have never been evaluated. In this study, it is hypothesized that these materials should offer some positive effects, for example the stimulation of growth to cell/tissue generation during REPs.

CHAPTER 3

Materials and Methods

3.1 Sample preparation

Tooth preparation

135 intact, single-rooted, human premolars from healthy patients aged 15-25 years that were extracted for orthodontic purposes were included. The teeth were stored in 0.5% chloramine-T (Chloramine T Trihydrate, A.R.; HiMedia Laboratories Pvt., Mumbai, India). The crowns of the teeth were removed 1 mm above the cemento-enamel juction (CEJ) and the apices of the teeth also were removed to provide root segments 10 mm in length. The periodontal ligament was removed using a scalpel blade. After that, the root segments were instrumented using Mtwo® rotary files (VDW, Munich, Germany) Nos. 10-25, followed by Peeso reamers up to size 3 in order to create tooth models with large root canals. All canals were irrigated with normal saline during preparation. To remove the smear layer, all prepared samples were immersed in 17% EDTA for one minute and 1.25% NaOCl for one minute. All samples were finally ultrasonicated with phosphate-buffered saline (PBS) for one minute. The ultrasonication was performed three times.

In order to imitate the exact clinical procedure, all root segments were medicated with 1000 mg/mL calcium hydroxide paste (Univar; Ajax Finechem, Sydney, Australia) for one week and kept in a 37°C humidified atmosphere of 95% air and 5% CO_2 before the experiment. Then, all samples were rinsed with 5 mL PBS to remove medicament and irrigated with 17% EDTA using passive ultrasonic irrigation for five minutes. A 30-gauge side-vented needle (Max-I-Probe; Dentsply Maillefer, Ballaigues, Switzerland) with a 1 mL tuberculin syringe was used to deliver the irrigant. After the canal was filled with irrigant, a No. 20 Irrisafe® ultrasonic tip (Acteon, Merignac, France) connected to a piezo-electric ultrasonic unit (P5 Newtron; Acteon) was inserted through the canal 1 mm short of the root apex and activated at the unit's power four setting for

five minutes. Then, the tooth segments were split longitudinally and kept in pairs for later use.

3.2 Part 1: The effects of ProRoot® MTA and Biodentine™ on TGF- ß1 release from dentin

Eighteen root segments were covered with nail varnish at the outer surfaces and randomly divided into three groups as shown in Table 3.1. ProRoot® MTA (Dentsply Tulsa Dental Specialties, Memphis, TN, USA) and Biodentine™ (Septodont, Saint-Maur-des-Fossés, France) were mixed according to the manufacturer's instructions. Then, one of the materials was placed into the coronal 3 mm of the root canal. The samples were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for four hours. After the initial setting, all root segments were placed in 24-well polystyrene plates containing 500 µl of PBS, and stored at 4°C. Then, 200 µl of solution was collected from each well on day 14, and kept at -80°C until used for evaluation. The concentration of TGF-ß1 was quantified using the sandwich enzyme-linked immunosorbent assay (ELISA) test system (Human TGF-ß1 Quantikine ELISA Kit, R&D Systems™, Wiesbaden, Germany) following the manufacturer's protocol provided. The results were calculated and statistically analyzed using the Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test.

Table 3.1 Experimental design, Part 1

Group	Materials
1 (Control)	Prepared root segments without materials
2	Prepared root segments with ProRoot® MTA
3	Prepared root segments with Biodentine™

3.3 Part 2: The effect of ProRoot® MTA and Biodentine™ on the mineralization potential of human APCs

Preparation and culture of primary human APCs

Three patients aged 16-20 years without any underlying disease, undergoing removal of impacted third molars at the Oral and Maxillofacial Surgery Clinic, Faculty

of Dentistry, Chiang Mai University were recruited and informed regarding the research project. Apical papilla cells were isolated from apical papilla tissues of non-carious immature mandibular third molars. After the patients or guardians consented to the experiment and following the extractions, the crowns and roots were rinsed with sterile saline, the apical papilla tissues were gently detached from the root apex, and immediately stored in alpha modification of Eagle's medium transport medium (Sigma-Aldrich, St Louis, MO, USA). The tissues were digested using a combination of Collagenase I (Gibco/Invitrogen, Gaithersburg, MD, USA) and Dispase II (Sigma-Aldrich), incubated in a 37°C humidified atmosphere of 95% air and 5% CO_2 for 45 minutes. After that, the cell suspension was centrifuged at 1500 rpm at 25^{o}C for five minutes and then cultured in complete alpha-minimum essential medium (α-MEM) (Sigma-Aldrich) containing 10% fetal bovine serum (Gibco/Invitrogen), 1% penicillin-streptomycin (Sigma-Aldrich), and 100 mol/L L-ascorbic acid (Sigma-Aldrich). The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 with the medium changed every three days. Cells at the second to third passages were used. Then, the cells were sub-cultured with 0.25% trypsin-EDTA (Gibco/Invitrogen) (Figure 3.1).

Dentinogenic/Osteogenic Differentiation Capacity

The tooth samples in this part of experimental were prepared using the same protocol as in the first part. However, the tooth samples were initially sterilized using gamma-radiation at 25 kGy. All technical steps were accomplished using a strict aseptic technique.

APCs, seeded into 24-well plates at 5,000 cells/well. When reaching 70% confluence, either tooth samples or material alone were placed together with the differentiation medium containing complete α-MEM, 50 µg/mL ascorbic acid (Sigma-Aldrich), 10 nmol/mL dexamethasone (Sigma-Aldrich), and 10 mmol/mL ß-glycerophosphate (Sigma-Aldrich). Then, cells were cultivated for 14 and 21 days in a humidified atmosphere of 95% air and 5% CO_2 with the medium changed every three days. The cells were monitored every day under an inverted-light microscope (DP12; OLYMPUS, Melville, NY). The experimental design was divided into seven groups which are describes in Table 3.2.

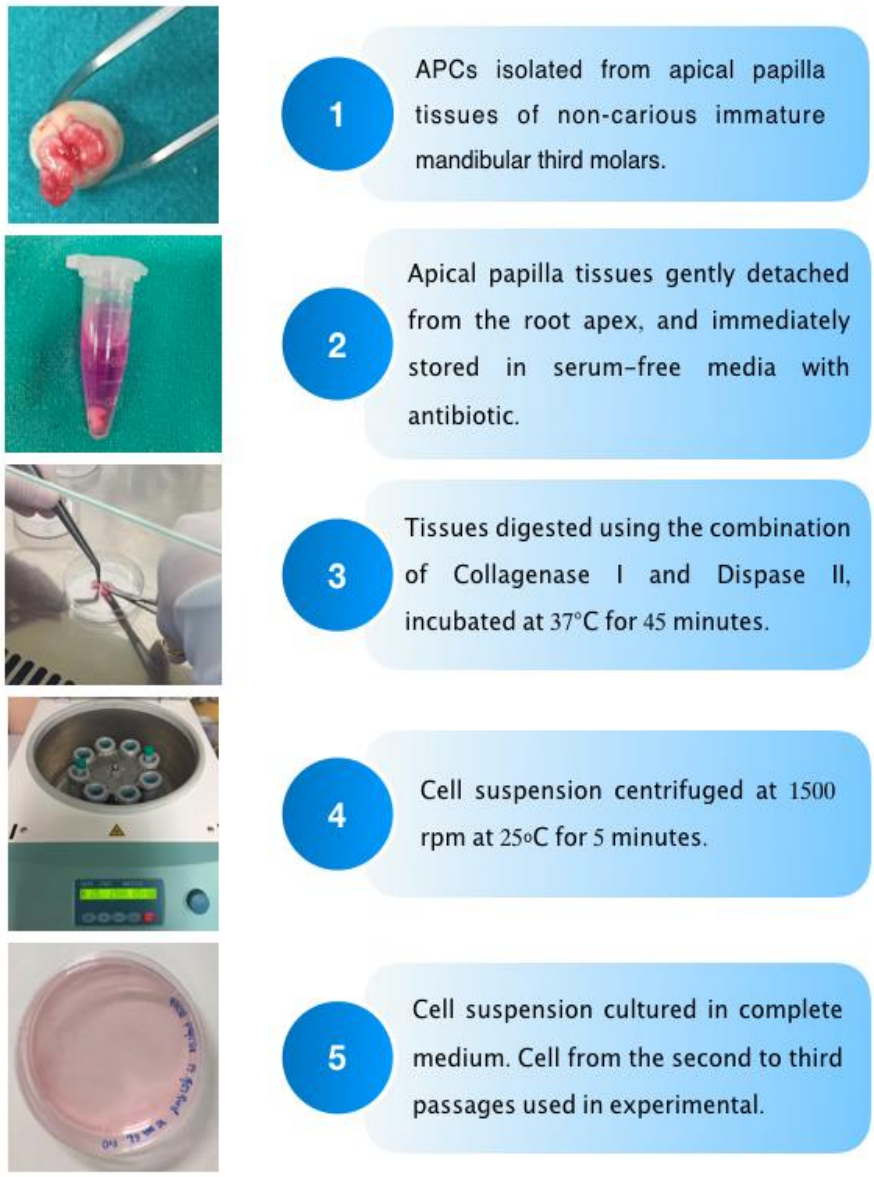


Figure 3.1 Preparation and culture of primary human APCs

Alizarin Red S Staining and Quantification Assay

Alizarin red S dye was used to identify calcium deposits in the cell culture. Experimental groups and control groups in dentinogenic differentiation medium were investigated at days 14 and 21. First, all tooth samples were washed gently three times with PBS before fixing with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Then, they were rinsed with 1 mL PBS/well, and 0.5 mL alizarin red S solution at pH of 4.2 (Sigma-Aldrich) was added into each well. The plates were

incubated at room temperature for five minutes with gentle shaking. After that, the excessive dye was thoroughly washed from the wells with deionized water. The APCs in all groups were visualised under an inverted-light microscope (OLYMPUS) and left to air-dry for one week. Subsequently, the amount of calcium deposit was quantitatively analyzed by destaining with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) in 10 mmol/L sodium phosphate at room temperature for 20 minutes. One hundred microliters of de-stained solution were transferred into 96-well plates, and measured spectrophotometrically at 550 nm. Then, the data were calculated and statistically analyzed (Figure 3.2).

Table 3.2 Experimental design, Part 2

Group	Materials	n
1	Control: APCs cultured in regular medium	6
2	APC+diff: APCs cultured in differentiation medium	6
3	APC+diff+MTA: APCs cultured in differentiation medium in the presence of ProRoot® MTA	6
4	APC+diff+BD: APCs cultured in differentiation medium in the presence of Biodentine™	6
5	APC+diff+tooth: APCs cultured in differentiation medium in the presence of root segments	6
6	APC+diff+tooth+MTA: APCs cultured in differentiation medium in the presence of root segments filled with ProRoot® MTA	6
7	APC+diff+tooth+BD: APCs cultured in differentiation medium in the presence of root segments filled with Biodentine™	6

* In all groups using root segments, the tooth (with/without material) was attached to the cell culture well, using a sterile orthodontic wire.

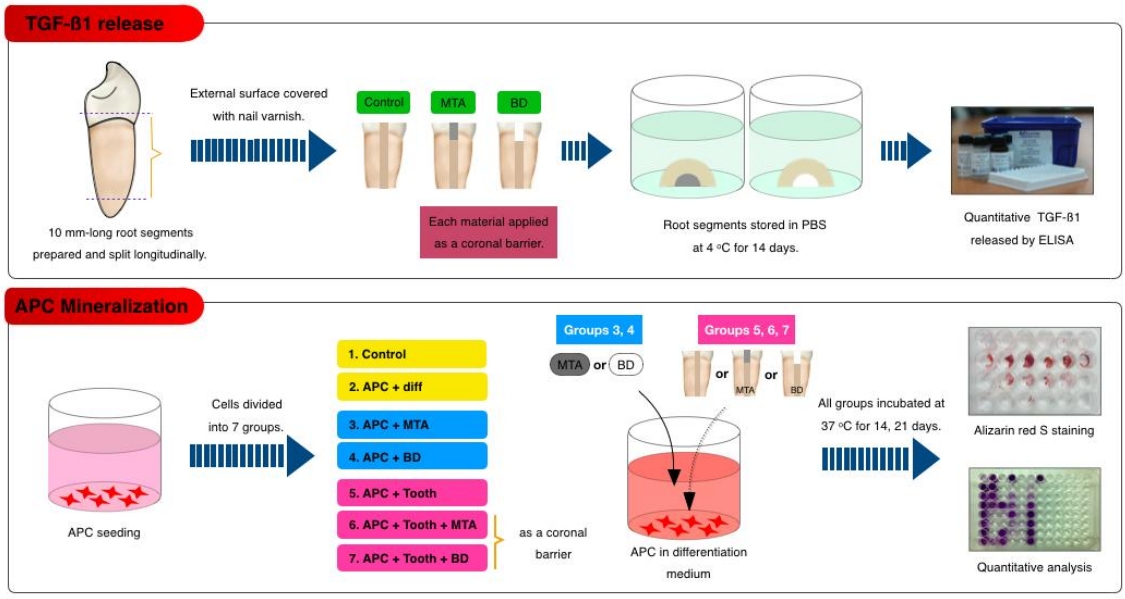


Figure 3.2 Schematic diagram demonstrating the experimental procedures. After the root segments were medicated and irrigated, the prepared root segments were used for the detection of TGF-ß1 release and for the investigation of APC mineralization.

3.4 Data Analysis

All experiments were conducted in triplicate and statistically analyzed using the Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test. The difference between experimental groups was considered to be statistically significant at $P < .05$.

CHAPTER 4

Results

4.1 TGF- ß1 release from dentin

Over 14 days, the concentration of TGF-ß1 released from dentin was greatest (1.35 ± 0.21 ng/ml) in the group in which Biodentine™ was used as a coronal barrier. The concentration of TGF-ß1 detected in the Biodentine™ group was significantly greater than that in either the control group (empty tooth) or the ProRoot® MTA group ($P < .05$). When compared to the control group (0.02 ± 0.046 ng/ml), the concentration of TGF-ß1 observed in the ProRoot® MTA group (0.07 ± 0.11 ng/ml) was not significantly different ($P >. 05$) (Figure 4.1).

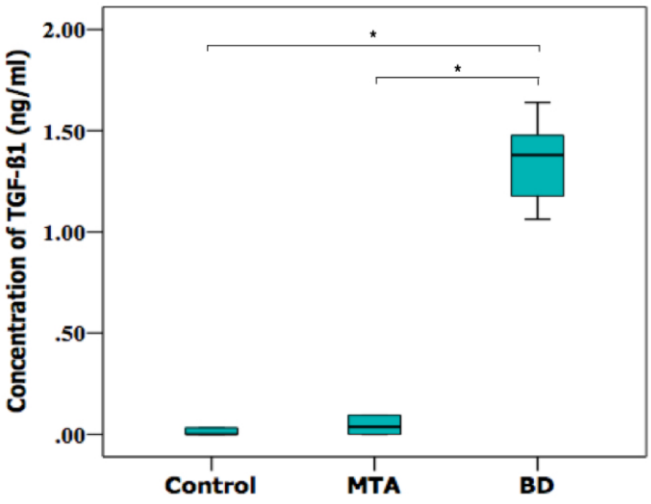


Figure 4.1 Effect of ProRoot® MTA and Biodentine™ when used as a coronal barrier on TGF-ß1 released from root canal dentin over 14 days. The greatest concentration of TGF-ß1 was detected in the Biodentine™ group (*$P < .05$).

4.2 Mineralization potential of human APCs

4.2.1 Primary human APCs culture

During the first day, most of the cells had a round cellular morphology. The cells started to attach to the cell culture plate (Figure 4.2*A*). After four days, cells began to form scattered clusters containing small spindle-shaped cells (Figure 4.2*B*). The cell

clusters expanded continuously and connected to each other. Around day seven, cells had a fibroblastic-like morphology, showing elongated bipolar or multipolar processes (Figure4.2*C*). After 10 days, the density of the cells reached about 50% confluence. Cells attached to the culture well in a monolayer (Figure 4.2*D*). Approximately two weeks after cell seeding, cells density reached up to 70% confluence. The cells appeared to be fibroblastic (Figure 4.2*E*).

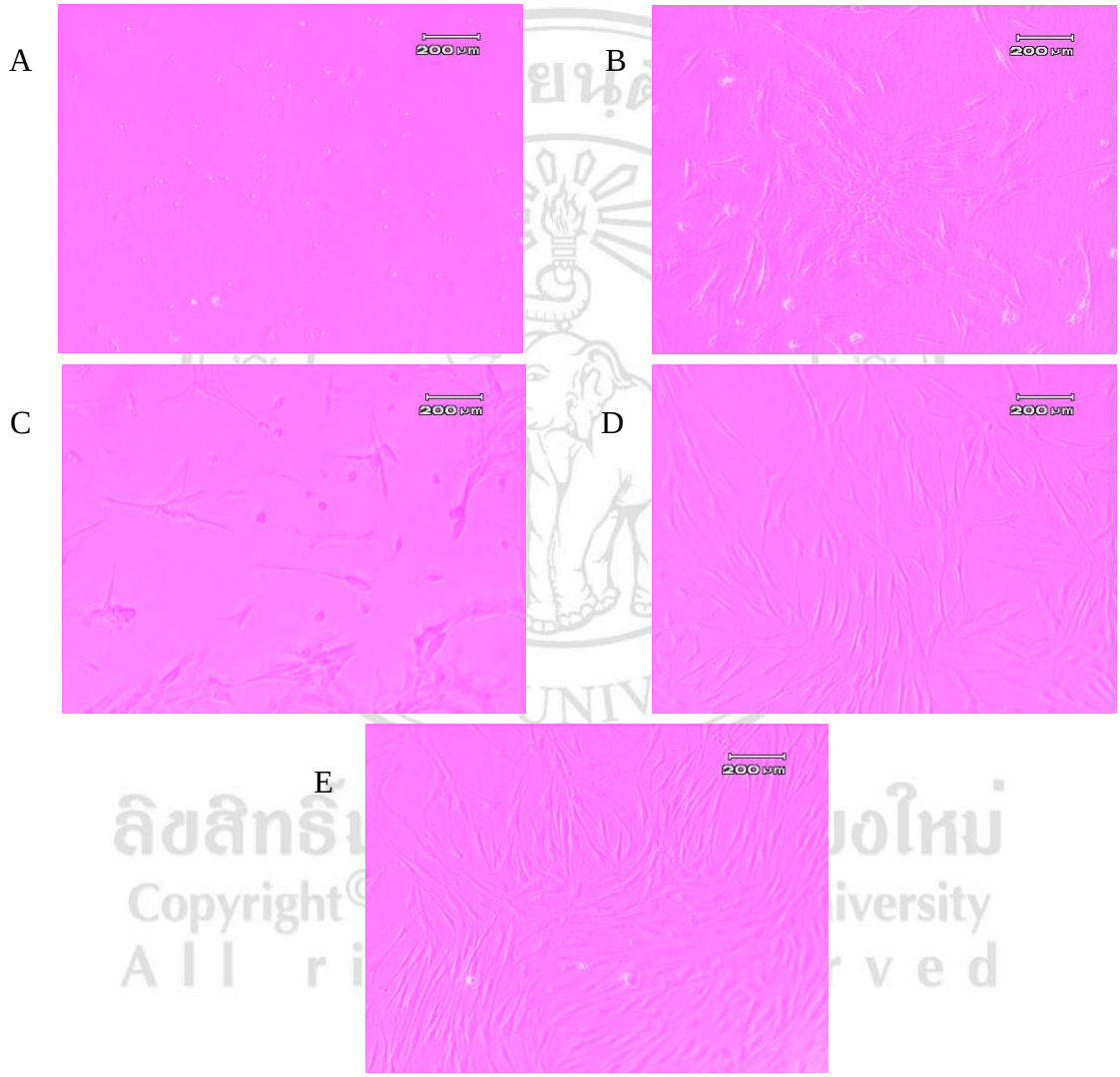
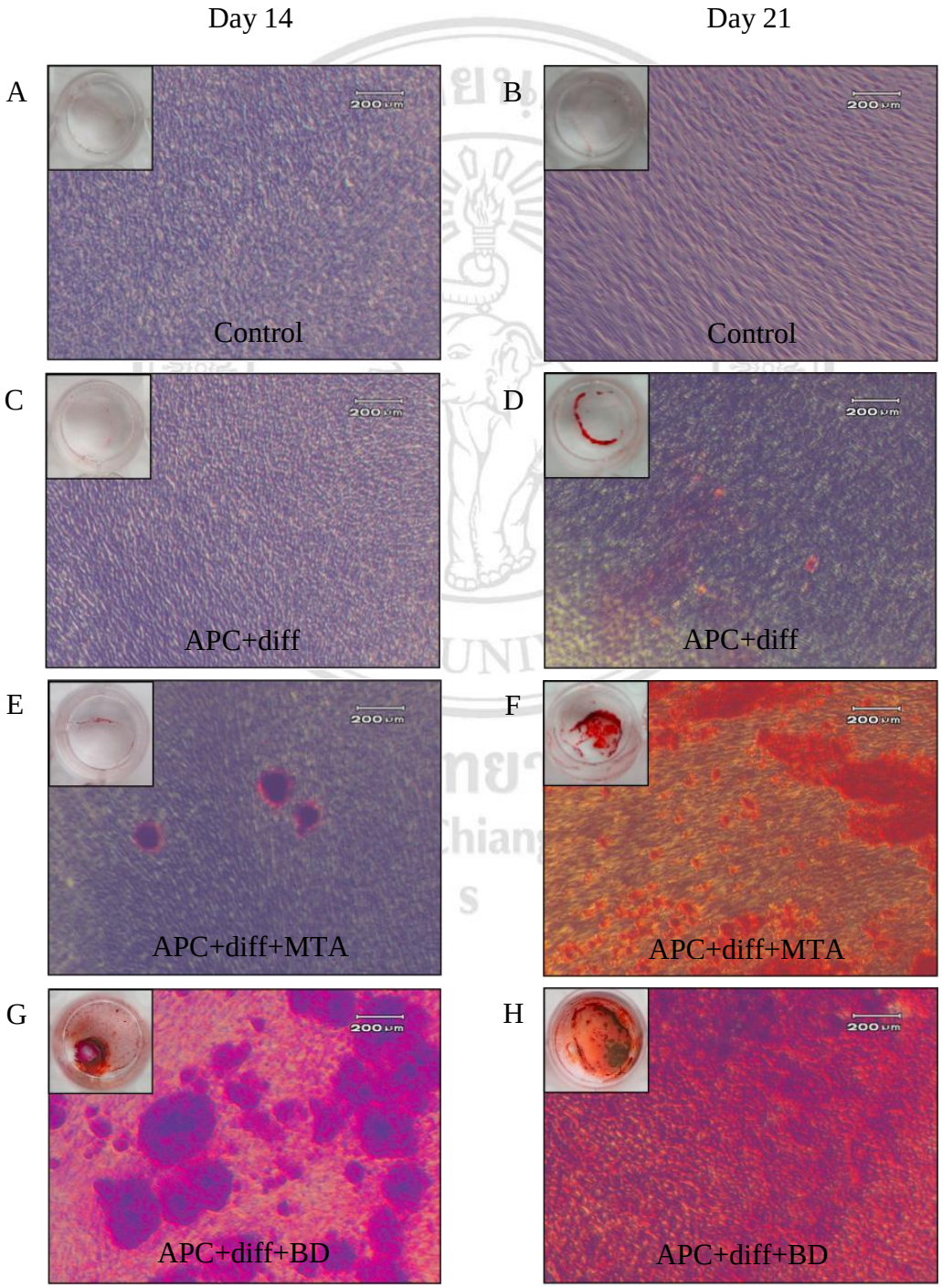


Figure 4.2 Inverted-microscope images showing primary APC culture from day 1 to day 14 at 4x magnification. (*A*) Round cellular morphology appeared on day 1. (*B*) Cells began to form scattered clusters on day 4 and, (*C*) continuously elongated bipolar or multipolar processes around day 7. (*D*) Cell density reached about 50% confluence after 10 days, and (*E*) subsequently reached 70% after two weeks.

4.2.2 Dentinogenic/Osteogenic Differentiation Capacity

4.2.2.1 Alizarin Red S Staining

The findings from Alizarin Red S staining revealed mineralized nodule formation in all experimental groups on both days 14 and 21, indicated by detection of pinkish-red clusters (Figure 4.3).



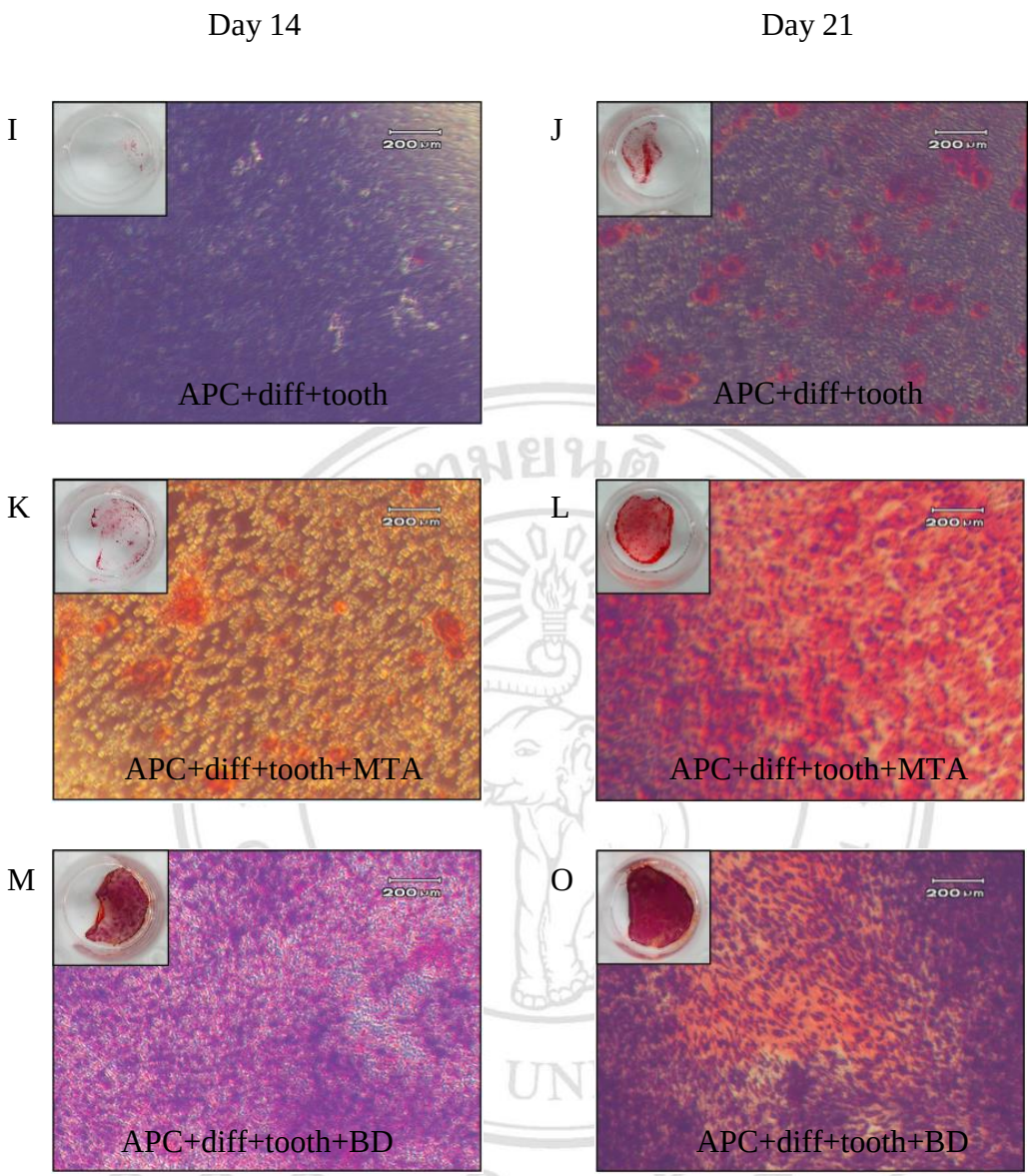
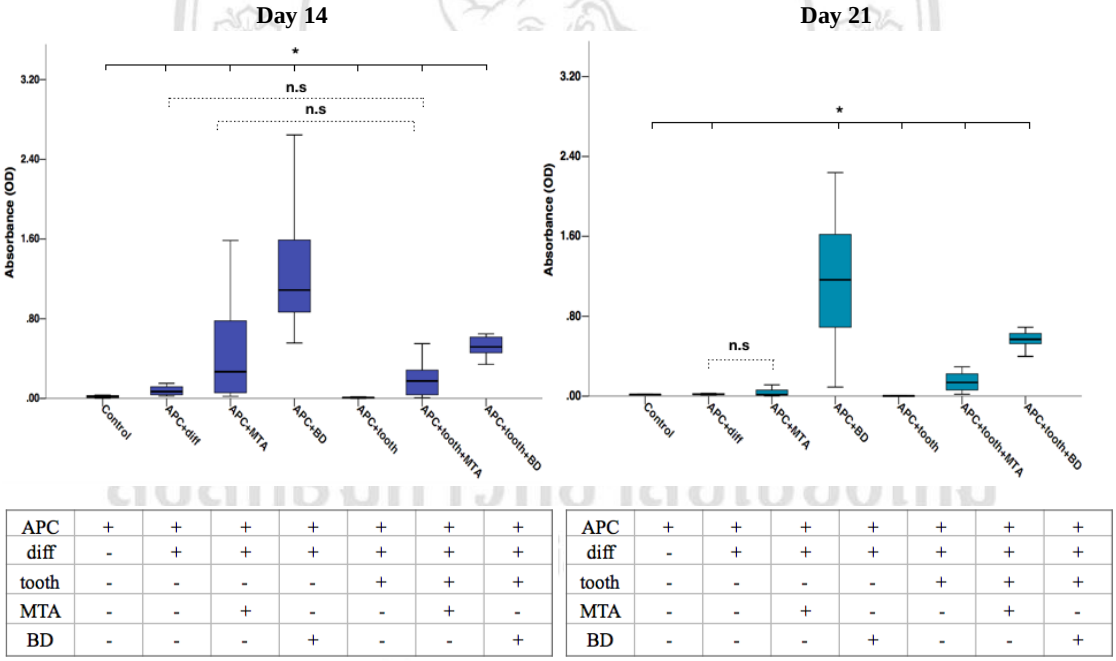


Figure 4.3 Inverted microscope images showing APCs with mineralized nodules stained with alizarin red S at 4x magnification and insert photographs representing all experimental groups. Left and right columns represent results on days 14 and 21, respectively. *(A, B) Control group*: no mineralization was observed at either time point. (*C, D*) *APC+diff*: small mineralization nodules were detected. *(E, F) APC+diff+MTA*: mineralized nodules were observed in greater amounts than in the control group and APC+diff group. (*G, H*) *APC+diff+BD*: the greatest mineralized nodules formation was observed. *(I, J) APC+diff+tooth*: mineralized formation was obvious only on day 21. *(K, L) APC+diff+tooth+MTA* and *(M, N) APC+diff+tooth+BD*: mineralized nodules were noticed to a greater extent than in the group using tooth without materials.

4.2.2.2 Quantitative analysis

When quantitatively analyzed, the amount of calcium deposits was highest in the APC+diff+BD groups, at both time-points. When considering the use of Biodentine™ as a coronal barrier (APC+diff+tooth+BD), mineralization was detected, but in significantly smaller amounts than in the group in which Biodentine™ was used alone (APC+diff+BD), at both time periods ($P < .05$). In the APC+diff+MTA group, mineralization was significantly greater than in the control group, only on day 21 ($P < .05$). When ProRoot®MTA was applied as a coronal barrier (APC+diff+tooth+MTA), mineralization was significantly greater than in the control group at both time points ($P < .05$). Considering the mineralization capacity between the two materials, Biodentine™, either used alone or as a coronal barrier, had better mineralization potential than did ProRoot®MTA at both time points (Figure 4.4*A*, *B*).



APC	+	+	+	+	+	+	+
diff	-	+	+	+	+	+	+
tooth	-	-	-	-	+	+	+
MTA	-	-	+	-	-	+	-
BD	-	-	-	+	-	-	+

APC	+	+	+	+	+	+	+
diff	-	+	+	+	+	+	+
tooth	-	-	-	-	+	+	+
MTA	-	-	+	-	-	+	-
BD	-	-	-	+	-	-	+

Figure 4.4 Box plots depicting the quantitative analysis of the calcium deposits among groups. Mineralization was significantly increased when Biodentine™ was used, either alone or as a coronal barrier, on both days 14 (*A*) and 21 (*B*). However, the mineralization effect was reduced, especially in the Biodentine™ group, when used as a coronal barrier compared to the materials alone. *****Significant difference compared to the control group ($P<.05$). ns = no statistical differences.

CHAPTER 5

Discussion

This study investigated the effect of bioactive materials, when applied as a coronal barrier, on growth factor release from root dentin, and on the mineralization of APCs. The results demonstrate that Biodentine™ stimulated the release of TGF-ß1 in a greater concentration than did ProRoot® MTA. Moreover, Biodentine™ used either alone or as a coronal barrier produced significantly greater mineralization than did ProRoot® MTA.

Currently, regenerative endodontics is a common procedure, applying the tissue engineering concept, for treating necrotic immature permanent teeth with apical periodontitis. However, the constant observation of intracanal calcification after REPs has drawn some attentions, since it may affect long-term outcome, and also upcoming treatments (19, 20). Various factors have been hypothesized to play roles in the intracanal calcification process. Among those, the use of bioactive materials, which have the potential to stimulate excessive mineralization, is a concern, since one cannot be sure, whether these materials would exert positive or negative effects on the long-term clinical outcome. Therefore, it is essential to examine the basic role of these currently used bioactive materials in mineralization.

This study shows that Biodentine™ has significant potential to stimulate TGF- ß1 release from the root dentin. The detection of high levels of TGF- ß1 can be explained by the concept that the alkaline property of the studied materials helps to solubilize non-collagenous dentin matrix components (28). Although, both ProRoot® MTA and Biodentine™ exhibited high pH, a recent study has shown that the pH of Biodentine™ was highest on day 14, whereas the pH of ProRoot® MTA progressively decreased over time (126).

Considering the effect of these materials on mineralization, this study shows that Biodentine™ has greater mineralization potential than does ProRoot® MTA. Our findings

are comparable to those of previous studies, which have reported that Biodentine™ promotes greater odontoblastic differentiation showing higher expression of ALP, DSPP and DMP-1 genes than does ProRoot® MTA on days 14 and 21 (127, 128). One of the reasons for the greater mineralization potential might have been the greater concentration of TGF-ß1 released from dentin when Biodentine™ was used. Moreover, the alkaline environment, created by this material activated a latent form of TGF-ß1 into an active form, which enhanced cell differentiation and mineralization (129). Other studies also have suggested that the increase in mineralization may be associated with the secretion of TGF-ß1 (28, 30, 35).

Recent studies have investigated the pathway of mineralization, focusing on the influx of calcium ions through the MAPK pathway (130, 131). Those studies have revealed that both ProRoot® MTA and Biodentine™ induce cell differentiation and mineralization using this pathway (130, 131). Earlier studies have reported that the numbers of calcium ions released from Biodentine™ are significantly greater than those released from MTA (119, 126, 132). Thus, the greater cell differentiation and mineralization produced by Biodentine™ when compared to ProRoot® MTA can also be explained by the larger amount of calcium ions released from these materials. Moreover, some histological studies have shown hard tissue formation and distribution of mineralized nodules when Biodentine™ was used as a pulp capping material (121, 133).

To date, there have been no scientific studies examining the effect of these materials when directly applied to the tooth. Most of the studies investigated the effect of materials when used alone as set materials or as solutions (131, 134, 135). Therefore, since these materials are commonly used in contact with the tooth, the potential influence of tooth dentin on these materials is of interest. In this study, either Biodentine™ or ProRoot® MTA was applied to the coronal part of the root canal, mimicking the final step of REP, in which a capping material is used to seal the root canal in the regenerative phase. Then, the mineralization potential of these materials, when used as a coronal barrier, was compared to that in the groups in which the material was used alone and the control group, in which no barrier material was used. The results from the second part of the experiment revealed that cell mineralization was detected in the groups in which the materials were used as a coronal barrier, but in smaller amounts

than in the groups in which the materials were used alone. However, a significant difference was observed only with Biodentine™, at both time periods ($P < .05$). These findings may be explained by the effect of dentin buffering capacity, by which protons released from the carboxyl groups of amino acids in the dentin interact with the hydroxyl ions from the materials. Such a buffering effect decreases the pH of the environment and may interrupt the mineralization process (136). However, further studies regarding this buffering effect are required to investigate this idea.

Another association regarding decreased mineralization in teeth to which Biodentine™ is applied is the interaction of calcium ions released from Biodentine™ (120). Previous studies have demonstrated that the hydroxyapatite that forms between the materials, Biodentine™ or MTA, and dentin is enriched with calcium carbonate. This hydroxyapatite formation may impact the release of calcium ions from dentin and may affect mineralization (119). Moreover, the use of Biodentine™ creates longer tag-like hydroxyapatite structures containing more calcium than those observed when MTA was used (119, 120). Therefore, the effect of mineralization of Biodentine™ in the presence of tooth dentin may have been strongly diminished.

Recently, another interesting aspect related to the mineralization and differentiation effects of these bioactive materials has been published (137). The study examined the effect of material position, either direct or indirect contact, on cell differentiation. The results showed that direct contact of cells with the materials promoted greater cell differentiation than did indirect contact. In this study, in the groups in which the materials were used alone, the material was directly applied to the cells, whereas in the groups in which the materials were used as a coronal barrier, the material was attached to the tooth, which was presumably not in direct contact with the cells. Therefore, it can be implied that the greater mineralization observed in the groups in which the materials were used alone was because of the direct contact between the materials and the cells. The findings from this study also assure the important role of material positioning in cell differentiation.

To summarize, it can be speculated that using Biodentine™ would quickly generate mineralization. However, further investigation is required in order to observe, whether or not, these consequences will positively or negatively support the clinical outcome.

CHAPTER 6

Conclusions

Biodentine™, when was used as a coronal barrier, exhibited an increase in TGF-ß1 release from root canal dentin at 14 days, and this increase resulted in greater apical papilla cell differentiation and mineralization at 14 and 21 days than when ProRoot® MTA was used. Further long-term clinical studies are required to evaluate the effect of both materials on hard tissue formation.

REFERENCES

[1] Huang GT. A paradigm shift in endodontic management of immature teeth: conservation of stem cells for regeneration. J Dent. 2008;36(6):379-86.

[2] Ding RY, Cheung GS, Chen J, Yin XZ, Wang QQ, Zhang CF. Pulp revascularization of immature teeth with apical periodontitis: a clinical study. J Endod. 2009;35(5):745-9.

[3] Nosrat A, Seifi A, Asgary S. Regenerative endodontic treatment (revascularization) for necrotic immature permanent molars: a review and report of two cases with a new biomaterial. J Endod. 2011;37(4):562-7.

[4] Geisler TM. Clinical considerations for regenerative endodontic procedures. Dent Clin North Am. 2012;56(3):603-26.

[5] Hargreaves KM, Diogenes A, Teixeira FB. Treatment options: biological basis of regenerative endodontic procedures. J Endod. 2013;39(3 Suppl): S30-43.

[6] Chueh LH, Huang GT. Immature teeth with periradicular periodontitis or abscess undergoing apexogenesis: a paradigm shift. J Endod. 2006;32(12):1205-13.

[7] Petrino JA, Boda KK, Shambarger S, Bowles WR, McClanahan SB. Challenges in regenerative endodontics: a case series. J Endod. 2010;36(3):536-41.

[8] Cehreli ZC, Isbitiren B, Sara S, Erbas G. Regenerative endodontic treatment (revascularization) of immature necrotic molars medicated with calcium hydroxide: a case series. J Endod. 2011;37(9):1327-30.

[9] Jeeruphan T, Jantarat J, Yanpiset K, Suwannapan L, Khewsawai P, Hargreaves KM. Mahidol study 1: comparison of radiographic and survival outcomes of immature teeth treated with either regenerative endodontic or apexification methods: a retrospective study.

J Endod. 2012;38(10):1330-6.

[10] Kahler B, Mistry S, Moule A, Ringsmuth AK, Case P, Thomson A, et al. Revascularization outcomes: a prospective analysis of 16 consecutive cases. J Endod. 2014;40(3):333-8.

[11] Li L, Pan Y, Mei L, Li J. Clinical and Radiographic Outcomes in Immature Permanent Necrotic Evaginated Teeth Treated with Regenerative Endodontic Procedures. J Endod. 2017;43(2):246-51.

[12] Chen MY, Chen KL, Chen CA, Tayebaty F, Rosenberg PA, Lin LM. Responses of immature permanent teeth with infected necrotic pulp tissue and apical periodontitis/abscess to revascularization procedures. Int Endod J. 2012;45(3):294-305.

[13] Saoud TM, Zaazou A, Nabil A, Moussa S, Lin LM, Gibbs JL. Clinical and radiographic outcomes of traumatized immature permanent necrotic teeth after revascularization/revitalization therapy. J Endod. 2014;40(12):1946-52.

[14] Chan EK, Desmeules M, Cielecki M, Dabbagh B, Ferraz Dos Santos B. Longitudinal Cohort Study of Regenerative Endodontic Treatment for Immature Necrotic Permanent Teeth. J Endod. 2017;43(3):395-400.

[15] Silujjai J, Linsuwanont P. Treatment Outcomes of Apexification or Revascularization in Nonvital Immature Permanent Teeth: A Retrospective Study. J Endod. 2017;43(2):238-45.

[16] Wang X, Thibodeau B, Trope M, Lin LM, Huang GT. Histologic characterization of regenerated tissues in canal space after the revitalization/revascularization procedure of immature dog teeth with apical periodontitis. J Endod. 2010;36(1):56-63.

[17] Shimizu E, Jong G, Partridge N, Rosenberg PA, Lin LM. Histologic observation of a human immature permanent tooth with irreversible pulpitis after revascularization/regeneration procedure. J Endod. 2012;38(9):1293-7.

[18] Khoshkhounejad M, Shokouhinejad N, Pirmoazen S. Regenerative Endodontic Treatment: Report of Two Cases with Different Clinical Management and Outcomes. J Dent (Tehran). 2015;12(6):460-8.

[19] Song M, Cao Y, Shin SJ, Shon WJ, Chugal N, Kim RH, et al.

Revascularization-associated Intracanal Calcification: Assessment of Prevalence and Contributing Factors. J Endod. 2017;43(12):2025-33.

[20] Kahler B, Kahler SL, Lin LM. Revascularization-associated Intracanal Calcification: A Case Report with an 8-year Review. J Endod. 2018;44(12):1792-5.

[21] Chuensombat S, Khemaleelakul S, Chattipakorn S, Srisuwan T. Cytotoxic effects and antibacterial efficacy of a 3-antibiotic combination: an in vitro study. J Endod. 2013;39(6):813-9.

[22] Ruparel NB, Teixeira FB, Ferraz CC, Diogenes A. Direct effect of intracanal medicaments on survival of stem cells of the apical papilla. J Endod. 2012;38(10):1372-5.

[23] AAE. AAE Clinical Considerations for a Regenerative Procedure Revised 6-8-16 [Available from:http://www.aae.org/specialty/wp-content/uploads/sites/2/2017/06/currentregenerativeendodonticconsiderations.pdf.

[24] Phumpatrakom P, Srisuwan T. Regenerative capacity of human dental pulp and apical papilla cells after treatment with a 3-antibiotic mixture. J Endod. 2014;40(3):399-405.

[25] Kitikuson P, Srisuwan T. Attachment Ability of Human Apical Papilla Cells to Root Dentin Surfaces Treated with Either 3Mix or Calcium Hydroxide. J Endod. 2016;42(1):89-94.

[26] Galler KM, Buchalla W, Hiller KA, Federlin M, Eidt A, Schiefersteiner M, et al. Influence of root canal disinfectants on growth factor release from dentin. J Endod. 2015;41(3):363-8.

[27] Zeng Q, Nguyen S, Zhang H, Chebrolu HP, Alzebdeh D, Badi MA, et al. Release of Growth Factors into Root Canal by Irrigations in Regenerative Endodontics. J Endod. 2016;42(12):1760-6.

[28] Graham L, Cooper PR, Cassidy N, Nor JE, Sloan AJ, Smith AJ. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. Biomaterials. 2006;27(14):2865-73.

[29] Moodley DS, Peck C, Moodley T, Patel N. Management of necrotic pulp of immature permanent incisor tooth: A regenerative endodontic treatment protocol: case report. S Afr dent j. 2017;72(n.3 Johannesburg

Apr. 2017).

[30] Tomson PL, Grover LM, Lumley PJ, Sloan AJ, Smith AJ, Cooper PR. Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. J Dent. 2007;35(8):636-42.

[31] Smith AJ, Lesot H. Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair? Crit Rev Oral Biol Med. 2001;12(5):425-37.

[32] Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. Arch Oral Biol. 2000;45(11):1013-6.

[33] Melin M, Joffre-Romeas A, Farges JC, Couble ML, Magloire H, Bleicher F. Effects of TGFbeta1 on dental pulp cells in cultured human tooth slices. J Dent Res. 2000;79(9):1689-96.

[34] He H, Yu J, Liu Y, Lu S, Liu H, Shi J, et al. Effects of FGF2 and TGFbeta1 on the differentiation of human dental pulp stem cells in vitro. Cell Biol Int. 2008;32(7):827-34.

[35] Laurent P, Camps J, About I. Biodentine(TM) induces TGF-beta1 release from human pulp cells and early dental pulp mineralization. Int Endod J. 2012;45(5):439-48.

[36] Widbiller M, Eidt A, Hiller KA, Buchalla W, Schmalz G, Galler KM. Ultrasonic activation of irrigants increases growth factor release from human dentine. Clin Oral Investig. 2017;21(3):879-88.

[37] Diogenes A, Ruparel NB. Regenerative Endodontic Procedures: Clinical Outcomes. Dent Clin North Am. 2017;61(1):111-25.

[38] About I. Biodentine: from biochemical and bioactive properties to clinical applications. Giornale Italiano di Endodonzia. 2016;30:81-8.

[39] Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative endodontics: a review of current status and a call for action. J Endod. 2007;33(4):377-90.

[40] Rafter M. Apexification: a review. Dent Traumatol. 2005;21(1):1-8.

[41] Torabinejad M, Chivian N. Clinical applications of mineral trioxide aggregate. J Endod. 1999;25(3):197-205.

[42] Iwaya SI, Ikawa M, Kubota M. Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. Dent

Traumatol. 2001;17(4):185-7.

[43] Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod. 2004;30(4):196-200.

[44] Thibodeau B, Teixeira F, Yamauchi M, Caplan DJ, Trope M. Pulp revascularization of immature dog teeth with apical periodontitis. J Endod. 2007;33(6):680-9.

[45] Berkhoff JA, Chen PB, Teixeira FB, Diogenes A. Evaluation of triple antibiotic paste removal by different irrigation procedures. J Endod. 2014;40(8):1172-7.

[46] Diogenes A, Ruparel NB, Shiloah Y, Hargreaves KM. Regenerative endodontics: A way forward. J Am Dent Assoc. 2016;147(5):372-80.

[47] Alobaid AS, Cortes LM, Lo J, Nguyen TT, Albert J, Abu-Melha AS, et al. Radiographic and clinical outcomes of the treatment of immature permanent teeth by revascularization or apexification: a pilot retrospective cohort study. J Endod. 2014;40(8):1063-70.

[48] Bose R, Nummikoski P, Hargreaves K. A retrospective evaluation of radiographic outcomes in immature teeth with necrotic root canal systems treated with regenerative endodontic procedures. J Endod. 2009;35(10):1343-9.

[49] Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. J Endod. 2005;31(10):711-8.

[50] Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res. 2009;88(9):792-806.

[51] Ruparel NB, de Almeida JF, Henry MA, Diogenes A. Characterization of a stem cell of apical papilla cell line: effect of passage on cellular phenotype. J Endod. 2013;39(3):357-63.

[52] Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, et al. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One. 2006;1:e79.

[53] Lovelace TW, Henry MA, Hargreaves KM, Diogenes A. Evaluation of

the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. J Endod. 2011;37(2):133-8.

[54] Bansal R, Jain A, Mittal S. Current overview on challenges in regenerative endodontics. J Conserv Dent. 2015;18(1):1-6.

[55] Tran-Hung L, Laurent P, Camps J, About I. Quantification of angiogenic growth factors released by human dental cells after injury. Arch Oral Biol. 2008;53(1):9-13.

[56] Baker SM, Sugars RV, Wendel M, Smith AJ, Waddington RJ, Cooper PR, et al. TGF-beta/extracellular matrix interactions in dentin matrix: a role in regulating sequestration and protection of bioactivity. Calcif Tissue Int. 2009;85(1):66-74.

[57] Smith AJ, Tobias RS, Plant CG, Browne RM, Lesot H, Ruch JV. In vivo morphogenetic activity of dentine matrix proteins. J Biol Buccale. 1990;18(2):123-9.

[58] Tziafas D, Alvanou A, Panagiotakopoulos N, Smith AJ, Lesot H, Komnenou A, et al. Induction of odontoblast-like cell differentiation in dog dental pulps after in vivo implantation of dentine matrix components. Arch Oral Biol. 1995;40(10):883-93.

[59] Peters OA, Galicia J, Arias A, Tolar M, Ng E, Shin SJ. Effects of two calcium silicate cements on cell viability, angiogenic growth factor release and related gene expression in stem cells from the apical papilla. Int Endod J. 2016;49(12):1132-40.

[60] Luo Z, Li D, Kohli MR, Yu Q, Kim S, He WX. Effect of Biodentine on the proliferation, migration and adhesion of human dental pulp stem cells. J Dent. 2014;42(4):490-7.

[61] Lee SJ, Monsef M, Torabinejad M. Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. J Endod. 1993;19(11):541-4.

[62] Torabinejad M, Parirokh M. Mineral trioxide aggregate: a comprehensive literature review--part II: leakage and biocompatibility investigations. J Endod. 2010;36(2):190-202.

[63] Asgary S, Parirokh M, Eghbal MJ, Brink F. Chemical differences

between white and gray mineral trioxide aggregate. J Endod. 2005;31(2):101-3.

[64] Kratchman SI. Perforation repair and one-step apexification procedures. Dent Clin North Am. 2004;48(1):291-307.

[65] Kim M, Yang W, Kim H, Ko H. Comparison of the biological properties of ProRoot MTA, OrthoMTA, and Endocem MTA cements. J Endod. 2014;40(10):1649-53.

[66] Camilleri J. The chemical composition of mineral trioxide aggregate. J Conserv Dent. 2008;11(4):141-3.

[67] Sluyk SR, Moon PC, Hartwell GR. Evaluation of setting properties and retention characteristics of mineral trioxide aggregate when used as a furcation perforation repair material. J Endod. 1998;24(11):768-71.

[68] Schmitt D, Lee J, Bogen G. Multifaceted use of ProRoot MTA root canal repair material. Pediatr Dent. 2001;23(4):326-30.

[69] Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root-end filling material. J Endod. 1995;21(7):349-53.

[70] Camilleri J. Mineral trioxide aggregate: present and future developments. Endodontic Topics. 2015;32(1):31-46.

[71] Kaur M, Singh H, Dhillon JS, Batra M, Saini M. MTA versus Biodentine: Review of Literature with a Comparative Analysis. J Clin Diagn Res. 2017;11(8):ZG01-ZG5.

[72] Coleman NJ, Li Q. The impact of zirconium oxide radiopacifier on the early hydration behaviour of white Portland cement. Mater Sci Eng C Mater Biol Appl. 2013;33(1):427-33.

[73] Islam I, Chng HK, Yap AU. Comparison of the physical and mechanical properties of MTA and portland cement. J Endod. 2006;32(3):193-7.

[74] Vivan RR, Zapata RO, Zeferino MA, Bramante CM, Bernardineli N, Garcia RB, et al. Evaluation of the physical and chemical properties of two commercial and three experimental root-end filling materials. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010;110(2):250-6.

[75] Danesh G, Dammaschke T, Gerth HU, Zandbiglari T, Schafer E. A comparative study of selected properties of ProRoot mineral trioxide

aggregate and two Portland cements. Int Endod J. 2006;39(3):213-9.

[76] Matt GD, Thorpe JR, Strother JM, McClanahan SB. Comparative study of white and gray mineral trioxide aggregate (MTA) simulating a one- or two-step apical barrier technique. J Endod. 2004;30(12):876-9.

[77] Watts JD, Holt DM, Beeson TJ, Kirkpatrick TC, Rutledge RE. Effects of pH and mixing agents on the temporal setting of tooth-colored and gray mineral trioxide aggregate. J Endod. 2007;33(8):970-3.

[78] Chogle S, Mickel AK, Chan DM, Huffaker K, Jones JJ. Intracanal assessment of mineral trioxide aggregate setting and sealing properties. Gen Dent. 2007;55(4):306-11.

[79] Nekoofar MH, Adusei G, Sheykhrezae MS, Hayes SJ, Bryant ST, Dummer PM. The effect of condensation pressure on selected physical properties of mineral trioxide aggregate. Int Endod J. 2007;40(6):453-61.

[80] Holt DM, Watts JD, Beeson TJ, Kirkpatrick TC, Rutledge RE. The anti-microbial effect against enterococcus faecalis and the compressive strength of two types of mineral trioxide aggregate mixed with sterile water or 2% chlorhexidine liquid. J Endod. 2007;33(7):844-7.

[81] Dammaschke T, Gerth HU, Zuchner H, Schafer E. Chemical and physical surface and bulk material characterization of white ProRoot MTA and two Portland cements. Dent Mater. 2005;21(8):731-8.

[82] Reyes-Carmona JF, Felippe MS, Felippe WT. The biomineralization ability of mineral trioxide aggregate and Portland cement on dentin enhances the push-out strength. J Endod. 2010;36(2):286-91.

[83] Reyes-Carmona JF, Felippe MS, Felippe WT. Biomineralization ability and interaction of mineral trioxide aggregate and white portland cement with dentin in a phosphate-containing fluid. J Endod. 2009;35(5):731-6.

[84] Torabinejad M, Hong CU, Pitt Ford TR, Kaiyawasam SP. Tissue reaction to implanted super-EBA and mineral trioxide aggregate in the mandible of guinea pigs: a preliminary report. J Endod. 1995;21(11):569-71.

[85] Thomson TS, Berry JE, Somerman MJ, Kirkwood KL. Cementoblasts maintain expression of osteocalcin in the presence of mineral trioxide aggregate. J Endod. 2003;29(6):407-12.

[86] Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD. Cytotoxicity of four root end filling materials. J Endod. 1995;21(10):489-92.

[87] Zhu Q, Haglund R, Safavi KE, Spangberg LS. Adhesion of human osteoblasts on root-end filling materials. J Endod. 2000;26(7):404-6.

[88] Camp MA, Jeansonne BG, Lallier T. Adhesion of human fibroblasts to root-end-filling materials. J Endod. 2003;29(9):602-7.

[89] Bonson S, Jeansonne BG, Lallier TE. Root-end filling materials alter fibroblast differentiation. J Dent Res. 2004;83(5):408-13.

[90] Mitchell PJ, Pitt Ford TR, Torabinejad M, McDonald F. Osteoblast biocompatibility of mineral trioxide aggregate. Biomaterials. 1999;20(2):167-73.

[91] Keiser K, Johnson CC, Tipton DA. Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. J Endod. 2000;26(5):288-91.

[92] Ha WN, Nicholson T, Kahler B, Walsh LJ. Mineral Trioxide Aggregate-A Review of Properties and Testing Methodologies. Materials (Basel). 2017;10(11).

[93] Tani-Ishii N, Hamada N, Watanabe K, Tujimoto Y, Teranaka T, Umemoto T. Expression of bone extracellular matrix proteins on osteoblast cells in the presence of mineral trioxide. J Endod. 2007;33(7):836-9.

[94] Niu LN, Zhang W, Pashley DH, Breschi L, Mao J, Chen JH, et al. Biomimetic remineralization of dentin. Dent Mater. 2014;30(1):77-96.

[95] Prati C, Gandolfi MG. Calcium silicate bioactive cements: Biological perspectives and clinical applications. Dent Mater. 2015;31(4):351-70.

[96] Sarkar NK, Caicedo R, Ritwik P, Moiseyeva R, Kawashima I. Physicochemical basis of the biologic properties of mineral trioxide aggregate. J Endod. 2005;31(2):97-100.

[97] Ford TR, Torabinejad M, Abedi HR, Bakland LK, Kariyawasam SP. Using mineral trioxide aggregate as a pulp-capping material. J Am Dent Assoc. 1996;127(10):1491-4.

[98] Tziafas D, Pantelidou O, Alvanou A, Belibasakis G, Papadimitriou S. The dentinogenic effect of mineral trioxide aggregate (MTA) in short-

term capping experiments. Int Endod J. 2002;35(3):245-54.

[99] Holland R, de Souza V, Murata SS, Nery MJ, Bernabe PF, Otoboni Filho JA, et al. Healing process of dog dental pulp after pulpotomy and pulp covering with mineral trioxide aggregate or Portland cement. Braz Dent J. 2001;12(2):109-13.

[100] Nair PN, Duncan HF, Pitt Ford TR, Luder HU. Histological, ultrastructural and quantitative investigations on the response of healthy human pulps to experimental capping with mineral trioxide aggregate: a randomized controlled trial. Int Endod J. 2008;41(2):128-50.

[101] Faraco IM, Jr., Holland R. Response of the pulp of dogs to capping with mineral trioxide aggregate or a calcium hydroxide cement. Dent Traumatol. 2001;17(4):163-6.

[102] Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review--Part III: Clinical applications, drawbacks, and mechanism of action. J Endod. 2010;36(3):400-13.

[103] Guven G, Cehreli ZC, Ural A, Serdar MA, Basak F. Effect of mineral trioxide aggregate cements on transforming growth factor beta1 and bone morphogenetic protein production by human fibroblasts in vitro. J Endod. 2007;33(4):447-50.

[104] Marciano MA, Costa RM, Camilleri J, Mondelli RF, Guimaraes BM, Duarte MA. Assessment of color stability of white mineral trioxide aggregate angelus and bismuth oxide in contact with tooth structure. J Endod. 2014;40(8):1235-40.

[105] Malkondu O, Karapinar Kazandag M, Kazazoglu E. A review on biodentine, a contemporary dentine replacement and repair material. Biomed Res Int. 2014;2014:160951.

[106] Septodont. Research and development department. Biodentine™; Active Biosilicate TechnologyTM.: Internal data; scientific file, 2009.

[107] Laurent P, Camps J, De Meo M, Dejou J, About I. Induction of specific cell responses to a Ca(3)SiO(5)-based posterior restorative material. Dent Mater. 2008;24(11):1486-94.

[108] Grech L, Mallia B, Camilleri J. Investigation of the physical properties of tricalcium silicate cement-based root-end filling materials. Dent

Mater. 2013;29(2):e20-8.

[109] Guneser MB, Akbulut MB, Eldeniz AU. Effect of various endodontic irrigants on the push-out bond strength of biodentine and conventional root perforation repair materials. J Endod. 2013;39(3):380-4.

[110] Setbon HM, Devaux J, Iserentant A, Leloup G, Leprince JG. Influence of composition on setting kinetics of new injectable and/or fast setting tricalcium silicate cements. Dent Mater. 2014;30(12):1291-303.

[111] Kaup M, Schafer E, Dammaschke T. An in vitro study of different material properties of Biodentine compared to ProRoot MTA. Head Face Med. 2015;11:16.

[112] Butt N, Talwar S, Chaudhry S, Nawal RR, Yadav S, Bali A. Comparison of physical and mechanical properties of mineral trioxide aggregate and Biodentine. Indian J Dent Res. 2014;25(6):692-7.

[113] Aggarwal V, Singla M, Miglani S, Kohli S. Comparative evaluation of push-out bond strength of ProRoot MTA, Biodentine, and MTA Plus in furcation perforation repair. J Conserv Dent. 2013;16(5):462-5.

[114] Camilleri J, Grech L, Galea K, Keir D, Fenech M, Formosa L, et al. Porosity and root dentine to material interface assessment of calcium silicate-based root-end filling materials. Clin Oral Investig. 2014;18(5):1437-46.

[115] Wang Z. Bioceramic materials in endodontics. Endodontic Topics. 2015;32(1 May 2015):3-30.

[116] Attik GN, Villat C, Hallay F, Pradelle-Plasse N, Bonnet H, Moreau K, et al. In vitro biocompatibility of a dentine substitute cement on human MG63 osteoblasts cells: Biodentine versus MTA((R)). Int Endod J. 2014;47(12):1133-41.

[117] Jung S, Mielert J, Kleinheinz J, Dammaschke T. Human oral cells' response to different endodontic restorative materials: an in vitro study. Head Face Med. 2014;10:55.

[118] Camilleri J, Sorrentino F, Damidot D. Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. Dent Mater. 2013;29(5):580-93.

[119] Han L, Okiji T. Bioactivity evaluation of three calcium silicate-based

endodontic materials. Int Endod J. 2013;46(9):808-14.

[120] Atmeh AR, Chong EZ, Richard G, Festy F, Watson TF. Dentin-cement interfacial interaction: calcium silicates and polyalkenoates. J Dent Res. 2012;91(5):454-9.

[121] Shayegan A, Jurysta C, Atash R, Petein M, Abbeele AV. Biodentine used as a pulp-capping agent in primary pig teeth. Pediatr Dent. 2012;34(7):e202-8.

[122] Tran XV, Gorin C, Willig C, Baroukh B, Pellat B, Decup F, et al. Effect of a calcium-silicate-based restorative cement on pulp repair. J Dent Res. 2012;91(12):1166-71.

[123] Nowicka A, Wilk G, Lipski M, Kolecki J, Buczkowska-Radlinska J. Tomographic Evaluation of Reparative Dentin Formation after Direct Pulp Capping with Ca(OH)2, MTA, Biodentine, and Dentin Bonding System in Human Teeth. J Endod. 2015;41(8):1234-40.

[124] Nikfarjam F, Beyer K, Konig A, Hofmann M, Butting M, Valesky E, et al. Influence of Biodentine(R) - A Dentine Substitute - On Collagen Type I Synthesis in Pulp Fibroblasts In Vitro. PLoS One. 2016;11(12):e0167633.

[125] Mullaguri H, Suresh N, Surendran S, Velmurugan N, Chitra S. Role of pH Changes on Transforming Growth Factor-beta1 Release and on the Fibrin Architecture of Platelet-rich Fibrin When Layered with Biodentine, Glass Ionomer Cement, and Intermediate Restorative Material. J Endod. 2016;42(5):766-70.

[126] Zeid SA, Alothmani OS, Yousef MK. Biodentine and mineral trioxide aggregate: an analysis of solubility, pH changes and leaching elements. Life Sci J. 2015;12:18-23.

[127] Miller AA, Takimoto K, Wealleans J, Diogenes A. Effect of 3 Bioceramic Materials on Stem Cells of the Apical Papilla Proliferation and Differentiation Using a Dentin Disk Model. J Endod. 2018;44(4):599-603.

[128] Wongwatanasanti N, Jantarat J, Sritanaudomchai H, Hargreaves KM. Effect of Bioceramic Materials on Proliferation and Odontoblast Differentiation of Human Stem Cells from the Apical Papilla. J Endod.

2018;44(8):1270-5.

[129] Smith AJ, Scheven BA, Takahashi Y, Ferracane JL, Shelton RM, Cooper PR. Dentine as a bioactive extracellular matrix. Arch Oral Biol. 2012;57(2):109-21.

[130] Woo SM, Hwang YC, Lim HS, Choi NK, Kim SH, Kim WJ, et al. Effect of nifedipine on the differentiation of human dental pulp cells cultured with mineral trioxide aggregate. J Endod. 2013;39(6):801-5.

[131] Jung JY, Woo SM, Lee BN, Koh JT, Nor JE, Hwang YC. Effect of Biodentine and Bioaggregate on odontoblastic differentiation via mitogen-activated protein kinase pathway in human dental pulp cells. Int Endod J. 2015;48(2):177-84.

[132] Gandolfi MG, Siboni F, Botero T, Bossu M, Riccitiello F, Prati C. Calcium silicate and calcium hydroxide materials for pulp capping: biointeractivity, porosity, solubility and bioactivity of current formulations. J Appl Biomater Funct Mater. 2015;13(1):43-60.

[133] Kim J, Song YS, Min KS, Kim SH, Koh JT, Lee BN, et al. Evaluation of reparative dentin formation of ProRoot MTA, Biodentine and BioAggregate using micro-CT and immunohistochemistry. Restor Dent Endod. 2016;41(1):29-36.

[134] Chang SW, Lee SY, Ann HJ, Kum KY, Kim EC. Effects of calcium silicate endodontic cements on biocompatibility and mineralization-inducing potentials in human dental pulp cells. J Endod. 2014;40(8):1194-200.

[135] Daltoe MO, Paula-Silva FW, Faccioli LH, Gaton-Hernandez PM, De Rossi A, Bezerra Silva LA. Expression of Mineralization Markers during Pulp Response to Biodentine and Mineral Trioxide Aggregate. J Endod. 2016;42(4):596-603.

[136] Teoh YY, Athanassiadis B, Walsh LJ. Comparison of Commercial Calcium Hydroxide Pastes for Prolonged Antibacterial Effect using a Colourimetric Assessment. Materials (Basel). 2018;11(3).

[137] Loison-Robert LS, Tassin M, Bonte E, Berbar T, Isaac J, Berdal A, et al. In vitro effects of two silicate-based materials, Biodentine and BioRoot RCS, on dental pulp stem cells in models of reactionary and reparative

dentinogenesis. PLoS One. 2018;13(1):e0190014.

APPENDIX A

Ethics Committee Approval Letter

NO. 19 / 2018

CERTIFICATE OF ETHICAL CLEARANCE

Human Experimentation Committee
Faculty of dentistry
Chiang Mai University
Chiang Mai, Thailand

Title of project or study : Release of transforming growth factor beta-1 from human tooth dentin after application of either Biodentine™ or ProRoot® MTA as a coronal barrier.

Principal Investigator : Tanida Srisuwan

Participating Institution (S) : Faculty of Dentistry
Chiang Mai University
Chiang Mai, Thailand

Approved by the Faculty of Dentistry Human Experimentation Committee : April 19, 2018

Signature of the Chairman of the Committee :

.....
(Prof. Anak Iamaroon,D.D.S.,M.S.,Ph.D.)

Countersigned : .....
(Assist.Prof.Dr.Narumanas Korwanich, D.D.S, M.S., Ph.D.)
Dean, Faculty of Dentistry

APPENDIX B

Informed Consent

ใบยินยอมเข้าร่วมโครงการวิจัย (Informed consent)

1. โครงการวิจัยเรื่อง

(ภาษาไทย) การปลดปล่อยทรานส์ฟอร์มิงโกรทแฟกเตอร์ บีตา-1 จากเนื้อฟันมนุษย์ภายหลังการใช้ไบโอเดนทีนหรือโปรรูทเอ็มทีเอ เป็นวัสดุกั้นบริเวณคอฟัน

(ภาษาอังกฤษ) Release of transforming growth factor beta-1 from human tooth dentin afterapplication of either Biodentine™ or ProRoot® MTA as a coronal barrier

ท่านกำลังถูกทาบทามเพื่อเข้าร่วมในโครงการวิจัยเรื่องดังกล่าวข้างต้น ก่อนที่ท่านจะตัดสินใจว่าจะเข้าร่วมโครงการวิจัยนี้ เราต้องการจะอธิบายให้ท่านทราบถึงวัตถุประสงค์ของการวิจัยนี้ อันตรายที่ท่าน จะได้รับและสิ่งที่เราคาดหวังจากท่านเมื่อท่านตัดสินใจเข้าร่วมโครงการวิจัย เมื่อท่านตกลงใจที่จะเข้าร่วม โครงการเราจะขอท่านเซ็นชื่อในใบยินยอมต่อหน้าบุคคลซึ่งเป็นพยาน

การเข้าร่วมโครงการวิจัยนี้จะขึ้นอยู่กับความสมัครใจของท่านไม่มีการบังคับ ท่านอาจตัดสินใจที่จะไม่เข้าร่วมโครงการหรือถอนออกจากโครงการเวลาใดก็ได้ โดยท่านจะไม่สูญเสียประโยชน์ของท่านเกี่ยวกับ การดูแลรักษาตามมาตรฐาน

2. วัตถุประสงค์ของโครงการ

1) เพื่อศึกษาผลของไบโอเดนทีนและและโปรรูทเอ็มทีเอต่อการเพิ่มการปลดปล่อยทรานฟอร์มมิ่งโกรทแฟคเตอร์เบตา-1 จากคลองรากฟันมนุษย์
2) เพื่อศึกษาผลของไบโอเดนทีนและและโปรรูทเอ็มทีเอต่อการเพิ่มจำนวนของการพอกพูน
3) แร่ธาตุในเนื้อเยื่อปลายรากฟันมนุษย์

3. วิธีการ

ทางกลุ่มจะทำการคัดเลือกฟันจากผู้ป่วยที่นัดผ่าฟันคุดและยินยอมรับการรักษาเรียบร้อยแล้ว

โดยผู้ทำการถอนฟันหรือผ่าฟันคุดจะเป็นนักศึกษาทันตแพทย์ชั้นปีที่ 5 ภายใต้การดูแลของอาจารย์ทันตแพทย์ ก่อนทำการถอนฟันทางกลุ่มวิจัยจะทำการขอฟันคุดที่ถอนแล้วเพื่อใช้ในงานวิจัยและอธิบายถึงขั้นตอนการทำวิจัยให้ผู้ป่วยรับทราบว่าฟันที่ถอนออกไปจะถูกนำเนื้อเยื่อรอบปลายรากฟันและนำเซลล์ในเนื้อเยื่อนั้นไปศึกษาการพอกพูนแร่ธาตุในเซลล์ หากผู้ป่วยไม่ยินยอม ทางกลุ่มจะไม่นำมาพิจารณาใช้ในการวิจัย ก่อนทำการถอนฟันหรือผ่าฟันคุด นักศึกษาทันตแพทย์ผู้ทำการผ่าตัด จะอธิบายถึงความเสี่ยงทั้งหมดที่อาจเกิดขึ้น พร้อมทั้งขั้นตอนการแก้ไข

4. ความเสี่ยงและหรือความไม่สบายต่าง ๆ ที่อาจเกิดขึ้น

4.1 ภาวะแทรกซ้อนที่อาจเกิดก่อนทำการผ่าฟันคุดและการแก้ไข

ภาวะข้างเคียงของการใช้น้ำยาบ้วนปากชนิดคลอเฮกซิดีน 0.12% ได้แก่ staining บนผิวฟันและ oral surfaces, calculus formation รวมถึงการเปลี่ยนแปลง taste perception ของผู้ป่วย ผู้ป่วยสามารถทำความสะอาดได้เองตามคำแนะนำจากทันตแพทย์ ส่วนหินน้ำลายที่ไม่สามารถกำจัดได้อาจต้องได้รับการขูดหินน้ำลายโดยทันตแพทย์

4.2 ภาวะแทรกซ้อนที่อาจเกิดขณะทำการผ่าฟันคุดและการแก้ไข

1) เลือดออกมากผิดปกติ คือ อาจเกิดจากผู้ป่วยมีอัตราการไหลและอัตราการหยุดของเลือดผิดปกติ การรักษาจะให้ prophylactic therapy ก่อนทำ เพื่อเตรียมผู้ป่วยให้พร้อมรับการรักษา หากเลือดออกมากให้ใช้ผ้าก๊อซซับตรงตำแหน่งที่เลือดออกทิ้งไว้สักครู่ ถ้ายังไม่หยุดให้ใช้ bone wax อัดลงไปยังจุดที่เลือดออก แล้วใช้ผ้าก๊อซกัดอีกที

2) อันตรายต่อเส้นประสาท Mandibular ซึ่งจะเกิดจากการที่ฟันนั้นอยู่ชิดกับ Mandibular canal ทำให้ผู้ป่วยชาบริเวณที่เส้นประสาทนี้ไปเลี้ยง ซึ่งได้แก่ ริมฝีปากล่างและคาง nerve injury มักจะหายได้เอง โดยอาการชาจะดีขึ้นเรื่อย ๆ การรักษาอาจพิจารณาให้ Vitamin B complex นอกจากบางรายอาจต้องทำ nerve repa

3) อันตรายต่อเส้นประสาท Lingual จะเกิดเมื่อฟันคุดอยู่ชิดทางด้านลิ้น ใต้Mylohyoid ridge คนไข้จะมีอาการชาบริเวณปลายลิ้น การหายอาจช้ากว่าเส้นประสาท mandibular การรักษาอาจพิจารณาให้ Vitamin B complex นอกจากบางรายอาจต้องทำ nerve repair

4) ปลายรากฟันหัก ถ้ารากนั้นอยู่ชิดกับ Mandibular canal หรือ Maxillary sinus ก็อาจดันเข้าไปใน canal หรือ sinus ได้ ดังนั้นต้องทำด้วยความระมัดระวังอย่างสูง ถ้าเกิดปัญหาขึ้นการรักษาจะนำส่วนรากฟันออก ร่วมกับให้ยาปฏิชีวนะป้องกันการติดเชื้อ

5) ฟันข้างเคียงอาจได้รับการกระทบกระเทือนจนโยกหรือหลุดถ้าโยกไม่มากไม่ต้อง

รักษาอะไร การรักษาแนะนำคนไข้ไม่ให้เคี้ยวอาหารเหนียวหลายวัน ถ้าโยกมากให้ยึดฟันไว้นาน 2-4 อาทิตย์

6) เครื่องมือทำฟัน เช่น หัว Bur หักฝังเข้าในกระดูกหรือปลาย Elevator หักค้างฝังอยู่ในกระดูก การรักษาให้เอาส่วนที่หักออกให้หมด

7) Alveolar process หักทำให้เลือดออกมาก การรักษาต้องพยายามห้ามเลือดและเย็บให้เรียบร้อย

8) Maxillary tuberosity หักทำให้เลือดออกมาก การรักษาต้องพยายามเอาส่วนที่หักออกและเย็บแผลให้เรียบร้อย

9) ขากรรไกรล่างหัก ซึ่งพบมากในผู้สูงอายุเพราะฟันมักจะมี Ankylosis และเปราะ การรักษาโดยทำ intermaxillary fixation โดยใช้ arch bar หรือ ivy loops

4.3 ภาวะแทรกซ้อนที่อาจเกิดหลังทำการผ่าฟันคุดและการแก้ไข

1) การบวม ซึ่งอาจพบได้ในลักษณะบวมน้ำ หรืออาการห้อเลือด การบวมจะมากที่สุดในวันที่ 2 หลังผ่า แต่จะยุบลงเรื่อย ๆ และหายได้เอง กินเวลาประมาณ 1 อาทิตย์ การรักษาควรใช้น้ำแข็งประคบหลังผ่าทันทีจะลดการบวมได้

2) การติดเชื้อภายหลังการผ่าตัด โดยมีการบวมมากผิดปกติ และไม่แสดงอาการของการยุบบวมภายหลังวันที่ 3-4 หลังการผ่าตัด อาจมีหนองสะสม ควรรีบให้การรักษา การรักษาได้แก่การผ่าและระบายหนอง พร้อมให้ยาปฏิชีวนะ ยาแก้ปวด และให้ผู้ป่วยพักผ่อนมาก ๆ

3) อาการปวด พบได้ตามปกติภายหลังการผ่าตัด 1-2 วันแรก เกิดจากระคายเคืองต่อบาดแผลหรือเกิดจากการติดเชื้อ ปัญหาแทรกซ้อนอื่น ๆ เช่น dry socket การรักษาจะรักษาตามสาเหตุ เช่น curette เอาเศษกระดูกที่ตกค้างออก ใช้ bone file แต่งให้เรียบ หรือใช้น้ำอุ่นประคบในกรณีที่เกิดจาก trauma และ มีการบวมร่วมด้วยร่วมกับยาแก้ปวด

4) กระดูกเบ้าฟันอักเสบ เกิดได้จากเบ้าฟันไม่มีลิ่มเลือด พบหลังผ่าตัด 3-4 วัน การรักษาโดยใช้น้ำอุ่นหรือน้ำเกลือล้างเบ้าฟันให้สะอาดเพื่อล้างเอา necrotic debris ออกไปใช้ก๊อซซับให้แห้ง แล้ว pack เบ้าฟันหลวม ๆ ด้วย gauze strip ที่ชุบยาแก้ปวดหรือ antiseptic (เช่น clove oil (Eugenol) ผสมวาสลิน หรือ Iodoform paste) ผู้ป่วยจะหายปวด เปลี่ยน pack วันเว้นวันจนกระทั่งเบ้าฟันตื้นขึ้น

5) การอ้าปากได้จำกัด การรักษาต้องให้ผู้ป่วยหัดอ้าปากกว้าง ๆ

5. ประโยชน์ที่คาดว่าจะได้รับ

1) เป็นองค์ความรู้ในการอธิบายการทำงานของเซลล์เนื้อเยื่อปลายรากฟันของมนุษย์
2) สามารถนำองค์ความรู้นี้มาพัฒนาให้เกิดแนวทางปฏิบัติในการรักษา

6. ค่าใช้จ่าย

ท่านยินยอมชำระค่ารักษาในการถอนฟันและผ่าฟันคุดอย่างง่ายในอัตราปกติ ตามประกาศของคณะทันตแพทยศาสตร์ ดังนี้ ค่าถอนฟัน 50 บาท ค่าผ่าฟันคุดอย่างง่าย 150 บาท ภายหลังการทำการรักษาทางศัลยศาสตร์ เพื่อนำฟันคุดออกแล้ว ผู้ป่วยจะได้รับชุดอุปกรณ์เพื่อดูแลรักษาสุขภาพช่องปาก ได้แก่ แปรงฟัน ยาสีฟัน และน้ำยาบ้วนปาก

7. การได้รับบาดเจ็บที่เกี่ยวข้องกับการวิจัย

ผู้ป่วยได้รับการบาดเจ็บจากการผ่าตัดฟันกรามซี่ที่สามเพื่อเก็บตัวอย่างเนื้อเยื่อรอบปลายรากฟัน

8. บุคคลที่ท่านสามารถติดต่อเมื่อมีปัญหาหรือคำถามเกี่ยวกับโครงการวิจัยนี้

หากท่านมีปัญหาหรือคำถามเกี่ยวกับโครงการวิจัย นี้ท่านสามารถติดต่อ

ผศ.ทพญ.ดร. ธนิดา ศรีสุวรรณ

ภาควิชาทันตกรรมบูรณะ คณะทันตแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ โทร. (053) 944457

กรณีที่ผู้ป่วยมีอายุในช่วง 13 ถึงไม่เกิน 18 ปี ผู้วิจัยได้มีการขอความยินยอมจากเด็ก โดยเอกสารขอความยินยอมจากเด็กเป็นเอกสารเดียวกับของบิดามารดาหรือผู้ปกครอง ร่วมด้วยมีการชี้แจงด้วยภาษา ที่เข้าใจง่าย และหากท่านได้อ่านใบยินยอม หรือมีผู้อ่านและอธิบายใบยินยอมนี้ให้ท่านฟัง และท่านเข้าใจและสมัครใจที่จะเข้าร่วมโครงการวิจัยนี้ กรุณาเซ็นชื่อของท่าน ข้างล่างนี้

…….. ……..

( ) วัน/เดือน/ ปี

ชื่ออาสาสมัครหรือผู้ปกครอง

…… ……..

( ) วัน/เดือน/ปี

พยาน

APPENDIX C

Certificated of Irradiation Treatment

ห้องปฏิบัติการวัดปริมาณรังสีระดับสูง (High-Dose Dosimetry Laboratory)
ศูนย์ฉายรังสี สถาบันเทคโนโลยีนิวเคลียร์แห่งชาติ (องค์การมหาชน)
เลขที่ 9/9 หมู่ที่ 7 ต.ทรายมูล อ.องครักษ์ จ.นครนายก 26120
โทร. : 02-401-9889 ต่อ 3003 แฟกซ์ : 037-392913 www.tint.or.th

Serial no. : IC-HD-86/61 Page 1/1

ใบรับรองการฉายรังสี

เลขที่คำขอ (ศส) :	794/61 ลงวันที่ : 6 มิถุนายน 2561
ชื่อ/ที่อยู่ผู้ขอรับบริการ	ทพญ.กุนท์ลดา วัฒนาภัควงศ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ถนนสุเทพ ตำบลสุเทพ อำเภอเมือง จังหวัดเชียงใหม่ 50200
ชนิดของตัวอย่าง	ฟันถอนมนุษย์
จำนวน	1 กล่อง
วันที่ส่งตัวอย่าง	7 มิถุนายน 2561
วันที่ทำการฉายรังสี	7 มิถุนายน 2561
ชนิดของรังสี	แกมมา
ชนิดเครื่องวัดปริมาณรังสี (Dosimeter)	Amber perspex
Maximum dose (kGy)	30.67
Minimum dose (kGy)	21.30
Dose uniformity	1.44

ผู้วัดปริมาณรังสี : .....
(เหนือตะวัน อำรงสิริภาคย์)
นักวิทยาศาสตร์นิวเคลียร์

ผู้ทวนสอบ/อนุมัติ : .....
(นงค์นุช แจ้งสว่าง)
หัวหน้าฝ่ายพัฒนาและบริการ

FM-IC-17 Rev. No: 02 EFF. Date: 07/03/2018

APPENDIX D

Standard Protocols

1. Powdered Media Preparation (M0644 SIGMA - Minimum Essential Medium Eagle)

1. Measure out 90% of final volume of tissue culture grade water. Water should be at room temperature.
2. While gently stirring the water, add the powdered medium or salt mixture. Stir until dissolved. Do not heat water.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution.
4. To the solution, add 2.2 grams of sodium bicarbonate for each liter of final volume of medium being prepared. Stir until dissolved.
5. Bring medium to final volume with tissue culture grade water.
6. Sterilize immediately by filtration using a membrane with a porosity of 0.2 micrometers or less.
7. Aseptically dispense into sterile containers. Store liquid medium refrigerated at 2-8°C and in the dark.

2. Preparation of 1000 mL of alpha-MEM complete medium

1. Measure 989 mL of MEM in beaker.
2. Add 1 mL of L-ascorbic acid.
3. Add 10 mL of Antibiotics (Penicillin and Streptomycin).
4. Add 100 mL of Fetal bovine serum.
5. Aseptically dispense into sterile containers. Store liquid medium refrigerated at 2-8 °C and in the dark

3. Reconstitution of PBS

One tablet of PBS (P4417 SIGMA) dissolved in 200 mL of deionized water

yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.

4. Preparation 10 mL of 1 M ß-glycerophosphate (Sigma-Aldrich)

1. Measure 10 mL of PBS in 15 mL centrifuge tube.
2. Add 2.16 g of ß-glycerophosphate.
3. Aseptically dispense into sterile containers. Store at 2-8 °C.

5. Preparation 10 mL of 5 mg/mL ascorbic acid (Sigma-Aldrich)

1. Measure 10 mL of PBS in 15 mL centrifuge tube.
2. Add 50 mg ascorbic acid.
3. Aseptically dispense into sterile containers. Store at 2-8 °C and in the dark.

6. Preparation of 20 µg/mL dexamethasone (Sigma-Aldrich)

1. Add 1 mg dexamethasone in 1 ml of 100% methanol and gently swirl.
2. Add 49 mL of alpha-MEM complete medium.
3. Store at -20 °C and avoid repeat freeze/thaw.

7. Preparation of 100 mL of differentiation medium

1. Measure 100 mL of alpha-MEM complete medium in beaker.
2. Add 1 mL of 1 M ß-glycerophosphate.
3. Add 1 mL of 5 mg/mL ascorbic acid.
4. Add 0.05 mL 20 µg/mL dexamethasone.
5. Mix well before use.

8. Preparation 500 ml of 40 mM Alizarin Red S (Sigma-Aldrich)

1. Add 6.846 g Alizarin Red S in 500 ml of deionized water.
2. Filter solution via 0.2 µm and titer pH to 4.2 with 1 N NaOH.
3. Store at 2-8 °C and in the dark.

9. Preparation of 4% Paraformaldehyde (Sigma-Aldrich) 1 Liter

1. Add 800 ml of 1X PBS to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C.
2. Add 40 g of paraformaldehyde powder to the heated PBS solution.
3. Slowly raise the pH by adding 1 N NaOH dropwise from a pipette until the

solution clears.

4. Once the paraformaldehyde is dissolved, the solution should be cooled and filtered.
5. Adjust the volume of the solution to 1 L with 1X PBS.
6. Recheck the pH, and adjust it with small amounts of dilute HCl to approximately 6.9. The solution can be aliquoted and frozen or store at 2-8 °C for up to one month.

10. Preparation 100 ml of 1 N Sodium hydroxide (NaOH, Sigma-Aldrich)

Dissolve 4 g of NaOH in deionized water to make volume 100 ml.

11. Preparation 100 ml of 1 N Hydrochloric (HCl)

To 91.67 mL of deionized water, slowly add 8.33 mL of 12 N HCl. Mix well.

12. Preparation 1000 mL of 10 mM Sodium phosphate (NaH_2PO_4)

1. Dissolve 1.2 g of NaH_2PO_4 in deionized water 900 ml.
2. Adjust pH to 7 and add deionized water to make volume 1000 ml.

13. ELISA assay procedure following the manufacturer's protocol

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, standard dilutions, and activated samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-21 (for cell culture supernate and urine samples) or Assay Diluent RD1-73 (for serum/plasma samples) to each well.
4. Add 50 µL of standard, control, or activated sample* per well. Tap the plate gently to mix. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid

at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µL of TGF-β1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

14. Protocol for Alizarin Red S staining and destaining

1. Aspirate medium and wash twice cultures with PBS 1 mL/well.
2. Fix for 15 minutes at room temperature in 4% paraformaldehyde 500 µL in PBS.
3. Wash 3 times with PBS 1mL/well.
4. Stain for 5 minutes at room temperature with filtered alizarin red solution (40 mM, pH 4.2) and gentle rocking in dark.
5. Wash excessive dye with deionized water 4 times, then add PBS 500 µL/well.
6. Visualize all samples under an inverted-light microscope and left to air-dry for 1 week.
7. Destaining for 20 minutes at room temperature with 10% cetylpyridinium chloride monohydrate in 10 mM NaH_2PO_4 1 mL/well.
8. Transfer 100 mL of destaining solution to 96 well plate. Measured spectrophotometrically at 550 nm

APPENDIX E

Statistical datasheets

1. Comparison between ProRoot®MTA and Biodentine™ on TGF- ß1 release from dentin.

Tests of Normality

	group	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
OD	control	.368	6	.011	.644	6	.001
	MTA	.264	6	.200*	.763	6	.027
	BD	.169	6	.200*	.981	6	.955

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Kruskal-Wallis Test

Ranks

	group	N	Mean Rank
OD	control	6	5.83
	MTA	6	7.17
	BD	6	15.50
	Total	18	

Test Statistics[a,b]

	OD
Chi-Square	12.264
df	2
Asymp. Sig.	.002

a. Kruskal Wallis Test

b. Grouping Variable: group

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD	control	6	5.83	35.00
	MTA	6	7.17	43.00
	Total	12		

Test Statistics[a]

	OD
Mann-Whitney U	14.000
Wilcoxon W	35.000
Z	-.714
Asymp. Sig. (2-tailed)	.475
Exact Sig. [2*(1-tailed Sig.)]	.589[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD	control	6	3.50	21.00
	BD	6	9.50	57.00
	Total	12		

Test Statistics[a]

	OD
Mann-Whitney U	.000
Wilcoxon W	21.000
Z	-2.934
Asymp. Sig. (2-tailed)	.003
Exact Sig. [2*(1-tailed Sig.)]	.002[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD	MTA	6	3.50	21.00
	BD	6	9.50	57.00
	Total	12		

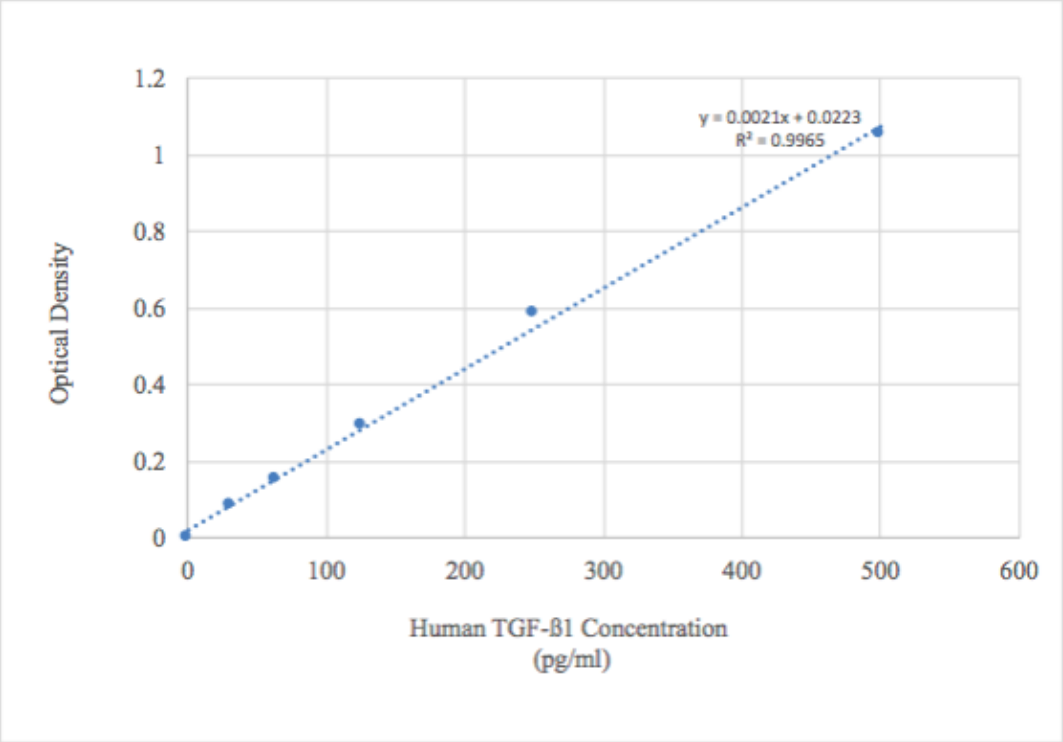
Test Statistics[a]

	OD
Mann-Whitney U	.000
Wilcoxon W	21.000
Z	-2.903
Asymp. Sig. (2-tailed)	.004
Exact Sig. [2*(1-tailed Sig.)]	.002[b]

a. Grouping Variable: group

b. Not corrected for ties.

Standard curve of samples



2. Quantitative analysis of Dentinogenic/Osteogenic Differentiation Capacity

- Comparison between groups on day 14 and 21

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	group	Statistic	df	Sig.	Statistic	df	Sig.
OD_D14	negative control	.140	18	.200*	.956	18	.526
	Positive control	.387	18	.000	.510	18	.000
	ProRoot MTA	.283	18	.001	.618	18	.000
	Biodentine	.132	18	.200*	.965	18	.691
	Tooth	.379	18	.000	.448	18	.000
	ToothMTA	.114	18	.200*	.952	18	.462
	ToothBD	.181	18	.122	.930	18	.198
OD_D21	negative control	.205	18	.044	.709	18	.000
	Positive control	.121	18	.200*	.921	18	.135
	ProRoot MTA	.187	18	.094	.833	18	.005
	Biodentine	.199	18	.058	.890	18	.038
	Tooth	.390	18	.000	.474	18	.000
	ToothMTA	.163	18	.200*	.876	18	.022
	ToothBD	.236	18	.009	.850	18	.009

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Kruskal-Wallis Test

Ranks

	group	N	Mean Rank
OD_D14	negative control	18	38.56
	Positive control	18	51.39
	ProRoot MTA	18	44.67
	Biodentine	18	112.19
	Tooth	18	16.69
	ToothMTA	18	77.69
	ToothBD	18	103.31
	Total	126	
OD_D21	negative control	18	27.17
	Positive control	18	52.53
	ProRoot MTA	18	75.53
	Biodentine	18	113.94
	Tooth	18	19.67
	ToothMTA	18	62.53
	ToothBD	18	93.14
	Total	126	

Test Statistics[a,b]

	OD_D14	OD_D21
Chi-Square	100.914	93.567
df	6	6
Asymp. Sig.	.000	.000

a. Kruskal Wallis Test

b. Grouping Variable: group

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	negative control	18	13.97	251.50
	Positive control	18	23.03	414.50
	Total	36		
OD_D21	negative control	18	11.03	198.50
	Positive control	18	25.97	467.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	80.500	27.500
Wilcoxon W	251.500	198.500
Z	-2.588	-4.257
Asymp. Sig. (2-tailed)	.010	.000
Exact Sig. [2*(1-tailed Sig.)]	.009[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	negative control	18	18.28	329.00
	ProRoot MTA	18	18.72	337.00
	Total	36		
OD_D21	negative control	18	10.64	191.50
	ProRoot MTA	18	26.36	474.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	158.000	20.500
Wilcoxon W	329.000	191.500
Z	-.127	-4.478
Asymp. Sig. (2-tailed)	.899	.000
Exact Sig. [2*(1-tailed Sig.)]	.913[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	negative control	18	9.50	171.00
	Biodentine	18	27.50	495.00
	Total	36		
OD_D21	negative control	18	9.50	171.00
	Biodentine	18	27.50	495.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	.000	.000
Wilcoxon W	171.000	171.000
Z	-5.132	-5.127
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	negative control	18	25.14	452.50
	Tooth	18	11.86	213.50
	Total	36		
OD_D21	negative control	18	22.14	398.50
	Tooth	18	14.86	267.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	42.500	96.500
Wilcoxon W	213.500	267.500
Z	-3.804	-2.078
Asymp. Sig. (2-tailed)	.000	.038
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.037[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	negative control	18	9.67	174.00
	ToothMTA	18	27.33	492.00
	Total	36		
OD_D21	negative control	18	11.86	213.50
	ToothMTA	18	25.14	452.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	3.000	42.500
Wilcoxon W	174.000	213.500
Z	-5.038	-3.782
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	negative control	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		
OD_D21	negative control	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	.000	.000
Wilcoxon W	171.000	171.000
Z	-5.132	-5.127
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Positive control	18	20.06	361.00
	ProRoot MTA	18	16.94	305.00
	Total	36		
OD_D21	Positive control	18	14.25	256.50
	ProRoot MTA	18	22.75	409.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	134.000	85.500
Wilcoxon W	305.000	256.500
Z	-.887	-2.421
Asymp. Sig. (2-tailed)	.375	.015
Exact Sig. [2*(1-tailed Sig.)]	.389[b]	.014[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Positive control	18	9.56	172.00
	Biodentine	18	27.44	494.00
	Total	36		
OD_D21	Positive control	18	9.50	171.00
	Biodentine	18	27.50	495.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	1.000	.000
Wilcoxon W	172.000	171.000
Z	-5.095	-5.126
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Positive control	18	25.61	461.00
	Tooth	18	11.39	205.00
	Total	36		
OD_D21	Positive control	18	25.56	460.00
	Tooth	18	11.44	206.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	34.000	35.000
Wilcoxon W	205.000	206.000
Z	-4.069	-4.023
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Positive control	18	11.14	200.50
	ToothMTA	18	25.86	465.50
	Total	36		
OD_D21	Positive control	18	15.25	274.50
	ToothMTA	18	21.75	391.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	29.500	103.500
Wilcoxon W	200.500	274.500
Z	-4.194	-1.851
Asymp. Sig. (2-tailed)	.000	.064
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.064[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Positive control	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		
OD_D21	Positive control	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	.000	.000
Wilcoxon W	171.000	171.000
Z	-5.127	-5.126
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	ProRoot MTA	18	9.78	176.00
	Biodentine	18	27.22	490.00
	Total	36		
OD_D21	ProRoot MTA	18	11.61	209.00
	Biodentine	18	25.39	457.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	5.000	38.000
Wilcoxon W	176.000	209.000
Z	-4.969	-3.923
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	ProRoot MTA	18	25.06	451.00
	Tooth	18	11.94	215.00
	Total	36		
OD_D21	ProRoot MTA	18	26.44	476.00
	Tooth	18	10.56	190.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	44.000	19.000
Wilcoxon W	215.000	190.000
Z	-3.754	-4.529
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	ProRoot MTA	18	12.17	219.00
	ToothMTA	18	24.83	447.00
	Total	36		
OD_D21	ProRoot MTA	18	21.00	378.00
	ToothMTA	18	16.00	288.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	48.000	117.000
Wilcoxon W	219.000	288.000
Z	-3.608	-1.424
Asymp. Sig. (2-tailed)	.000	.155
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.161[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	ProRoot MTA	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		
OD_D21	ProRoot MTA	18	14.86	267.50
	ToothBD	18	22.14	398.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	.000	96.500
Wilcoxon W	171.000	267.500
Z	-5.127	-2.072
Asymp. Sig. (2-tailed)	.000	.038
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.037[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Biodentine	18	27.44	494.00
	Tooth	18	9.56	172.00
	Total	36		
OD_D21	Biodentine	18	27.50	495.00
	Tooth	18	9.50	171.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	1.000	.000
Wilcoxon W	172.000	171.000
Z	-5.116	-5.131
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Biodentine	18	26.39	475.00
	ToothMTA	18	10.61	191.00
	Total	36		
OD_D21	Biodentine	18	27.33	492.00
	ToothMTA	18	9.67	174.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	20.000	3.000
Wilcoxon W	191.000	174.000
Z	-4.493	-5.031
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Biodentine	18	23.69	426.50
	ToothBD	18	13.31	239.50
	Total	36		
OD_D21	Biodentine	18	26.22	472.00
	ToothBD	18	10.78	194.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	68.500	23.000
Wilcoxon W	239.500	194.000
Z	-2.958	-4.398
Asymp. Sig. (2-tailed)	.003	.000
Exact Sig. [2*(1-tailed Sig.)]	.002[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Tooth	18	9.94	179.00
	ToothMTA	18	27.06	487.00
	Total	36		
OD_D21	Tooth	18	11.31	203.50
	ToothMTA	18	25.69	462.50
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	8.000	32.500
Wilcoxon W	179.000	203.500
Z	-4.893	-4.103
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	Tooth	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		
OD_D21	Tooth	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	.000	.000
Wilcoxon W	171.000	171.000
Z	-5.148	-5.131
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
OD_D14	ToothMTA	18	9.50	171.00
	ToothBD	18	27.50	495.00
	Total	36		
OD_D21	ToothMTA	18	11.78	212.00
	ToothBD	18	25.22	454.00
	Total	36		

Test Statistics[a]

	OD_D14	OD_D21
Mann-Whitney U	.000	41.000
Wilcoxon W	171.000	212.000
Z	-5.125	-3.828
Asymp. Sig. (2-tailed)	.000	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: group

b. Not corrected for ties.

CURRICULUM VITAE

Author's Name Ms. Kunlada Wattanapakkavong

Date/Year of Birth 17 September 1989

Place of Birth Bangkok, Thailand

Education

2008 - 2014 Doctor of Dental Surgery (D.D.S.), The Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand

Experience

2014 - now Dentist at The Department of Dental Public Health, Srisangworn Sukhothai Hospital, Sukhothai Provincial Health Office, Sukhothai, Thailand