ผลของไวท์โปรรูทเอ็มทีเอและพอร์ตแลนด์ซีเมนต์สองชนิดที่ผลิตในประเทศไทย
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EFFECT OF WHITE PROROOT® MTA AND TWO THAI WHITE PORTLAND CEMENTS

MIXED WITH BISMUTH OXIDE ON CEMENTOBLASTIC DIFFERENTIATION IN HUMAN

CEMENTOBLAST-LIKE CELL LINE

FLIGHT LIEUTENANT TREESUKHON EAKBANNASINGH

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for the Degree of Master of Science Program in Endodontology

Department of Operative Dentistry

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Thesis Title	EFFECT OF WHITE PROROOT$^{®}$ MTA AND TWO THAI WHITE PORTLAND CEMENTS MIXED WITH BISMUTH OXIDE ON CEMENTOBLASTIC DIFFERENTIATION IN HUMAN CEMENTOBLAST-LIKE CELL LINE
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ตรีสุคนธ์ เอกบรรณสิงห์ : ผลของไวท์โปรรูทเอ็มทีเอและพอร์ตแลนด์ซีเมนต์สองชนิดที่ผลิตในประเทศไทยที่ผสมบิสมัตออกไซด์ต่อการเปลี่ยนสภาพของเซลล์สร้างเคลือบรากฟันชนิดซีเมนโตบลาสไลค์เซลล์ไลน์ของมนุษย์. (EFFECT OF WHITE PROROOT$^{®}$ MTA AND TWO THAI WHITE PORTLAND CEMENTS MIXED WITH BISMUTH OXIDE ON CEMENTOBLASTIC DIFFERENTIATION IN HUMAN CEMENTOBLAST-LIKE CELL LINE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ทญ.ดร.ชุติมา ระติสุนทร, 84 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของพอร์ตแลนด์ซีเมนต์สีขาวที่ผลิตในประเทศไทยตราช้างและตรากิเลนที่ผสมบิสมัตออกไซด์และ ไวท์โปรรูทเอ็มทีเอต่อการแสดงออกของยีน   และการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตสต่อเซลล์สร้างเคลือบรากฟันชนิดซีเมนโตบลาสไลค์เซลล์ไลน์ของมนุษย์   โดยเลี้ยงเซลล์สร้างเคลือบรากฟันชนิดซีเมนโตบลาสไลค์เซลล์ไลน์ของมนุษย์   ด้วยอาหารเลี้ยงเซลล์ที่สกัดจากพอร์ตแลนด์ซีเมนต์ตราช้างและตรากิเลนและไวท์โปรรูทเอ็มทีเอ ในช่วงเวลาที่แตกต่างกันคือเป็นเวลา 1 3 และ 7 วัน วัดการแสดงออกของยีนอัลคาไลน์ฟอสฟาเตส โบนไซอะโลโปรตีน คอลลาเจนชนิดที่ 1 และออสทีโอแคลซินด้วยวิธีเรียลไทม์พอลิเมอเรสเชนรีแอกชัน   และวัดการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตสด้วยวิธีวิเคราะห์เอนไซม์ วิเคราะห์ความแตกต่างของสัดส่วนการแสดงออกของยีนและการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตส ด้วยสถิติการทดสอบของครัสคัล-วอลลิสที่ระดับนัยสำคัญ 0.05 จากการศึกษาพบว่าพอร์ตแลนด์ซีเมนต์ตราช้างกระตุ้นการแสดงออกของยีนอัลคาไลน์ฟอสฟาเตสอย่างมีนัยสำคัญทางสถิติในวันที่ 1 และ 3 ส่วนไวท์โปรรูทเอ็มทีเอจะกระตุ้นการแสดงออกของยีนอัลคาไลน์ฟอสฟาเตสเฉพาะวันที่ 3 วัสดุทั้งสามกลุ่มกระตุ้นการแสดงออกของยีนโบนไซอะโลโปรตีนในวันที่ 3 และพอร์ตแลนด์ซีเมนต์ตรากิเลนสามารถกระตุ้นการแสดงออกของยีนโบนไซอะโลโปรตีนในวันที่ 7 ด้วย พอร์ตแลนด์ซีเมนต์ตราช้างและกิเลนกระตุ้นการแสดงออกของยีนคอลลาเจนชนิดที่ 1 ในวันที่ 1   แต่การแสดงออกของยีนออสทีโอแคลซินจะลดลงอย่างมีนัยสำคัญทางสถิติในทั้งสามกลุ่มทดลองในวันที่ 3 และ 7 นอกจากนั้นพอร์ตแลนด์ซีเมนต์ตราช้างและตรากิเลนจะกระตุ้นการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตสได้มากกว่าไวท์โปรรูทเอ็มทีเอและกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติในวันที่ 3 และ 7 จากผลการศึกษาในเซลล์สร้างเคลือบรากฟันชนิดซีเมนโตบลาสไลค์เซลล์ไลน์ของมนุษย์นี้แสดงให้เห็นว่า พอร์ตแลนด์ซีเมนต์ตราช้างกระตุ้นการแสดงออกของยีนอัลคาไลน์ฟอสฟาเตสและโบนไซอะโลโปรตีนลักษณะเดียวกับไวท์โปรรูทเอ็มทีเอ พอร์ตแลนด์ซีเมนต์ตรากิเลนไม่กระตุ้นการแสดงออกของยีนอัลคาไลน์ฟอสฟาเตส   แต่กระตุ้นการแสดงออกของยีนโบนไซอะโลโปรตีนได้มากกว่าพอร์ตแลนด์ซีเมนต์ตราช้างและไวท์โปรรูทเอ็มทีเอ   และการทำงานของเอ็นไซม์อัลคาไลน์ฟอสฟาเตสจะถูกกระตุ้นโดยพอร์ตแลนด์ซีเมนต์ตราช้างและตรากิเลนเท่านั้น   ไม่ถูกกระตุ้นโดยไวท์โปรรูทเอ็มทีเอ

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KEYWORDS :   CEMENTOBLAST / MINERAL TRIOXIDE AGGREGATE / WHITE PORTLAND CEMENT / GENE EXPRESSION / ALKALINE PHOSPHATASE ACTIVITY

TREESUKHON EAKBANNASINGH: EFFECT OF WHITE PROROOT$^{®}$ MTA AND TWO THAI WHITE PORTLAND CEMENTS MIXED WITH BISMUTH OXIDE ON CEMENTOBLASTIC DIFFERENTIATION IN HUMAN CEMENTOBLAST-LIKE CELL LINE. ADVISOR: ASST.PROF. CHOOTIMA RATISOONTORN, Ph.D., 84 pp.

The purpose of this study was to investigate the effects of two Thai white Portland cement mixed with bismuth oxide (Chang and Kilan brands) and white ProRoot$^{®}$ MTA on gene expression and alkaline phosphatase activity of human cementoblast-like cell lines. Human cementoblast-like cell lines were exposed to material extracts for 1, 3, and 7 days. The expression of alkaline phosphatase, bone sialoprotein, type I collagen and osteocalcin were examined by quantitative real time polymerase chain reaction. Alkaline phosphatase activity was determined by enzymatic assay. Differences in relative expression ratio and alkaline phosphatase activity were analyzed by Kruskal-Wallis test ($p < 0.05$). Chang statistically significantly upregulated alkaline phosphatase at days 1 and 3 and white ProRoot$^{®}$ MTA stimulated alkaline phosphatase at day 3. All materials significantly stimulated bone sialoprotein at day 3 and Kilan also upregulated bone sialoprotein at day 7. Both Chang and Kilan significantly increased type I collagen expression at day 1 but gene expression of osteocalcin was significantly decreased by all materials at days 3 and 7. In addition, Chang and Kilan statistically significantly induced alkaline phosphatase activity more than white ProRoot$^{®}$ MTA and control at days 3 and 7. In conclusion, this study of human cementoblast-like cell lines showed that Chang induced alkaline phosphatase and bone sialoprotein expression in a similar manner to white ProRoot$^{®}$ MTA. Kilan could not upregulate alkaline phosphatase but could increase more bone sialoprotein expression than Chang and white ProRoot$^{®}$ MTA. Alkaline phosphatase activity could be upregulated by Chang and Kilan but not by white ProRoot$^{®}$ MTA.

Department :..... Operative Dentistry..................   Student's Signature............................

Field of Study :...Operative Dentistry..................   Advisor's Signature............................

Academic Year : ..2011…………………………..

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LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
BSP	bone sialoprotein
B2M	beta-2-microglobulin
Chang	White Portland cement (Chang brand) mixed with bismuth oxide
COL I	type I collagen
DMSO	dimethyl sulfoxide
DPSC	human dental pulp stromal cells
FBS	fetal bovine serum
HCEM	human cementoblast-like cell line
IRM	intermediate restorative material
Kilan	White Portland cement (Kilan brand) mixed with bismuth oxide
MC3T3	osteoblast-like cell line
MEM	minimum essential medium
MG63	human osteosarcoma cell line
MTA	Mineral trioxide aggregate
mRNA	messenger ribonucleic acid
OCCM	murine cementoblastic cell
OCN	osteocalcin

OPN	osteopontin
PCR	polymerase chain reaction
pNPP	p-nitrophenylphosphate
RGD	Arg-Gly-Asp
RBM	rat bone marrow cells
RT	reverse transcriptase
TIS	Thai Industrial Standard

CHAPTER I

INTRODUCTION

Background and rational

Ideally, retrofilling materials should permit healing of the periradicular tissue via regeneration of cementum, periodontal ligament and alveolar bone across the resected root-end surface (1). A key tissue in the initial process of periodontal regeneration is cementum (2). MTA has been shown to have a capacity to induce cementum covering the resected root surface (3-5). Cementum may have originated from both remaining periodontal ligament and growing connective tissue from bone (5). Although the precise mechanisms of MTA mineralized tissue-inducing activity remain uncertain, several studies detected that MTA upregulates the mRNA expression related to specific protein markers suggestive of mineralization (6-10).

According to its manufacturer, the chemical composition of ProRoot$^{®}$ MTA (Tulsa Dental, OK, USA) is quite similar to of Portland cement, except bismuth oxide which has been added to ProRoot$^{®}$ MTA for radio-opacity (11). MTA and Portland cement have similar physical properties, pH, setting time, solubility, dimensional change, compressive strength and antibacterial activity (12, 13). Both of them form crystals and calcified tissue next to the filling material when they are subcutaneously implanted in rats, suggesting that mechanism of their actions to form a mineralize tissue is similar (14). Furthermore, results of reverse transcriptase polymerase chain reaction showed that Portland cement allows the expression of mineralization related genes on cultured human pulp cell (15).

Because MTA is very expensive material, Portland cement, which is much cheaper, may be used as an alternative retrofilling material. The previous study showed that two Thai white Portland cements mixed with bismuth oxide have similar chemical

constituents and physical properties to white ProRoot$^{®}$ MTA(16) and are also biocompatible with primary human alveolar bone osteoblast (17).

According to the deposition of cementum after endodontic periapical surgery is considered a desired healing response and a pre-requisite for the reformation of a functional periodontal attachment (18). However, the effect of two Thai white Portland cements mixed with bismuth oxide on the expression of mineralization markers has not been investigated. Therefore, the purpose of present study is to investigate the effect of White ProRoot$^{®}$ MTA and two Thai white Portland cements mixed with bismuth oxide on the expression of markers of cementoblastic differentiation in Human cementoblast-like cell line, as well as to examine the effect of these materials on cellular alkaline phosphatase activity as a potential indicator of cementogenesis.

Research question

Do white ProRoot$^{®}$ MTA and two Thai white Portland cements mixed with bismuth oxide affect ALP, BSP, COL I and OCN gene expression and alkaline phosphatase activity of HCEM ?

Research objective

To evaluate effects of white ProRoot$^{®}$ MTA and two Thai white Portland cements mixed with bismuth oxide on ALP, BSP, COL I and OCN gene expression and alkaline phosphatase activity of HCEM.

Hypothesis

      1. Null hypothesis H_0 : ALP, BSP, COL I and OCN gene expression of HCEM cultured in extracts of white ProRoot® MTA and two Thai white Portland cements mixed with bismuth oxide are similar to those culture in regular media with 95% confidence interval. Alternative hypothesis H_a : ALP, BSP, COL I and OCN gene expression of HCEM cultured in extracts of white ProRoot® MTA and two Thai white Portland cements mixed with bismuth oxide are not similar to those culture in regular media with 95% confidence interval.

      2. Null hypothesis H_0 : Alkaline phosphatase activity of HCEM cultured in white ProRoot® MTA and two Thai white Portland cements mixed with bismuth oxide extracts is similar to those culture in regular media with 95% confidence interval. Alternative hypothesis H_a : Alkaline phosphatase activity of HCEM cultured in white ProRoot® MTA and two Thai white Portland cements mixed with bismuth oxide extracts is not similar to those culture in regular media with 95% confidence interval.

Field of Research

      To investigate the cementoblastic differentiation mRNA marker and alkaline phosphatase activity in human cementoblast-like cell line.

Keywords

      Cementoblast, Mineral trioxide aggregate, White portland cement, Gene expression, Alkaline phosphatase activity

Research design

Laboratory Experimental Research

Limitations of research

1. This is an in *vitro* study which may not represent clinical situations.

2. The number of samples is limited due to the cost white ProRoot$^®$ MTA.

3 The underlying biological regulatory mechanisms of HCEM may differ fundamentally from cementoblasts *in vivo*.

Obstacles

1. Bacterial contamination.

2. Material samples are broken when being removed from plastic molds.

Expected benefit & Application

1. If Thai White Portland cements with bismuth oxide induce cementoblastic differentiation of Human cementoblast like cell line equally or better than MTA, these Portland cements may be used as a substitution of ProRoot$^®$ MTA in order to reduce the cost of Endodontic surgery or other procedures

2. To obtain *in vivo* properties of the materials for further animal and clinical studies will be needed.

Ethical consideration

The study was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University.

CHAPTER II
LITERATURE REVIEW

Ideal retrograde filling material

Surgical endodontic therapy is performed to prevent the egress of irritants from root canal system into the periradicular tissue (19). This procedure includes the exposure of involved apex, resection of apical end of root, preparation of class I cavity and insertion of a root end filling material. Appropriate retrograde filling material is an important prerequisite to obtain a successful therapeutic result (20, 21).

The requirements of an ideal root-end filling material include bonding to the tooth tissue and sealing the root three-dimensionally, preferably inhibiting the growth of pathogenic microorganism, creating a dimensional stability with no effects by moisture either set or unset state, being well tolerated by periradicular tissues with no inflammatory reactions and non-toxic both locally and systematically, not producing corrosive product and staining the tooth or soft tissue, having a high radiopacity, stimulating the regeneration of periodontium, and having a long shelf life and being easy to manipulate (22-24).

Many materials have been suggested as retro-filling materials including gutta percha, amalgam, polycarboxylate cement, zinc phosphate cement, IRM, EBA, Cavit, glass ionomer, resin composite, MTA, gold foil, cyanoacrylate, Diaket, Titanium screw and Teflon (25). However, there are no materials having all ideal properties of a root end filling material (26).

Mineral Trioxide Aggregate (MTA)

Mineral Trioxide Aggregate (MTA) is a biomaterial that had been investigated for endodontic applications since early 1990s at Loma Linda University, California, USA. It was given an approval for endodontic used by the U.S. Food and Drug Administration in 1998 (27). Initially, MTA was introduced in grey-color but it caused discoloration of the overlying tissues (28). Therefore, a white MTA was developed for this reason and became commercially available as ProRoot® MTA (Tulsa Dental Products, Tulsa). The principal components of the grey formula are tricalcium silicate ($3CaO \cdot SiO_2$), bismuth oxide, dicalcium silicate ($2CaO \cdot SiO_2$), tricalcium aluminate ($3CaO \cdot Al_2O_3$), tetracalcium aluminoferrite ($4CaO \cdot Al_2O_3$) and calcium sulfate dehydrate(29). The major difference between grey and white MTA is the concentration of carborundum (Al_2O_3), periclase (MgO) and particularly FeO which are lower in the white MTA than in grey MTA (30). Both formulas of grey and white MTA are 75% Portland cement, 20% bismuth oxide (to make the aggregate radiographically identifiable)(11) and 5% gypsum by weight (31).

At present, MTA is widely used in the endodontic therapy. It has given a good result when being applied for both surgical and non-surgical endodontic treatments such as root–end filling (25), pulp capping (32), pulpotomy (33), apexification (34), internal resorption (35) and perforation repair (36). It is more popular as retro-filling material than other materials because it has *in vitro* and *in vivo* biocompatibility (37), good sealing ability (38, 39), antimicrobial activity (40) and dimensional stability (26) and encourages hard-tissue deposition (41). MTA powder consists of fine hydrophilic particles. Hydration of MTA powder results in a colloidal gel that solidifies into a hard structure (26). Although MTA has many advantages, it is more expensive, has longer setting time and poorer handing characteristics comparing to other retrofilling materials.

Portland cement

Portland cement is hydraulic cement that is produced by heating a homogeneous mixture of limestone and clay into a sintering temperature which is about 1450 °C. It is a basic ingredient of concrete, mortar or stucco. The main chemical compound constituents of White Portland cement is similar to of white ProRoot® MTA as shown in Table 1. When Portland cement is mixed with water, its chemical compounds will undergo a series of chemical reactions that cause it to set.

Table 1. Chemical constituents of White Portland cement

Chemical Name	Chemical Formula	Shorthand Notation	Percent by Weight
Tricalcium Silicate	$3CaO×SiO_2$	C_3S	60
Dicalcium Silicate	$2CaO×SiO_2$	C_2S	19
Tricalcium Aluminate	$3CaO×Al_2O_3$	C_3A	11
Tetracalcium Aluminoferrite	$4CaO×Al_2O_3×Fe_2O_3$	C_4AF	1
Other			9

(From: American Society for Testing and Materials. Portland cement. ASTM C150)

Since Portland cement is a slightly radiopaque, it does not meet the minimum requirement for radiopacity set out in ISO 6876/2001, which is the major disadvantage of Portland cement if it is clinically used (12). Therefore, bismuth oxide is added to enable a radiographic assessment (42).

Many studies compared MTA with Portland cement and indicated that they had a similar chemical composition except there was no detectable quantity of bismuth oxide in Portland cement (13, 43-45). Moreover, Portland cement and MTA have similar physical properties, pH, radiopacity, setting time, solubility, dimensional change,

compressive strength, *in vivo* and *in vitro* biocompatibility and antimicrobial effect (12, 13, 46). These suggest that Portland cement may be used as a substitute for MTA (46). From previous studies, white ProRoot® MTA and two Thai white Portland cements mixed with bismuth oxide have comparable chemical constituents and physical properties (16, 47). Besides, Thai white Portland cement mixed with bismuth oxide is biocompatible with human alveolar bone osteoblasts (17). However, more studies need to be conducted before a clinical use can be recommended.

Bismuth oxide

Bismuth oxide (Bi_2O_3) is a heavy yellow powder, no odor, insoluble in water and chemically inert. It is usually obtained as a by-product of the smelting of copper and lead ores.

It can be used in many applications such as plumbing, bullets, metal alloys, soldering, etc. Bismuth compounds could also be used in pharmaceutics and be incorporated into special polymers and materials for bone implants, dental prosthetic devices, catheters, sutures and surgical instruments to make them detectable by X-rays without any toxicity or carcinogenicity that is associated with other heavy metals.

It is required to add at least 15% of Bismuth oxide into white Portland cement in order to give a sufficient radiopacity for being used as an endodontic material (48). One part of bismuth oxide has been added to four parts of two Thai white Portland cements to make the aggregate radiographically similar to white ProRoot® MTA (16).

Cementum

Many histological studies have shown the presence of new cementum tissues in contact with the MTA surface when applying MTA as a retrofilling material onto defective tooth roots in monkeys (4) and dogs (3, 5, 49, 50). Similar findings were reported when MTA was used for repairing perforations in dogs' teeth (51) and root canal obturated with MTA exhibited apical closure by new cementum (52). These results suggested that MTA stimulated deposition of newly formed cementum in direct contact with the material which is a phenomenon not seen with other commonly used root end filling materials. In addition to a physical seal of the root-end filling, this cementum provides a biological seal and thereby creates a double seal (49).

Cementum is a mineralized tissue covering the roots of teeth. It shares many properties with bone, most notably a remarkable similarity on biochemical composition. It is approximately 50% hydroxyapatite and 50% collagen and noncollaganeous proteins (53). However, it has several distinct features: first, it is avascular; second, it has no innervation; third, it does not undergo significant remodeling as bone does; rather, there is a slow deposition of cementum: and fourth, it has different functions from of bone. The main function of cementum in health is to provide the attachment of periodontal ligament to roots and surrounding alveolar bone. After periodontal disease, cementum is thought to contribute to the process of repair (54). Some studies suggested that without the cementum the periodontium cannot be restored to health (55, 56). Thus, requirements for clinical regeneration of the periodontium, including formation of new bone and new cementum and connective tissue attachment, may include treatments directed at stimulating or augmenting cementum formation.

Major three cementum types different in their presence of cells and collagen fibers are distinguished in humans. *The acellular afibrillar cementum* covers teeth at and along the cemento-enamel junction; it consists of mineralized matrix but lacks of

collagen fibrils and embedded cells. The cells responsible for depositing this cementum have not been identified and its functional significance is unknown. The lack of collagen fibrils indicates that this cementum variety has no functions in tooth attachment(57). *Acellular extrinsic fiber cementum* was secreted from periodontal ligament fibroblast. It is usually confined to the coronal half of the root. It consists of a dense fringe of collagenous fibers implanted into dentinal matrix which are perpendicularly oriented to the root surface. In addition, it serves as an exclusive function of anchoring the root to periodontal ligament. *Cellular intrinsic fiber cementum* contains cementocytes embedded in a collagenous matrix of intrinsic collagen fibers. It was secreted from cementoblasts. These collagen fibers are oriented mostly parallel to the root surface. It is found in the furcation, on the apical root portion, in old resorption lacunae and in the root fracture site. It plays an important role as an adaptive tissue that brings and maintains the tooth in its proper position, as well as participates in the repair process (57).

Histologically, the light microscope revealed two types of surface reactions over MTA in which new cellular cementum overlays a crystalline-like structure. The pattern of newly formed cementum is not always from the margin of the resected dentin, but a small island of cementum is also found covering MTA which is totally isolated from the resected dentin (5).

Cementoblastic differentiation

There is an accumulative histological evidence in which cementum is critical for appropriate maturation of the periodontium, both during development and those associated with regeneration of periodontal tissues (53). Cementogenesis depends upon two crucial factors: (1) Cementoblasts, the cells responsible for cementum production and regeneration, and (2) the presence or absence of the cues and signals necessary to recruit and stimulate these cells (57).

Cementum is avascular and does not undergo remodeling. Therefore in the regeneration process, new cementoblasts must be continuously recruited from committed cementoprogenitor cells in order to replace cementoblasts that have reached the end of their life span. The origin of cementoblast precursors functioning in periodontal regeneration is not completely known. One possible source is the undifferentiated mesenchymal cells in the perivascular area of the adjacent periodontal ligament. According to previous study, it was revealed that fibroblast-like cells growing from the peripheral periodontal ligament may be differentiated into cementoblasts and may form cementum in regeneration of PDL attachment (58). Another potential origin is progenitor cells found in the marrow of adjacent osseous tissue (4).

The molecular factors that regulate new cell recruitment and differentiation are not mutually exclusive. A variety of chemotactic factors, adhesion molecules, growth factors, cytokine and ECM constituents participate together in regulating which receptors are expressed and which biochemical signaling events are induced (59). However, The precise molecular factors that regulate regenerative cementum are not well defined (53, 54).

Markers of cementoblastic differentiation

During cementum formation, cementoblasts synthesize several proteins and secrete them into the extracellular matrix. Cementum appears to contain factors in common with those associated with bone and to be developmentally controlled by similar factors. Although a number of genes that play a crucial role in cementogenesis have been identified, their precise functions are not clearly defined (53).

Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. As the name suggests, alkaline phosphatases are most effective in an alkaline

environment. Super saturation of phosphate ions, released from organic phosphate esters, would result in a precipitation of calcium phosphate salts (60-62). Although alkaline phosphatase exists in plasma membrane bound form, part of enzyme may also be bound to the extracellular matrix (63).

This enzyme is a glycoprotein thought to be involved in processes leading to mineral formation in tissues like bone and cementum. Alkaline phosphatase is believed to participate in cementum mineralization (53, 64-67). In rat molars, ALP is heterogeneously distributed in the periodontal ligament, with the highest activity being found in areas related to mineralization, adjacent to the alveolar bone and cementum. The enzyme activity was higher adjacent to cellular cementum than to acellular cementum and a significantly positive correlation was found between ALP activity and thickness of adjacent acellular cementum layer, which indicates a close relationship between local phosphate production and cementum formation rate (65). A histological examination of teeth from alkaline phosphatase-knockout mice suggests that alkaline phosphatase may have a more critical role in controlling formation of acellular cementum versus cellular cementum (68). Moreover, its activity is considered an important indicator of bone formation and a phenotypic marker of osteoblasts and upregulated in regenerating human periodontal cells (69).

Bone sialoprotein (BSP) is a phosphorylated and sulfated glycoproteins specially expressed in mineralized connective tissues, including bone, mineralizing cartilage, dentin, and cementum (70-72). It is prominently expressed in acellular extrinsic fiber cementum and acellular afibrillar cementum (73). BSP contains the Arg-Gly-Asp (RGD) sequence that acts as an adhesive domain, and the poly-glutamic acid residues. The RGD domain can bind both extracellular matrix proteins and integrins on cell surface. The poly-glutamic acid residues have a negative charge that can be bound to calcium ion molecules. Murine dentoalveolar tissue was analyzed by immunohistochemistry, and the northern blot analysis of dental follicle indicated that

BSP is expressed by dental follicle cells at the initiation of cementogenesis (71). *In situ* hybridization, it revealed distinct expression of BSP in surrounding bone at early stage of development and was specific to cell lining the root surface, with limited expression in the surrounding periodontal ligament region at stage of active cementogenesis (72). A comprehensive analysis of cementum formation has shown that the BSP gene is activated with the differentiation of cementoblast, and the high level of expression is associated with the early, rapid formation of the mineralizing cementum matrix (71, 72). Therefore, BSP is believed to serve an adhesion function for root-surface cells and to participate in initiating mineralization. It is also chemotactic to pre-cementoblasts and promotes their adhesion and differentiation (74). BSP capable to nucleate hydroxyapatite formation is also supported by the association of BSP expression and the pathological mineralization. There have been reported regarding the mineralization and BSP expression in ankylosing periodontal ligament (75). Most importantly, since the expression of BSP is highly specific for mineralizing tissues including bone, mineralizing cartilage, dentin, and cementum (76), this protein may be proven to be useful and reliable marker for cementoblast differentiation (77).

Type I Collagen (COL I) is a primary organic extracellular matrix component of cementum accounting for 90% of all collagens. The collagens are composed of three polypeptide alpha chains, two α1 chains and one α2 chain, coiled around each other to form the classic triple helix configuration. They are also found in fibrous supporting tissue, the dermis of skin, tendons, ligaments and bone in a variable arrangement from loose to dense according to the mechanical support required. Type I collagen is an essential component of the extracellular matrix that is required prior to mineralized matrix formation (78). In cementum, collagen promotes cellular attachment, functions to maintain the integrity of hard and soft tissue, and is active in their development, maturation, and repair (53). In mineralized tissue, they also provide a scaffold for the mineral crystals (79). COL I coating screw implants can enhance bone-implant contact (BIC) and peri-implant bone formation (80).

Osteocalcin (OCN) is a major noncollagenous protein found in abundance in the extracellular matrices of bone, dentin and cementum, and appears to be involved in the mineralization process (81). OCN is restricted to cells that have mineralizing capacity including osteoblast, odontoblast and cementoblast (53). Therefore, OCN is considered excellent markers for mineralized tissue-specific cells (82). *In situ* study has shown that OCN expression was selective to root lining cells and was not present throughout the periodontal ligament in CD-1 mice. These findings indicated that OCN is marker selective to cementoblast (2). Increased serum OCN levels correlate with bone cell synthesis and bone formation (83). Moreover, the transgenic mice lacking the osteocalcin gene develop a phenotype marked by enhanced bone formation without impairing bone resorption (84). Thus, it can be suggested that osteocalcin may be a negative regulator of mineral formation. In addition, it is possible that osteocalcin is one of the molecules that control the mineral-to-ligament ratio, allowing for formation of a periodontal ligament region versus an ankylosis situation (84).

Molecular analysis of the cellular response to Mineral trioxide aggregate and Portland cement

Several *in vitro* studies have examined the molecular responses of hard tissue-forming cells to MTA. Up-regulation of various types of cytokines and biologic markers has been reported in the presence of MTA or MTA extracts when being compared with control or other test materials. These studies were performed in several cell types including osteoblast, fibroblast, periodontal ligament cell and cementoblast.

The effect of MTA on cementoblast production was investigated in a tissue culture experiment. Reverse transcriptase polymerase chain reaction has verified that MTA permitted murine cementoblastic cell (OCCM-30) expressing mRNA involved in cementoblastic differentiation, COL-I, ALP, OCN and BSP (7). In a recent study, MTA

extracts at lower concentrations induced a biomineralization of these cells and caused the upregulation of the mRNA expression of COL I and BSP (10).

In other cell types, MTA induced alkaline phosphatase expression and activity in both periodontal ligament and gingival fibroblasts. It also induced an osteogenic phenotype such as osteonidogen, osteonectin, and osteopontin (6). The upregulation of COL I and OCN mRNA expression responded to osteoblast-like cell line (MC3T3-E1) treated with MTA (9). In human osteosarcoma cell line (MG63), MTA cements also showed the COL I, OCN, ALP, BSP and osteopontin (OPN) expression (85).

The cellular effects of Portland cement on cultured human pulp cells have been investigated. Portland cement is biocompatible, allowed the expression of mRNAs of a dentin-specific and noncollagenous protein involved in the mineralization such as dentin sialophosphoprotein and osteonectin on cultured human pulp cells (15). Moreover, the expression of osteonectin in the Portland cement group was similar to that of the dexamethasone-treated group suggested that Portland cement plays an important role in the mineralization of tooth structure (15). Subsequently, a study investigated whether Portland cement mixed with bismuth oxide facilitates the mineralization process in human dental pulp cells compared with pure Portland cement. The results indicate that both Portland cement mixed with bismuth oxide and pure Portland cement are biocompatible and induce high alkaline phosphatase levels of activity in relatively early stages of the mineralization. The expression of osteonectin and dentin sialophosphoprotein mRNAs in human pulp cells in the induction medium increased in a time-dependent manner. To detect mineralization nodules, alizarin red S staining showed a similar increase in Portland cement or Portland cement mixed with bismuth oxide compared with control. These results concluded that Portland cement and Portland cement mixed with bismuth oxide have similar effects in terms of mineralization and suggests that radiopaque Portland cement also has the potential to be used as a pulp-capping material (86). However, at

present, there is no study direct comparing the effect of MTA and Portland cement mixed with bismuth oxide on gene expression and cell differentiation in cementoblast.

Taken together, I hypothesize that MTA and Portland cements mixed with bismuth oxide promote a regenerative ability by stimulating hard tissue-forming cells including cementoblast to induce a matrix formation and mineralization.

Models of human cementoblastic cell culture

Using a variety of techniques has successfully isolated and cultured the cells from developing root surface of mice (2, 82, 87). However, human cementoblasts have been successfully grown from dissected fragments of healthy tooth root cementum treated with collagenase (88, 89). This system has a significant drawback because of a limited capacity to divide and reach cellular senescence and of a requirement that healthy teeth will be extracted for the cultures to be established. Also, it is still not clearly established whether cementoblastic cells can be reproducibly obtained, according to this approach, from teeth of aged and/or diseased patients. In addition, cells from a human cementoblastoma were isolated and cultured. However, it may not always be possible to extrapolate results from cementoblastoma cell cultures because its tumor characteristics may affect cellular behavior (90).

Recently, the human cementoblast-like (HCEM) cell lines were established from human extracted tooth by transfection with telomerase catalytic subunit *hTERT* gene. Two stable clones (HCEM-1 and -2) with high telomerase activity were obtained. These clones expressed mRNA for differentiation markers, type I collagen, alkaline phosphatase, runt-related transcription factor 2, osteocalcin, bone sialoprotein and cementum-derived protein. Moreover, these cells showed high activities of ALP and calcified nodule formation *in vitro* and subcutaneous transplanted HCEM produced the cementum-like materials on the surface of

hydroxyapatite granules *in vivo*. These cell lines can be useful cell models for investigating the characteristics of human cementoblasts (91).

As a result, Cementogenesis is considered a critical event for regenerating the periodontal tissues after an endodontic periapical surgery and the cementoblasts play important roles in the regeneration of cementum. Therefore, this study focuses on the effect of White ProRoot$^{®}$ MTA and two Thai White Portland cements mixed with Bismuth oxide on the markers of cementogenesis in Human cementoblast-like (HCEM) cell line.

CHAPTER III

MATERIALS AND METHODS

Materials

1. White Portland cement (Kilan brand, Universal White cement Co., LTD)

2. White Portland cement (White Elephant brand, The Siam White cement Co., LTD)

3. White ProRoot$^{®}$MTA (Dentsply, USA)

4. Bismuth oxide (Fluka, Spain)

5. Sterile Distilled water

6. Glass slab

7. Metal spatula

8. Plastic ring mold

9. Cover slip

10. 35-mm. tissue culture dish (Corning, USA)

11. 60-mm. Tissue culture dish (Corning, USA)

12. 24-well-flat-bottom plate (Nunc, Denmark)

13. 96-well-flat-bottom plate (Costar, USA)

14. 75 cm^2 cell culture flask (Corning, USA)

15. 25 cm^2 cell culture flask (Corning, USA)

16. CO_2 incubator

17. Laminar flow hood

18. Pasteur pipette

19. Pipette tip 20, 200, 1000 μl

20. Pipette 10, 25 ml

21. Hemocytometer

22. Microplate reader

23. Minimum essential medium alpha (α-MEM, Invitrogen, Grand Island, NY)

24. Fetal Bovine Serum (FBS) (Gibco BRL, USA)

25. 0.25% trypsin-EDTA (Gibco BRL, USA)

26. Dimethyl sulfoxide (DMSO)

27. Penicillin G (Gibco BRL, USA)

28. Streptomycin (Gibco BRL, USA)

29. Phosphate Buffer Saline (PBS)

30. RNeasy Mini Kit (Qiagen, K.K., Kyoto, Japan)

31. DNase I (Qiagen, Chatworth, CA, USA).

32. Improm- II reverse transcriptase (Improm-II™, Promega Corp, USA)

33. Lightcycler® 480 Real-Time PCR System (Roche Applied Science, Germany).

34. *p*-nitrophenylphosphate (pNPP) (Sigma- USA, product code. P7998)

35. 3 N NaOH solution

36. Microplate reader (Anthos, Zenyth 200rt)

Methods

1. <u>Cell culture</u>

An immortalized human cementoblast cell line (HCEM) was used in this study. The study was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University. These cells have been previously isolated and characterized (91). Cells were cultured in minimum essential medium alpha (α-MEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) plus penicillin G solution (10 U/ml) and streptomycin (10mg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C.
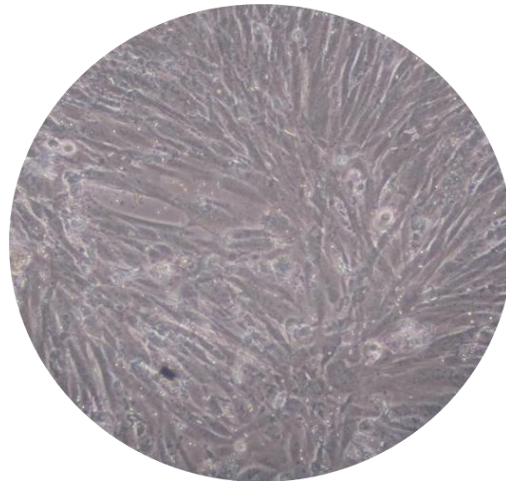
Figure 1 The morphology of HCEM in culture. (magnification 200X)

2. <u>**Preparation of Test Materials**</u>

Materials used in this study were white ProRoot[®] MTA of Dentsply Thailand Co., Ltd. and two commercial Thai White Portland cements which have been approved by TIS no. 133 2518 (1975), i.e. Thai White Portland cement, Chang brand of The Siam White cement Co., Ltd. and Kilan brand of Universal White cement Co., Ltd.

Thai White Portland cements were mixed with bismuth oxide (Fluka, Spain) at 4:1 ratio by weight using a grinding machine (RetschS1000 F, F.Kurt Retach GmbH&Co., KG, Germany) for creating homogeneous power, and then were sterilized with ethylene oxide gas. One gram of each white Portland cement mixed with bismuth oxide was mixed with 0.3 ml sterile distilled water and White ProRoot[®] MTA was mixed with distilled water according to the manufacturer's instruction.

Figure 2 Thai White Portland cements mixed with bismuth oxide

Figure 3 White ProRoot[®] MTA

Sixteen cylinder discs of 6 mm in diameter and 1 mm in height for each tested material were prepared under aseptic condition and then incubated for three hours in 95% humidity at 37°C. After removing specimens from plastic molds, all samples of each material were placed into a 60-mm tissue culture dish.
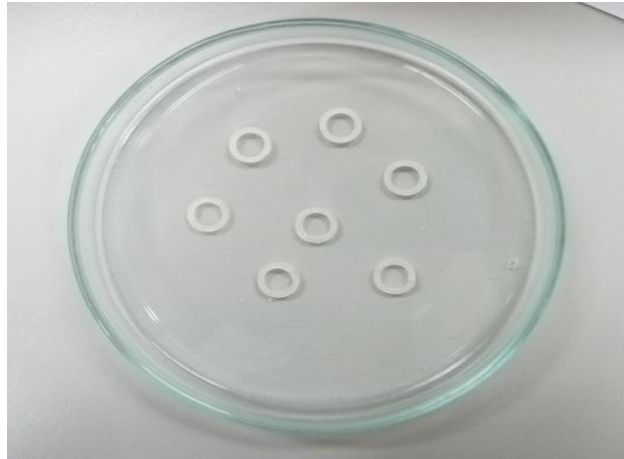
Figure 4 Plastic molds used in preparation of test materials

Figure 5 Chang or Kilan and sterile distilled water before mixing
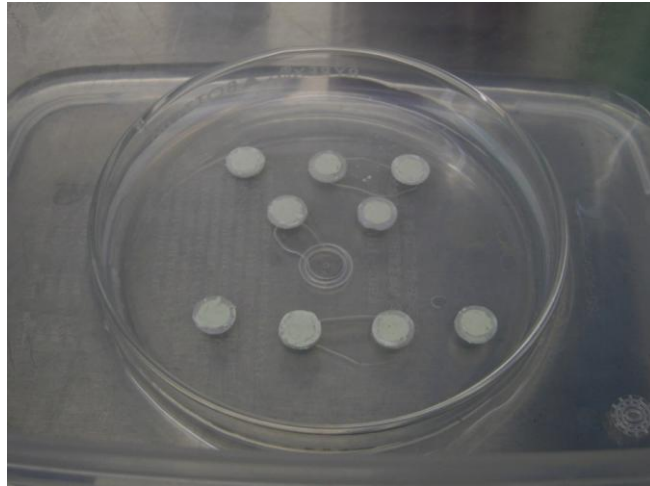
Figure 6 Plastic molds filled with material
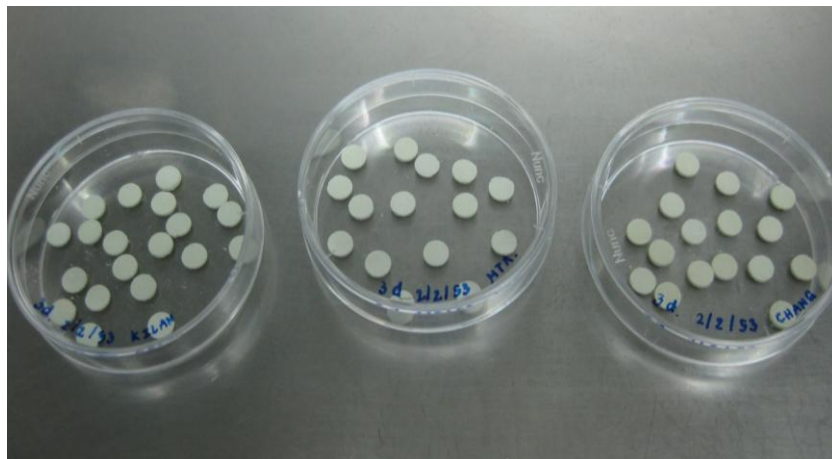
Figure 7 Samples were removed from plastic molds and placed into a 60-mm

tissue culture dish.

3. <u>Preparation of extract media</u>

The extraction ratio was adapted from International Standard ISO 10993-
12(1999). 220 µl/sample of minimum essential medium alpha (α-MEM) supplemented
with 10% fetal bovine serum (FBS) and penicillin G solution (10 U/ml), and streptomycin

was added into the culture dishes containing material discs and then incubated at the condition of 5% CO_2 , 37 °C (16 samples/ material/ day group). The extract medium was pipetted off the dish each day for usage. Subsequently, new medium was added to the dish for the following day extraction.
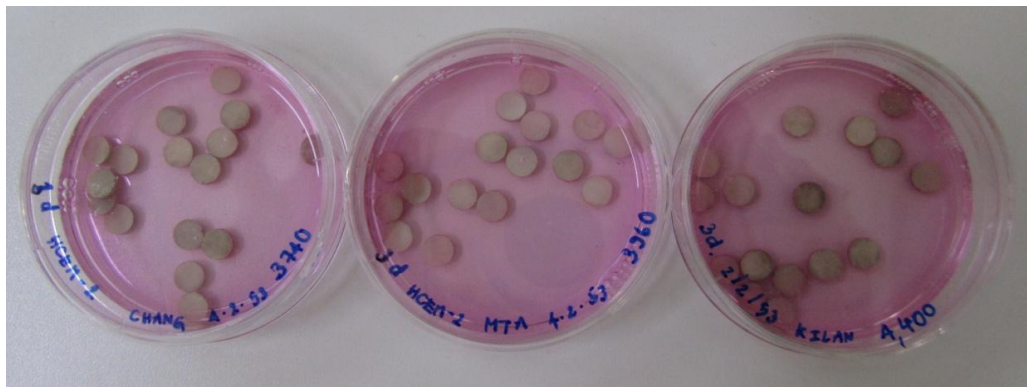
Figure 8 Samples in 60-mm culture dishes filled with culture medium.

4. <u>Real-time Polymerase chain reaction</u>

HCEM ($3X10^5$ cells/dish) was seeded in 60 mm dishes and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. for 24 hours to allow adhesion, and then culture media were replaced with extraction media every day.

At days 1,3 and 7, cells from one 60- mm culture dish were trypsinized. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, K.K., Kyoto, Japan) according to the manufacturer's instructions. The RNA samples were further purified by a successive treatment with DNase I (Qiagen, Chatworth, CA, USA). Then, they were spectrophotometrically quantitated at 260 and 280 nm and stored at -80 °C. A reverse transcription was performed using Improm- II reverse transcriptase (Improm-II™, Promega Corp, USA) as follows: 1 μg of RNA was added to 5 μl of reverse transcription mixture (1 μl of Oligo DT and Rnase-free water). The mixture was denatured at 70 °C for 5 min and chilled immediately on ice. Four

microliters of ImProm-II™ 5X Reaction Buffer, 4.8 µl of MgCl2, 1.0 µl of dNTP Mix (final concentration 0.5mM for each dNTP), 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor and 1.0 µl of ImProm-II™ and incubated at 25 °C for 5 min followed by 42 °C for 1 hour. The reactions were inactivated by heating at 70 °C for 15 min. A reaction without reverse transcriptase was used as a negative control.

Real-time polymerase chain reaction (PCR) was performed on a mixture of 5 µl of complementary DNA and 15 µL of master mix containing 10 µL SYBR Green PCR Master Mix (Invitrogen, USA), 1 mM of primers, and PCR-grade water using Lightcycler® 480 Real-Time PCR System (Roche Applied Science, Germany). The specific primers used in this study are alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen type I(COL-I), osteocalcin (OCN), and beta-2-microglobulin (B2M), as listed in Table 3. Beta-2-microglobulin (B2M) was used as the reference gene. Dnase free water was used as a negative control. A four-segment Light Cycler ™ PCR amplification and melting curve protocol were used. The program was set at 10-min denaturation step at 95 °C in order to activate the DNA polymerase followed by 45 amplification cycles at 90 °C, 60 °C and 72 °C for 10 s. Melting curve analysis was performed at 95 °C for 5 seconds, 65 °C for 1 minute and heating to 97°C using a ramp rate of 0.11°C/sec with continuous monitoring of fluorescence. The protocol was ended with a cooling segment (40 °C). Lightcycler® 480 software (Roche Applied Science, Germany) was used to estimate the threshold cycle numbers (crossing points; Cp). The standard curves were generated using the second derivative maximum method, in which the software based on default values determines the log-linear area. Data obtained by calibrator normalized relative quantification with efficiency correction based on the crossing points calculated by the Light- Cycler ™ software. (Roche Applied Science, Germany). The relative quantification in gene expression was determine using $2^{-\Delta\Delta Ct}$ method described previously(92). The quantification of each mRNA expression was evaluated by performing real-time PCR in triplicate.

Table 2. Primer sequences used for real time PCR

Gene	Sequence	Product size (basepair)	Reference
ALP - sense - antisense	5' GACAAGAAGCCCTTCACTGC 3' 5' AGACTGCGCCTGGTAGTTGT 3'	120	Shen et al. (93)
BSP - sense - antisense	5' GGGCAGTAGTGACTCATCCGAAG 3' 5' CTCCATAGCCCAGTGTTGTAGCAG 3'	208	Cordonnier et al. (94)
COL I - sense - antisense	5' AAGGTCATGCTGGTCTTGCT 3' 5' GACCCTGTTCACCTTTTCCA 3'	114	Ratisoontorn et al. (95)
OCN - sense - antisense	5' GAAGCCCAGCGGTGCA 3' 5' CACTACCTCGCTGCCCTCC 3'	70	Ratisoontorn et al. (95)
B2M - sense - antisense	5' GATGAGTATGCCTGCCGTGT 3' 5' CAATCCAAATGCGGCATCT 3'	114	Wellmann et al. (96)

5. <u>Alkaline Phosphatase activity</u>

HCEM were plated at 1×10^5 cells per well in 24-well plates. After an initial attachment period of 24 hours, they were exposed to an extracted media and changed every day. After 1, 3 and 7 days in culture, the extracted media were removed from wells and washed with Phosphate buffer solution for three times. The cells were lysed with 400 µl of cell lysis buffer and centrifuged at 300 X g for three minutes. The 100 µl of each cell lysate were added to 100 µl *p*-nitrophenylphosphate (pNPP) (Sigma-USA, product code. P7998) in 96-well plates. After thirty min of incubation at room temperature, the reaction was stopped by addition of 50 µl of 3 N NaOH solution, and the absorbance was read at 405 nm using a microplate reader (Anthos Zenyth 200rt, UK). To determine the specific activity of ALP, protein concentrations in each lysate were determined using the DC protein assay (Bio-Rad, USA). 5 µl of each cell lysate

were added into 96-well plates. After that, 25 µl of reagent A (an alkaline copper tartrate solution) mixed with 200 µl of reagent B (a dilute Folin Reagent) were added into each well. They were incubated for fifteen minutes at room temperature with gentle agitation. Absorbance was measured at 750 nm using the microplate reader. The protein concentration was calculated from a standard curve. The ALP-specific activity was determined using the following formula:

$$\text{ALP activity } (\mu mol\ \mu g^{-1}\ s^{-1}) = \frac{\text{ALP concentration } (\mu molmL^{-1})}{\text{Protein concentration } (\mu gmL^{-1})} \times \frac{1}{\text{ALP incubation time (s)}}$$

$$\text{ALP concentration } (\mu molmL^{-1}) = \frac{(\text{OD sample} - \text{ODblank}) \times \text{Reaction Volume}}{\varepsilon \times \text{Sample volume}}$$

(ε is the molar extinction coefficient ($M^{-1}.cm^{-1}$) which for pNPP is $\varepsilon = 18.8\ mM^{-1}.cm^{-1}$).

The experiment were performed in triplicate.

6. Calcium concentration

Material discs were incubated in medium without cells by the same extraction ratio as described above.Three material discs were placed into each cell culture dish containing 660 µL minimum essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin G solution (10 U/ml) and streptomycin. The extract medium was pipetted off the dish for experiment and new medium were replaced everyday.

Ca^{2+} in extract media was measured by using the Quantichrom Calcium Assay Kit (BioAssay Systems, Hayward, USA) everyday. 5 µL of extract media was mixed with 200 µL working reagents and tapped lightly to mix then read optical density

at 612 nm after 3 min incubation. The calcium concentration was calculated from a standard curve. The experiment were performed in triplicate.

7. Statistical analysis

All experiments were performed in triplicate. Data were expressed as the means of each group$\pm$ standard deviations (SD).

For statistical analysis, the Kruskal-Wallis test was performed by using the StatsDirect software version 2.7.8 to test the group differences in relative expression ratio of gene expression and alkaline phosphatase activity followed by Conover-Inman test for post hoc multiple comparisons at the 95% confidence interval.

8. Budgets

1. Cell culture	20,000 Baht
2. Material	80,000 Baht
3. Media and Reagent	10,000 Baht
4. Real time PCR	60,000 Baht
5. Alkaline phosphatase activity assay	10,000 Baht
6. Document and copying	3,000 Baht
Total	183,000 Baht

CHAPTER IV

Results

Cementoblastic differentiation marker gene expression by real-time quantitative RT-PCR

We examined the cementoblastic marker gene expression of HCEM at days 1, 3 and 7 in culture with regular medium (control), Chang extracted medium, Kilan extracted medium and MTA extracted medium. The levels of ALP, BSP, COL I and OCN were determined by real-time quantitative RT-PCR using B2M as reference gene. The normalized relative quantifications of each sample are shown in Figure 1- 4. In all experiments, the copy numbers of the individual housekeeping gene (B2M) were stable and constant. There are also no gene expressions observed both in the molecular biology grade water sample and the sample without reverse transcriptase (RT-). The melting peak generated represented the specific amplified product. All samples had only a single peak, indicating a pure product and no primer/dimer formation.

ALP   ALP expression was gradually increased at days 1 and 3 then decreased at day 7 in all groups. At day 1, only Chang extract significantly upregulated ALP expression while Kilan significantly reduced expression comparing to control. There was no statistically significant difference between MTA and control group. At day 3, the significant increase in ALP expression was occurred in white ProRoot[®] MTA and Chang-treated cells when compared with the control group. Kilan also stimulated ALP expression but there was no statistically significant difference when compared to the control group. At day 7, ALP gene expression of all groups decreased and there was no statistically significant difference between any groups. (Figure 9)

Figure 9 Relative expression ratio of ALP at days 1, 3 and 7. The bar represents the mean ± standard deviation. * = statistically significantly different at $p < 0.05$, ** = statistically significantly different at $p < 0.001$.

BSP BSP expression increased with culture time in all groups. At day 1, there was no significant difference in BSP expression between Chang and control group, but both Kilan and white ProRoot$^{®}$ MTA significantly decreased compared with the control and Chang groups. All experimental groups significantly increased BSP expression compared with the control group at day 3, of which Kilan showed highest BSP expression significantly more than Chang and MTA groups. The BSP expression at day 7 showed the same trend as day 3, but only Kilan group statistically significantly increased when compared with the control group. (Figure 10)

Figure 10 Relative expression ratio of BSP at days 1, 3 and 7. The bar represents the mean $\pm$ standard deviation. * = statistically significantly different at $p < 0.05$, ** = statistically significantly different at $p < 0.001$.

COL I Chang and Kilan significantly increased COL I expression compared with control and white ProRoot$^{®}$ MTA groups at day 1. However, there was a significantly decreased expression of all experimental groups compared with the control group at days 3 and 7 in all of experimental groups. At day 3, Chang significantly expressed COL I less than MTA, but Kilan significantly expressed COL I more than MTA. At day 7, both Chang and Kilan significantly expressed COL I less than MTA. (Figure 11)
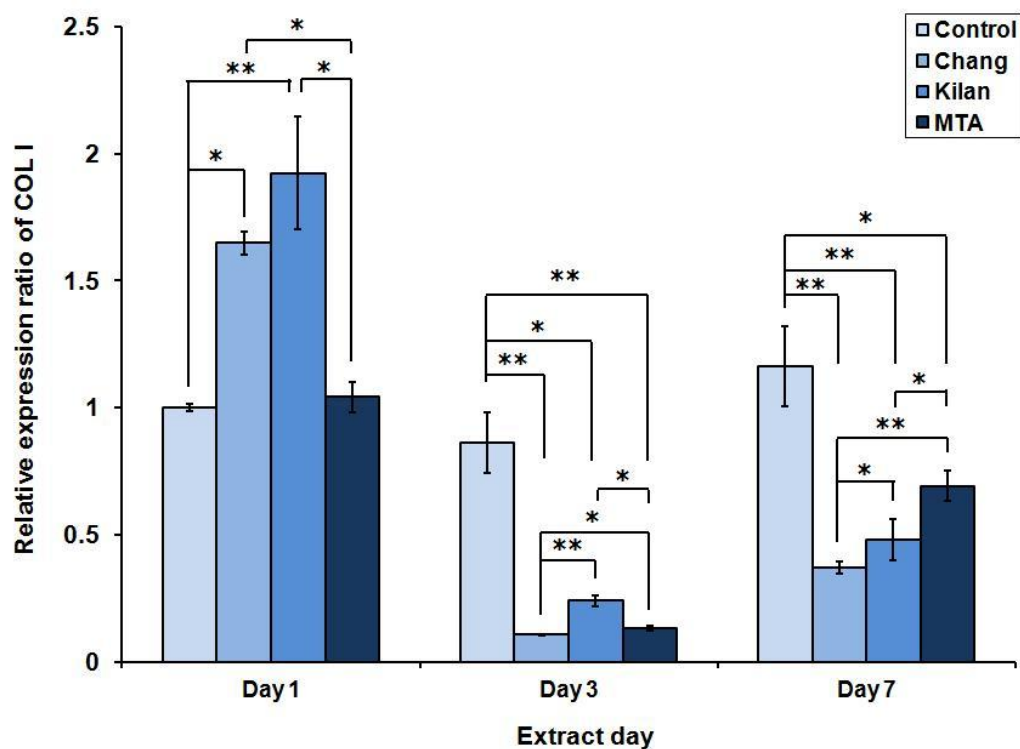
Figure 11 Relative expression ratio of COL I at days 1, 3 and 7. The bar represents the
mean $\pm$ standard deviation. * = statistically significantly different at $p < 0.05$,
** = statistically significantly different at $p < 0.001$.

OCN There was no difference among all groups at day 1, but all of experimental groups decreased expression compared with the control group at days 3 and 7. At day 3, MTA significantly expressed OCN less than Chang. (Figure 12)
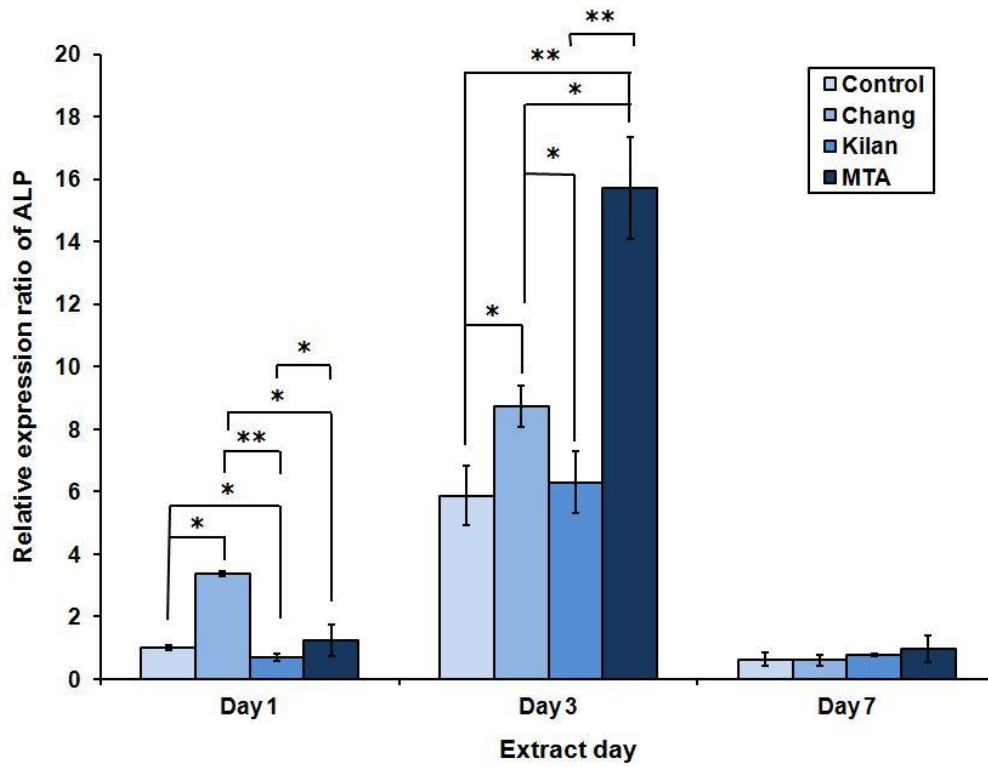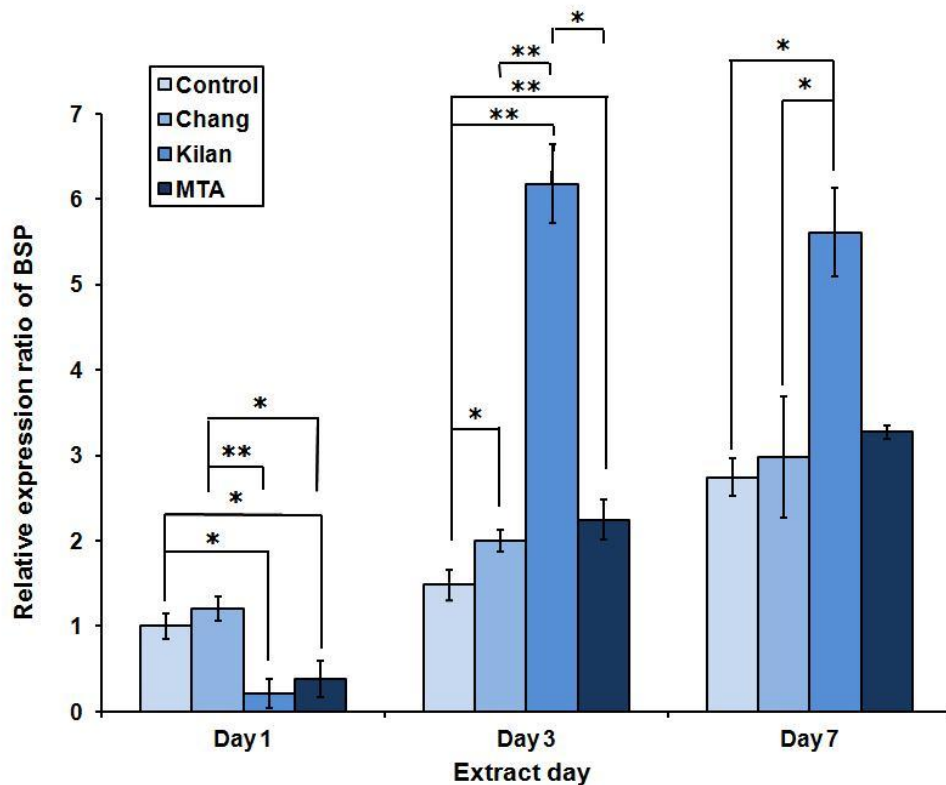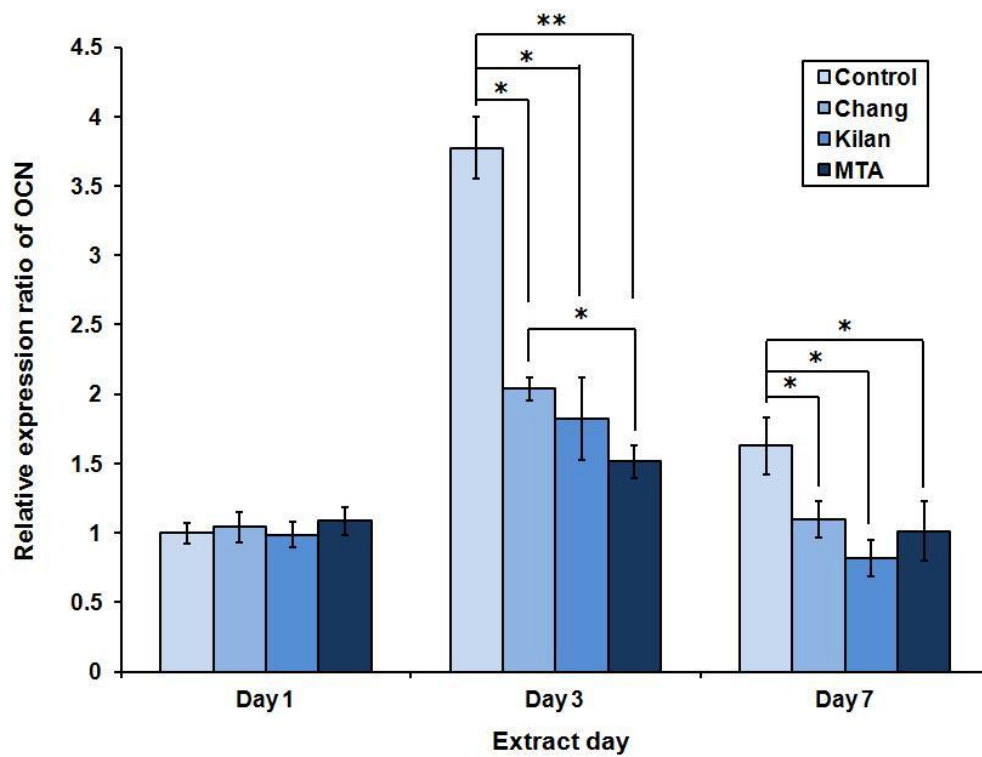

Figure 12 Relative expression ratio of OCN at days 1, 3 and 7. The bar represents the mean $\pm$ standard deviation. * = statistically significantly different at $p < 0.05$, ** = statistically significantly different at $p < 0.001$.

Alkaline phosphatase activity ALP activity is considered an important indicator of cementum mineralization. In our study, we determined ALP activity of HCEM cultured with Chang, Kilan and white ProRoot[®] MTA extracts at days 1, 3 and 7.

ALP activity of three groups was not detectable at day 1 (data not shown). ALP activity was observed after day 3 and increased in all groups at day 7. Chang showed the most expression of ALP activity followed by Kilan. The ALP activities at days 3 and 7 were statistically significantly higher than those of white ProRoot[®] MTA and control group. However, there was no significant difference between white ProRoot[®] MTA and control group. (Figure 13)
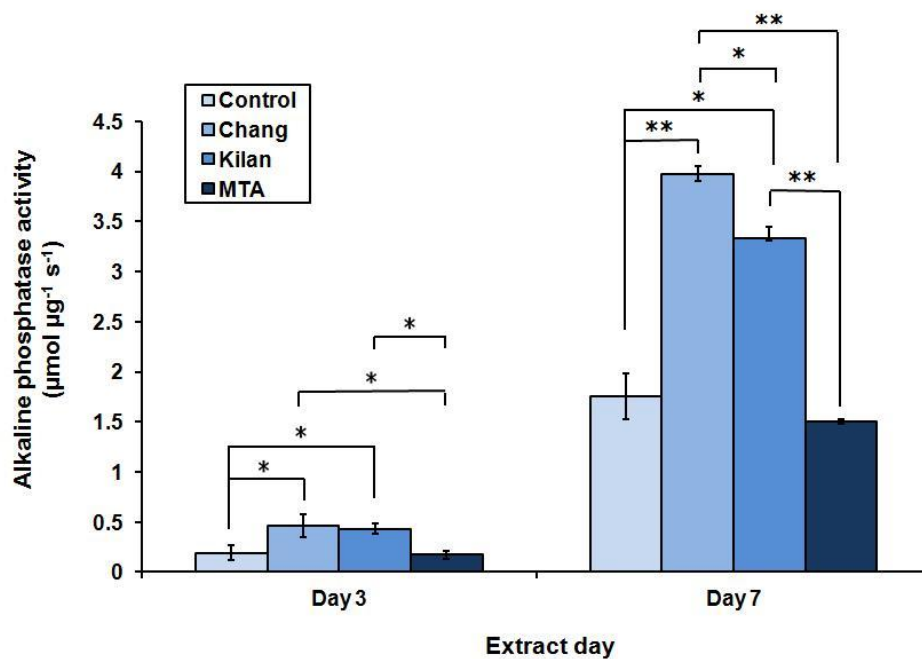
Figure 13 ALP activity of HCEM treated with material extracts and untreated control media at days 3 and 7. The bar represents the mean $\pm$ standard deviation. * = statistically significantly different at $p < 0.05$, ** = statistically significantly different at $p < 0.001$.

Calcium concentration At days 1 and 2, Chang significantly released more calcium ion than Kilan and MTA, respectively. After day 3, Kilan released more calcium ion than Chang and MTA. MTA significantly released calcium ion less than Chang and Kilan at all extract days. The a-MEM medium used in this study contained 0.2 mmol/L calcium ions. Thus, the more calcium ions released from the experimental materials into the culture medium. (Figure 14)
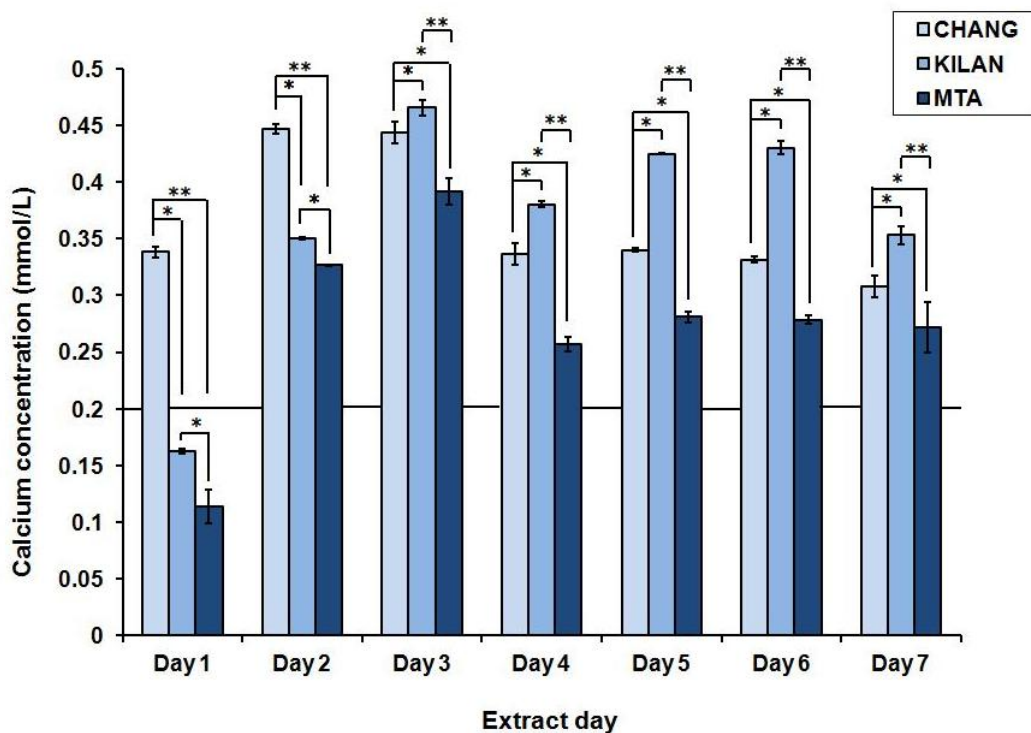
Figure 14 The amount of calcium ions in the extract media. The bar represents the mean $\pm$ standard deviation. * = statistically significantly different at $p <$ 0.05, ** = statistically significantly different at $p < 0.001$. Full line shows calcium ions containing in α-MEM supplemented with 10% FBS (0.2 mmol/L).

CHAPTER V

Discussion

Cementogenesis is a critical event for regeneration of periodontal tissues. The most appropriate cell type to study the effects of endodontic materials on cementogenesis is the cementoblast. This study utilizes the human cementoblast-like (HCEM) cell lines that have been established from human extracted tooth by tranfection of *hTERT* gene and shown to express bone/ cementum-related proteins, such as type I collagen, alkaline phosphatase, runt-related transcription factor 2, osteocalcin, bone sialoprotein and specific marker of cementum, cementum-derived protein.(CP-23). These cells also showed high activities of ALP and alizarin red staining. It is indicated that these cells produce mineralized calcified nodule. These cell lines were suggested that they are useful cell models for investigating the mechanism of differentiation of human cementoblasts(91).The advantages of using cell line are its reproducibility of the results, rapidly multiply, stable cell populations and large enough for biochemical analyses(97).

This study used extract media method to evaluate gene expression and ALP activity of HCEM. From histological and radiographic findings, mineralized tissue formation must take a long time; therefore, it may be influenced from indirect effect of material that gradual release rather than direct contact with materials. Furthermore, according to study in gingival and periodontal fibroblasts, it discovered that cell exposed to washed materials showed greater proliferation and maximal enzyme activity was detected earlier than those cultured with fresh materials(6). However, this method allows only for the assessment of the effects of diffusible components of the our Portland cements mixed with bismuth oxide and white ProRoot$^{®}$ MTA and may not represent the full effects of direct cell-material interactions. Under clinical (*in vivo*) conditions, cellular by-products are diluted in the interstitial tissue fluids and eliminated through the vascular. Therefore, this study have changed the solution daily to mimic the clinical situation.

The different concentrations of white ProRoot$^{®}$ MTA extract showed the different viabilities, morphologies and induced biomineralizations of OCCM-30 cell(10). Therefore, the concentrations of extract media may be a critical factor that influences the ALP activity and gene expression. The extraction ratio used in this study was adapted from International Standard ISO 10993-12(1999) in which previous study showed that this concentration was biocompatible with primary human alveolar bone osteoblast(17), as well as in HCEM(98).

To evaluate the effects of PC and white ProRoot$^{®}$ MTA on differentiation of HCEM, gene expression was measured by quantitative real-time PCR that provides quantitative measurements of gene expression changes over time, such as in the response of cell cultures with biomaterials and progression of cell differentiation. The advantages of this method are high precision, high sensitivity, real-time character, requiring less primary substance and simplicity. However, the relative quantification method did not determine the real copy number of transcript of interest. To compare gene expression between groups, each gene expression was normalized with housekeeping gene (B2M) and was analyzed as the change in expression of the target relative to calibrator (control group at day 1). B2M expression was stable and constant and seemed to be unregulated on extracted media or control substrates. Thus, it was suitable to be considered as a housekeeping gene in this study. In addition, in ALP activity experiment, normalization with protein concentration is requisite before comparing ALP activity between groups.

It is inconclusive whether cementoblasts are a unique cell type or a positional osteoblast regulated by its environment factors. Stein and Lian (99) demonstrated temporal manner of osteoblastic gene expression in three principle periods defining its developmental sequences: (1) proliferation, expressing proteins needed in cell divisions, such as histones, c-fos and c-myc and also COL I, (2) extracellular matrix

maturation, ALP is expressed immediately in post-proliferative period and declined in heavily mineralized cultures, and (3) mineralization, a differentiated osteoblast expresses OPN and BSP at the onset of mineralization and then expresses OCN in the late period correlated with calcium deposit and nodule formation. Although there have not been fully clarified regarding the detailed mechanism regulating cementoblastic differentiation but cementoblasts share many phenotypical features with osteoblasts. Therefore, there is a possibility that expression profile of molecular factor regulated cementoblastic differentiation is in similar manner as osteoblast.

ALP is a phosphate-releasing enzyme which is believed to participate in cementum mineralization(53, 64-67). There is a significantly positive correlation between ALP activity and thickness of adjacent acellular cementum layer, which indicates a close relationship between local phosphate production and cementum formation rate(65). Our result revealed ALP expression of both control and experimental groups which showed the peak level at day 3 and decresed at day 7. It may be explained by ALP expression of osteoblastic differentiation which indicated that ALP gene expression was elevated in a matrix maturation stage and declined in mineralization stage.

Many previous studies showed that MTA induced the ALP expression in many cell types such as human periodontal ligament fibroblast(6, 100), gingival fibroblast(6), human dental pulp stromal cells (DPSCs)(101), human osteosarcoma cell line (MG63)(85) and murine cementoblast cells (OCCM-30)(7). In this study, HCEM treated with MTA expressed ALP comparable with the control group at day 1, and MTA upregulated ALP expression at day 3 and decreased to a comparable level again at day 7. According to Nakayama et al.'s study(102), there was no difference in the expression of ALP mRNA in rat bone marrow cells (RBM) in the control and MTA group at the early stage of differentiation (days 1, 2 and 3). Morover, Bonson et al.'s study also showed that PDL fibroblasts cultured with MTA expressed ALP prematurely (at day 3), with

declining expression at days 7 and 14(6). This is similar to MTA treated cells also Chang treated cells in the study.

In the ALP activity aspect of this study, ALP activity was not detectable at day 1 because the high pH during setting stage of the materials may caused protein denature(103) or the amount of alkaline phosphatase protein is too small to detect the activity. The result of this study showed no significant difference of ALP activity between MTA and control groups which is similar to studies in rat bone marrow cells (RBM) and osteoblast-like cell line (MG-63) in which mineral trioxide aggregate maintained a level of ALP activity equivalent to unstimulated cells (102, 104). In addition, our results showed increased ALP activity at days 3 and 7 in Chang and Kilan groups consistent with those of Min et al. (86) which discovered that Portland cement and Portland cement mixed with bismuth oxide increased ALP activity.

Morover, a study found that high pH of calcium hydroxide can stimulate the mineralization through activation of tissue enzymes like ALP (105) whereas change in calcium ion does not appear to directly affect enzymatic activity, it may serve only as the cation which limits the solubility of hydroxyl ion(106). Thus, the possible mechanism that regulate ALP activity seems depending on state of alkalinity. The present study showed that Chang and Kilan groups significantly upregulate ALP activity at day 7 compared to control group but no significant difference between white ProRoot[®] MTA and control group. This may be due to the difference in their pH level. Although this study did not measure the pH value of the extracted media after different incubation times, previous study discovered that the pH value at the surface of fully set white ProRoot® MTA was 12.07 which was the highest pH value while Chang and Kilan were 11.43 and 11.46, respectively(47). While ALP activity was inhibited at the pH value of 11.9(106). Therefore, the ALP activity of white ProRoot[®] MTA may be suppressed by its highly alkaline agents. However, the pH value required for optimum activity *in vivo* may not be the same as that *in vitro* and the exact mechanism how two Thai white Portland cements

mixed with bismuth oxide stimulated ALP activity is still unclear. Further studies should be carried out to investigate this phenomenon.

We also found that ALP mRNA level in white ProRoot$^®$ MTA group was higher than in Chang and Kilan groups at days 3 and 7, but its enzyme activity was less. The inconsistent ALP gene expression and ALP activity in PDL and gingival fibroblasts have been previously reported(6). The correlation of transcript and protein stability with respect to the regulation of functional levels of phosphatase activity will need to be investigated.

BSP is one of major noncollagenous glycoproteins that are specially expressed in mineralized connective tissues(70-72). It is chemotactic to pre-cementoblasts and promotes their adhesion and differentiation(74). High level of BSP expression is associated with initiating formation of the mineralizing cementum matrix(71, 72) and this protein is a useful and reliable marker for cementoblast differentiation(53). The result of this study showed that BSP expression increased as the culture time increased in all experimental and control groups. This is the same manner like human osteoblast-like Saos2 cells(107) and human osteosarcoma cell line (MG63)(85).

We found that Chang, Kilan and white ProRoot$^®$ MTA upregulated BSP expression at day 3 when compared with the control group, and Kilan also upregulated this expression at day 7. These findings were consistent with previous study which BSP expression caused by osteoblast cells (MC3T3-E1) from 24 to 72 hours was evident in MTA-treated and control cultures (9), and BSP expression was also found to be upregulated in MTA-treated human dental pulp cells (108).

COL I is a primary organic extracellular matrix component of cementum that is required prior to the mineralized matrix formation(78). In cementum, collagen promotes cellular attachment, functions to maintain the integrity of hard and soft tissue, and is

active in their development, maturation, and repair(53). Our study discovered that total protein concentration was in similar trend as COL I expression because COL I is the major protein of the extracellular matrix. All of Chang, Kilan and MTA extract significantly decreased COL I expression at days 3 and 7. This is consistent with Nakayama et al.'s study (102) which mineral trioxide aggregate suppressed COL I expression in rat bone marrow osteoblast-like cells (RBM). However, COL I expression was upregulated only by Chang and Kilan at day 1. Therefore, Thai white cements mixed with bismuth oxide could induce more the COL I expression in the primary period of HCEM differentiation than MTA.

A study on murine immortalized cementoblast (OCCM-30), adding white ProRoot$^{®}$ MTA powder into cultured media at various concentrations found that 0.02 and 0.002 mg/ml concentrations could upregulate BSP and COL I expression at days 3 and 5 and slightly decreased OCN expressions were note in the MTA-treated groups at day 5(10). In this study, white ProRoot$^{®}$ MTA also increased BSP and decreased OCN expression in HCEM cell line at days 3 and 7. However, our result showed decreased COL I expression at days 3 and 7 which could be due to different methods of sample preparation.

An *in situ* hybridization study has shown that OCN expression was selective to root lining cells and was not present throughout the periodontal ligament in CD-1 mice. These findings indicated that OCN is marker selective to cementoblast(2). OCN plays a major role in the mineralization stage. Its expression is well correlated to the time of mineralized nodule formation. In this study, we could not detect the mineralized nodule even in a 28-day cell culture in alizarin red staining. Therefore, the 7-day gene expression study might be too short for detecting the OCN expression. However, we expected that OCN expression was gradually upregulated with the culture time, but we found high OCN expression in the control group at day 3 and it decreased at day 7. OCN expression in human alveolar bone osteoblast cell lines could be detected at day 3

and down-regulated at day 7 to day 14 then upregulated at day 21 to day 28 (109). Because this study investigated OCN expression of HCEM up to day 7, OCN down regulation may be similar to early stage of human alveolar bone osteoblasts.

There was no difference in OCN gene expression observed among all groups at day 1 but decrease expression was noted in the Chang, Kilan and MTA–treated groups compared with the untreated control group at days 3 and 7. This finding agrees with a report in mouse MC3T3-E1 osteoblast cells that OCN expression was inhibited in the presence of MTA(110). However, our study is inconsistent with Tani-ishii's study(9) which used transwell plates with filter membrane bottoms. Moreover, our finding does not agree with Thomson et al.'s study(7) which demonstrated that cementoblasts maintain expression of OCN in the presence of mineral trioxide aggregate by confocal microscopy and Koh et al.'s study(104) which used ELISA assays and found that OCN production was enhanced when osteoblasts (MG-63) were grown on mineral trioxide aggregate. The differences between these studies and ours could be because of different detection methods.

As previously mentioned that ALP, BSP, COL I and OCN are crucial for cementogenesis. These genes play important roles in proliferation, extracellular matrix maturation and mineralization stages during the differentiation of cementoblast. Therefore, upregulation of ALP and BSP gene and ALP activity of HCEM upon incubation with extract media indicated that these materials may induce cementoblastic differentiation.

The possible mechanism which both PC and white ProRoot$^®$ MTA contribute to increase the cementoblastic differentiation is the ions released from these materials. The main chemical composition of these materials is calcium. Many studies revealed that Portland cements and white ProRoot$^®$ MTA can release calcium ions (8, 111-113) which have been implicated in enhancing the osteoblastic differentiation(114), stimulating the

bone Morphogenetic Protein-2 expression and calcification in human periodontal ligament cells (113), upregulated the levels of BSP mRNA in Saos2 osteoblast-like cells (107) and modulating the cementogenesis via increased fibroblast growth factor-2 expression in OCCM-30(115). The result of this study shows that Chang, Kilan and MTA generally release calcium ions except Kilan and MTA groups at day 1 which calcium concentration was lower than those of minimum essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (FBS) used in this study which was approximately 0.2 mmol/L. This might not be because calcium ions were not released from materials. Previous study using x-ray analytical microscope to investigate chemical composition found that Calcium ions of Chang, Kilan and MTA were similar and maximum solubility was shown at day 1 and decreased at days 7 and 21. Although there is no statistical significant difference, the solubility of MTA is more than of Kilan and Chang, respectively(47). Therefore, the failure to detect calcium ions released from Kilan and white ProRoot® MTA at day 1 is thought to be the result of the calcium ions released from materials being incorporated with phosphate ions from the medium to form hydroxycarbonate apatite (HCA). Moreover, we did not detect calcium-sensing receptor (CaSR), class 3 G-protein-coupled receptor expression in HCEM by RT-PCR (data not shown). Therefore, we believe that CaSR may not be involved in modulating cementoblastic differentiation of HCEM. Nevertheless, calcium might be regulated via other pathways. Another possible ion that PC and white ProRoot® MTA contribute to increased cementoblastic differentiation is the Silicon (Si) ion which is released from calcium silicate, one of the main constituents in both PC and white ProRoot® MTA. This Si ion has been previously reported to increase gene expression of the differentiation markers, COL I ,OCN, ALP, and ALP activity of MG-63 osteoblast-like cells(116). Taken together, the ionic dissolution products releasing from PC and MTA are probably beneficial to the differentiation of HCEM. Further studies are necessary to be performed to indicate the bioactive components releasing from both PC and white ProRoot® MTA and its possible signaling pathways.

There are some limitations in the present study. The current study was an *in vitro* experiment using HCEM, the results from this study can only assess the effects of two Thai white Portland cements mixed with bismuth oxide and white ProRoot® MTA on the expression of markers of cementoblastic differentiation and alkaline phosphatase activity in HCEM. Although HCEM resembles cementoblast in some respects but their underlying biological regulatory mechanisms may differ fundamentally from those of primary cementoblasts. Moreover, periradicular wound healing is complex, involved various cellular and molecular factors. Animal studies and clinical investigations are needed before these Thai white Portland cements mixed with bismuth oxide can be used in clinical practices.

In conclusion, the results of this study demonstrated that Chang upregulated ALP at days 1 and 3 and white ProRoot® MTA upregulated ALP at day 3. All materials stimulated BSP expression at day 3 and Kilan also upregulated BSP at day 7. Both Chang and Kilan increased COL I expression at day 1 but gene expression of OCN was decreased by all materials at days 3 and 7. Moreover, Chang and Kilan induced ALP activity more than white ProRoot® MTA and control at days 3 and 7. These results suggest that two Thai white Portland cements mixed with bismuth oxide may be an alternative retrofilling materials that could be beneficial in cemental repair in endodontic periapical surgery.

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APPENDIX

Table 1   Relative expression ratio of ALP of HCEM treated with material extracts and untreated control media.

	Relative expression ratio of ALP (mean ± SD)		
Extract day / Material	Day 1	Day 3	Day 7
Control	1.000000016 ± 0.079811272	5.867418544 ± 0.958885629	0.634679456 ± 0.2259821
Chang	3.37873431 ± 0.077905004	8.722475811 ± 0.66289587	0.605428026 ± 0.17625693
Kilan	0.701228252 ± 0.113945441	6.291943226 ± 0.99126909	0.776692369 ± 0.03771933
White ProRoot®MTA	1.225066827 ± 0.512124824	15.729858 ± 1.625419	0.968047807 ± 0.423938478

Table 2   Relative expression ratio of BSP of HCEM treated with material extracts and untreated control media.

	Relative expression ratio of BSP (mean ± SD)		
Extract day / Material	Day 1	Day 3	Day 7
Control	0.999999561 ± 0.146338113	1.485169849 ± 0.17755659	2.744567374 ± 0.22318474
Chang	1.201865234 ± 0.145785	1.997198603 ± 0.123776	2.983766587 ± 0.706899
Kilan	0.208078 ± 0.167172	6.180297515 ± 0.458029481	5.610393031 ± 0.518628157
White ProRoot®MTA	0.381582713 ± 0.212675	2.247956576 ± 0.235479	3.272518193 ± 0.078449

Table 3 Relative expression ratio of COL I of HCEM treated with material extracts and untreated control media.

	Relative expression ratio of COL I (mean $\pm$ SD)		
Extract day / Material	Day 1	Day 3	Day 7
Control	1 $\pm$ 0.013862611	0.864018905 $\pm$ 0.119491655	1.163720806 $\pm$ 0.155899688
Chang	1.647896133 $\pm$ 0.04394484	0.106255746 $\pm$ 0.004363747	0.37136829 $\pm$ 0.023431124
Kilan	1.923788246 $\pm$ 0.222691328	0.240295222 $\pm$ 0.019930458	0.480703913 $\pm$ 0.080606091
White ProRoot®MTA	1.041484621 $\pm$ 0.058743978	0.131818723 $\pm$ 0.007992822	0.692972257 $\pm$ 0.058118514

Table 4 Relative expression ratio of OCN of HCEM treated with material extracts and untreated control media.

	Relative expression ratio of OCN (mean $\pm$ SD)		
Extract day / Material	Day 1	Day 3	Day 7
Control	1.000000075 $\pm$ 0.072633916	3.773305758 $\pm$ 0.223510016	1.62678821 $\pm$ 0.203546197
Chang	1.042529973 $\pm$ 0.108169648	2.03755858 $\pm$ 0.08019738	1.095031482 $\pm$ 0.131414394
Kilan	0.98752839 $\pm$ 0.093777693	1.8225524 $\pm$ 0.296329713	0.8172735 $\pm$ 0.130137567
White ProRoot®MTA	1.085031195 $\pm$ 0.102105825	1.515043558 $\pm$ 0.117875141	1.01252911 $\pm$ 0.21516601

Table 5   Total protein concentration of HCEM treated with material extracts and untreated control media.

	Total protein concentration (µg/mL) (mean$\pm$ SD)		
Extract day / Material	Day 1	Day 3	Day 7
Control	0.446725318 $\pm$ 0.101586558	0.938742261 $\pm$ 0.078404753	0.889866406 $\pm$ 0.073368069
Chang	0.638970349 $\pm$ 0.046195656	0.779081134 $\pm$ 0.211695091	0.762789182 $\pm$ 0.050162282
Kilan	0.583577713 $\pm$ 0.099207151	0.788856305 $\pm$ 0.042608983	0.987618117 $\pm$ 0.139731389
White ProRoot[®]MTA	0.547735419 $\pm$ 0.062845557	0.596611274 $\pm$ 0.05558397	0.599869664 $\pm$ 0.020348641

Table 6   ALP activity of HCEM treated with material extracts and untreated control media.

	Alkaline phosphatase activity (µmol µg^{-1} s^{-1}) (mean$\pm$ SD)	
Extract day / Material	Day 3	Day 7
Control	0.193491 $\pm$ 0.075676	1.751332 $\pm$ 0.230977
Chang	0.466205 $\pm$ 0.112062	3.976241 $\pm$ 0.070276
Kilan	0.430095 $\pm$ 0.05526	3.335838 $\pm$ 0.113737
White ProRoot[®]MTA	0.174541 $\pm$ 0.041793	1.507651 $\pm$ 0.023931

<u>Total protein concentration</u> The total protein concentration increased with the cultured time. All experimental materials upregulated total protein concentration at day 1 but they decreased this concentration at day 3. However, there was no statistically significant difference between all groups at days 1 and 3. At day 7, Chang and MTA statistically significantly reduced protein concentration as compared with the control group. Kilan increased protein concentration when compared with the control group but there was no statistically significant difference. (Figure 15)
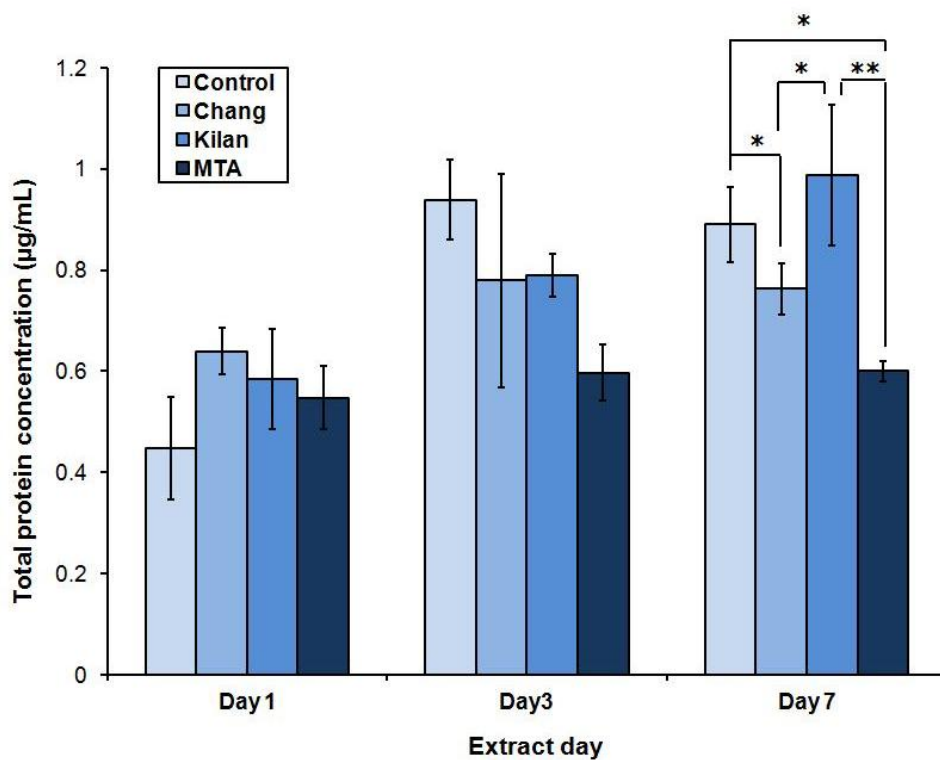

Figure 15 The total protein concentration released of HCEM treated with material extracts and untreated control media at days 1, 3 and 7. The bar represents the mean $\pm$ standard deviation. * = statistically significantly different at $p < 0.05$, ** = statistically significantly different at $p < 0.001$.

Alizarin red S staining of mineralized nodules The mineralized nodules could not be detected even in the 28-day cell culture. We also did not find any mineralization in cells grown in medium with or without ascorbic acid and dexamethasone. (Figure 16)
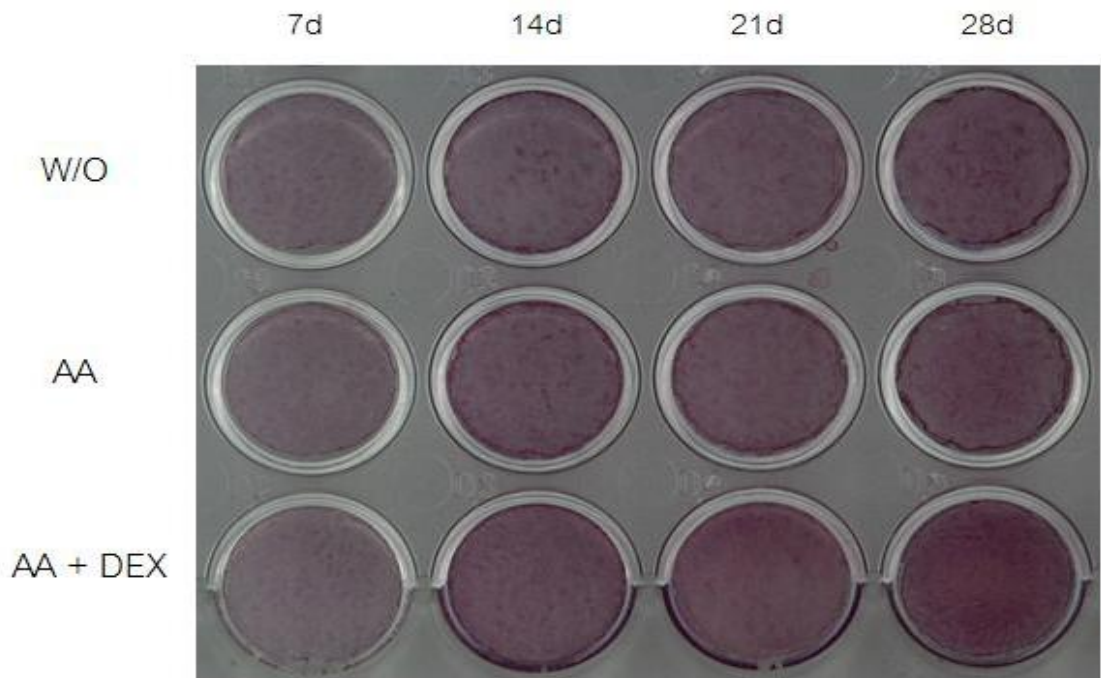

Figure 16 Alizarin red S staning. 1X 10^5 HCEM cells were cultured in the α-MEM supplemented with 10% FBS for 7, 14, 21and 28 days. W/O = cells grown in the medium without AA and Dexamethasone. AA = cells grown in the medium with ascorbic acid. AA + DEX = cells grown in the medium with ascorbic acid and Dexamethasone.

Statistic analysis

1. Kruskal-Wallis results of relative expression ratio of ALP gene expression in HCEM

   treated with material extracts medium at day 1

   Kruskal-Wallis test

   Variables: Control day1, Chang day1, Kilan day1, MTA day1

   Groups = 4
   df = 3
   Total observations = 12

   T = 3.923077
   P = 0.2699

2. Kruskal-Wallis results of relative expression ratio of ALP gene expression in HCEM

   treated with material extracts medium at day 3

   Kruskal-Wallis test

   Variables: Control day3, Chang day3, Kilan day3, MTA day3

   Groups = 4
   df = 3
   Total observations = 12

   T = 9.461538
   P = 0.0237

   Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
   Critical t (8 df) = 2.306004

Control day3 and Chang day3	significant	
(5 > 2.977039)	P = 0.0047	
Control day3 and Kilan day3	not significant	
(1 > 2.977039)	P = 0.4609	
Control day3 and MTA day3	significant	
(8 > 2.977039)	P = 0.0003	
Chang day3 and Kilan day3	significant	
(4 > 2.977039)	P = 0.0147	
Chang day3 and MTA day3	significant	
(3 > 2.977039)	P = 0.0486	
Kilan day3 and MTA day3	significant	
(7 > 2.977039)	P = 0.0006	

3. Kruskal-Wallis results of relative expression ratio of ALP gene expression in HCEM treated with material extracts medium at day 7

Kruskal-Wallis test

Variables: Control day7, Chang day7, Kilan day7, MTA day7

Groups = 4
df = 3
Total observations = 12

T = 5.615385
P = 0.1319

4. Kruskal-Wallis results of relative expression ratio of BSP gene expression in HCEM treated with material extracts medium at day 1

Kruskal-Wallis test

Variables: Control day 1, Chang day 1, Kilan day 1, MTA day 1

Groups = 4
df = 3
Total observations = 12

T = 8.948718
P = 0.03

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (8 df) = 2.306004

Control day 1 and Chang day 1	not significant	
(1.666667 > 3.437588)	P = 0.296	
Control day 1 and Kilan day 1	significant	
(6 > 3.437588)	P = 0.0038	
Control day 1 and MTA day 1	significant	
(4.333333 > 3.437588)	P = 0.0197	
Chang day 1 and Kilan day 1	significant	
(7.666667 > 3.437588)	P = 0.0009	
Chang day 1 and MTA day 1	significant	
(6 > 3.437588)	P = 0.0038	
Kilan day 1 and MTA day 1	not significant	
(1.666667 > 3.437588)	P = 0.296	

5. Kruskal-Wallis results of relative expression ratio of BSP gene expression in HCEM

   treated with material extracts medium at day 3

   Kruskal-Wallis test

   Variables: Control day 3, Chang day 3, Kilan day 3, MTA day 3

   Groups = 4
   df = 3
   Total observations = 12

   T = 9.974359
   P = 0.0188

   Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
   Critical t (8 df) = 2.306004

Control day 3 and Chang day 3	significant
(3.333333 > 2.430742)	P = 0.0133
Control day 3 and Kilan day 3	significant
(9 > 2.430742)	P < 0.0001
Control day 3 and MTA day 3	significant
(5.666667 > 2.430742)	P = 0.0007
Chang day 3 and Kilan day 3	significant
(5.666667 > 2.430742)	P = 0.0007
Chang day 3 and MTA day 3	not significant
(2.333333 > 2.430742)	P = 0.0578
Kilan day 3 and MTA day 3	significant
(3.333333 > 2.430742)	P = 0.0133

6. Kruskal-Wallis results of relative expression ratio of BSP gene expression in HCEM

   treated with material extracts medium at day 7

   Kruskal-Wallis test

   Variables: Control day 7, Chang day 7, Kilan day 7, MTA day 7

   Groups = 4
   df = 3
   Total observations = 12

   T = 7.820513
   P = 0.0499

   Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
   Critical t (8 df) = 2.306004

Control day 7 and Chang day 7	not significant
(1.333333 > 4.279763)	P = 0.4929

Control day 7 and Kilan day 7   significant
(7.666667 > 4.279763)   P = 0.0033
Control day 7 and MTA day 7   not significant
(3.666667 > 4.279763)   P = 0.0836
Chang day 7 and Kilan day 7   significant
(6.333333 > 4.279763)   P = 0.0092
Chang day 7 and MTA day 7   not significant
(2.333333 > 4.279763)   P = 0.2441
Kilan day 7 and MTA day 7   not significant
(4 > 4.279763)   P = 0.0633

7. Kruskal-Wallis results of relative expression ratio of COL I gene expression in HCEM treated with material extracts medium at day 1

Kruskal-Wallis test

Variables: Control day 1, Chang day 1, Kilan day 1, MTA day 1

Groups = 4
df = 3
Total observations = 12

T = 9.564103
P = 0.0227

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (8 df) = 2.306004

Control day 1 and Chang day 1   significant
(6 > 2.876092)   P = 0.0013
Control day 1 and Kilan day 1   significant
(8.333333 > 2.876092)   P = 0.0002
Control day 1 and MTA day 1   not significant
(2.333333 > 2.876092)   P = 0.0983
Chang day 1 and Kilan day 1   not significant
(2.333333 > 2.876092)   P = 0.0983
Chang day 1 and MTA day 1   significant
(3.666667 > 2.876092)   P = 0.0187
Kilan day 1 and MTA day 1   significant
(6 > 2.876092)   P = 0.0013

8. Kruskal-Wallis results of relative expression ratio of COL I gene expression in HCEM treated with material extracts medium at day 3

Kruskal-Wallis test

Variables: Control day 3, Chang day 3, Kilan day 3, MTA day 3

Groups = 4
df = 3
Total observations = 12

T = 10.384615
P = 0.0156

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (8 df) = 2.306004

Control day 3 and Chang day 3	significant	
(9 > 1.882844)	P < 0.0001	
Control day 3 and Kilan day 3	significant	
(3 > 1.882844)	P = 0.0063	
Control day 3 and MTA day 3	significant	
(6 > 1.882844)	P < 0.0001	
Chang day 3 and Kilan day 3	significant	
(6 > 1.882844)	P < 0.0001	
Chang day 3 and MTA day 3	significant	
(3 > 1.882844)	P = 0.0063	
Kilan day 3 and MTA day 3	significant	
(3 > 1.882844)	P = 0.0063	

9. Kruskal-Wallis results of relative expression ratio of COL I gene expression in HCEM treated with material extracts medium at day 7

Kruskal-Wallis test

Variables: Control day 7, Chang day 7, Kilan day 7, MTA day 7

Groups = 4
df = 3
Total observations = 12

T = 10.384615
P = 0.0156

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (8 df) = 2.306004

Control day 7 and Chang day 7	significant	
(9 > 1.882844)	P < 0.0001	
Control day 7 and Kilan day 7	significant	
(6 > 1.882844)	P < 0.0001	
Control day 7 and MTA day 7	significant	
(3 > 1.882844)	P = 0.0063	
Chang day 7 and Kilan day 7	significant	
(3 > 1.882844)	P = 0.0063	
Chang day 7 and MTA day 7	significant	

(6 > 1.882844)　　　　　　P < 0.0001
Kilan day 7 and MTA day 7　　significant
(3 > 1.882844)　　　　　　P = 0.0063

10. Kruskal-Wallis results of relative expression ratio of OCN gene expression in HCEM

   treated with material extracts medium at day 1

   Kruskal-Wallis test

   Variables: Control day 1, Chang day 1, Kilan day 1, MTA day 1

   Groups = 4
   df = 3
   Total observations = 12

   T = 1.769231
   P = 0.6217

11. Kruskal-Wallis results of relative expression ratio of OCN gene expression in HCEM

   treated with material extracts medium at day 3

   Kruskal-Wallis test

   Variables: Control day 3, Chang day 3, Kilan day 3, MTA day 3

   Groups = 4
   df = 3
   Total observations = 12

   T = 8.74359
   P = 0.0329

   Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
   Critical t (8 df) = 2.306004

   Control day 3 and Chang day 3　　significant
   (3.666667 > 3.605373)　　　　P = 0.047
   Control day 3 and Kilan day 3　　significant
   (6 > 3.605373)　　　　　　P = 0.005
   Control day 3 and MTA day 3　　significant
   (8.333333 > 3.605373)　　　　P = 0.0007
   Chang day 3 and Kilan day 3　　not significant
   (2.333333 > 3.605373)　　　　P = 0.1739
   Chang day 3 and MTA day 3　　significant
   (4.666667 > 3.605373)　　　　P = 0.0175
   Kilan day 3 and MTA day 3　　not significant
   (2.333333 > 3.605373)　　　　P = 0.1739

12. Kruskal-Wallis results of relative expression ratio of OCN gene expression in

    HCEM treated with material extracts medium at day 7

    Kruskal-Wallis test

    Variables: Control day 7, Chang day 7, Kilan day 7, MTA day 7

    Groups = 4
    df = 3
    Total observations = 12

    T = 7.820513
    P = 0.0499

    Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
    Critical t (8 df) = 2.306004

Control day 7 and Chang day 7	significant	
(4.333333 > 4.279763)	P = 0.0478	
Control day 7 and Kilan day 7	significant	
(8 > 4.279763)	P = 0.0026	
Control day 7 and MTA day 7	significant	
(5.666667 > 4.279763)	P = 0.0157	
Chang day 7 and Kilan day 7	not significant	
(3.666667 > 4.279763)	P = 0.0836	
Chang day 7 and MTA day 7	not significant	
(1.333333 > 4.279763)	P = 0.4929	
Kilan day 7 and MTA day 7	not significant	
(2.333333 > 4.279763)	P = 0.2441	

13. Kruskal-Wallis results of ALP activity of HCEM treated with material extracts medium

    at day 3

    Kruskal-Wallis test

    Variables: Control day 3, Chang day 3, Kilan day 3, MTA day 3

    Groups = 4
    df = 3
    Total observations = 12

    T = 8.362573
    P = 0.0391

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (8 df) = 2.306004

Control day 3 and Chang day 3	significant	
(6 > 3.919453)	P = 0.0077	
Control day 3 and Kilan day 3	significant	
(5.666667 > 3.919453)	P = 0.0103	
Control day 3 and MTA day 3	not significant	
(.333333 > 3.919453)	P = 0.8494	
Chang day 3 and Kilan day 3	not significant	
(.333333 > 3.919453)	P = 0.8494	
Chang day 3 and MTA day 3	significant	
(6.333333 > 3.919453)	P = 0.0058	
Kilan day 3 and MTA day 3	significant	
(6 > 3.919453)	P = 0.0077	

14. Kruskal-Wallis results of ALP activity of HCEM treated with material extracts medium

at day 7

Kruskal-Wallis test

Variables: Control day 7, Chang day 7, Kilan day 7, MTA day 7

Groups = 4
df = 3
Total observations = 12

T = 10.044601
P = 0.0182

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (8 df) = 2.306004

Control day 7 and Chang day 7	significant	
(6.333333 > 2.430742)	P = 0.0003	
Control day 7 and Kilan day 7	significant	
(3.333333 > 2.430742)	P = 0.0133	
Control day 7 and MTA day 7	not significant	
(2.333333 > 2.430742)	P = 0.0578	
Chang day 7 and Kilan day 7	significant	
(3 > 2.430742)	P = 0.0216	
Chang day 7 and MTA day 7	significant	
(8.666667 > 2.430742)	P < 0.0001	
Kilan day 7 and MTA day 7	significant	
(5.666667 > 2.430742)	P = 0.0007	

BIOGRAPHY

Flight Lieutenant Treesukhon Eakbannasingh was born on 26th November 1981 in Nakhon Si Thammarat. She graduated with D.D.S. (Doctor of Dental surgery) from the Faculty of Dentistry, Chulalongkorn University in 2006, and had worked as a dentist at Wing 56 hospital, Songkla for 3 years. She studied in a Master degree program in Endodontology at Graduate School, Chulalongkorn University in 2011. At the present, she has worked as a government official of the Directorate of Medical Services, Royal Thai Air Force.