การพัฒนาวัสดุปิดเนื้อเยื่อในโพรงฟันโดยมีฟลูโอซิโนโลน อะซีโทไนด์เป็นส่วนประกอบ

นายภูมิศักดิ์ เลาวกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาทันตชีววัสดุศาสตร์ (สหสาขาวิชา)
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DEVELOPMENT OF A PULP CAPPING MATERIAL CONTAINING FLUOCINOLONE ACETONIDE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Dental Biomaterial Science
(Interdisciplinary Program)
Graduate School
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Thesis Title	DEVELOPMENT OF A PULP CAPPING MATERIAL CONTAINING FLUOCINOLONE ACETONIDE
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Field of Study	Dental Biomaterials Science
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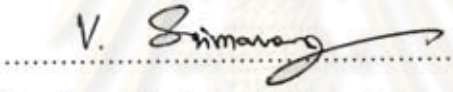

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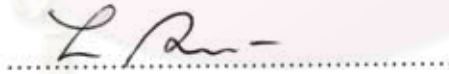

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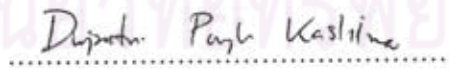

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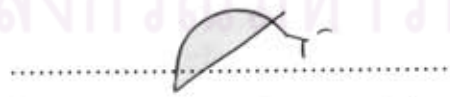

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ภูมิศักดิ์ เลาวกุล : การพัฒนาวัสดุปิดเนื้อเยื่อในโพรงฟันโดยมีฟลูโอซิโนโลน อะซีโทไนด์เป็นส่วนประกอบ. (DEVELOPMENT OF A PULP CAPPING MATERIAL CONTAINING FLUOCINOLONE ACETONIDE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร. วีระ เลิศจิราการ, 139 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวัสดุปิดเนื้อเยื่อในโพรงฟันโดยมีฟลูโอซิโนโลน อะซีโทไนด์เป็นส่วนประกอบ และทดสอบคุณสมบัติทางกายภาพ เชิงกล และชีววิทยาบางประการ วัสดุชนิดนี้เป็นซีเมนต์ชนิดแคลเซียมไฮดรอกไซด์ที่สามารถก่อตัวได้ และมีฟลูโอซิโนโลน อะซีโทไนด์ที่มีความเข้มข้น 50 มิลลิโมลาร์ต่อลิตรเป็นส่วนประกอบ ผู้วิจัยได้ทำการศึกษาการปลดปล่อยฟลูโอซิโนโลน อะซีโทไนด์จากวัสดุด้วยเทคนิคไฮเปอร์ฟอร์มานซ์ลิควิดโครมาโตกราฟี, วัดค่าความเป็นกรดด่างโดยใช้เครื่องวัดพีเอช, ทดสอบคุณสมบัติต่างๆ ของวัสดุตามมาตรฐานไอเอสโอ 3107:2004 ได้แก่ เวลาแข็งตัว, ความทนแรงอัด, การแตกตัว, และปริมาณของสารหนูที่ละลายได้ในกรด, ทดสอบความคงตัวของวัสดุในสภาวะเร่ง, ศึกษาการตอบสนองของเซลล์เพาะเลี้ยงจากเนื้อเยื่อในโพรงฟันมนุษย์ต่อสารที่ปลดปล่อยมาจากวัสดุ โดยทดสอบความเป็นพิษต่อเซลล์และการเพิ่มจำนวนเซลล์ด้วยวิธีเอ็มทีที, วัดปริมาณการสร้างอาร์เอ็นเอชนิดเดนทีนเซียโลฟอสโฟโปรตีนด้วยวิธีอาร์ทีพีซีอาร์ และโปรตีนชนิดเดนทีนเซียโลโปรตีนด้วยวิธีเวสเทิร์น, และศึกษาการต้านต่อการอักเสบโดยวัดการสร้างอาร์เอ็นเอชนิดไซโคลออกซีจีเนส 2 ทั้งนี้ได้ใช้วัสดุที่มีจำหน่ายในท้องตลาดในชื่อการค้า Dycal® เป็นวัสดุเปรียบเทียบ ผลการศึกษาพบว่า วัสดุต้นแบบสามารถปลดปล่อยฟลูโอซิโนโลน อะซีโทไนด์ได้ในความเข้มข้นที่เหมาะสมในระยะเวลา 5 วันแรก ค่าความเป็นด่าง, เวลาก่อตัว, และปริมาณสารหนูมีมากกว่าวัสดุควบคุมอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) แต่ค่าความทนแรงอัดและการแตกตัวไม่มีความแตกต่างกัน วัสดุมีความคงตัวที่ดีในสภาวะที่ใช้ทดลอง และเมื่อนำสารที่ปลดปล่อยออกมาจากวัสดุไปทดสอบกับเซลล์พบว่าสามารถเพิ่มจำนวนของเซลล์ได้ที่เวลา 72 ชั่วโมง และกระตุ้นการสร้างอาร์เอ็นเอชนิดเดนทีนเซียโลฟอสโฟโปรตีนและลดไซโคลออกซีจีเนส 2 ได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) โดยสรุปพบว่าวัสดุดังกล่าวอาจเป็นทางเลือกหนึ่งในการรักษาเนื้อเยื่อในที่มีการอักเสบได้

สาขาวิชา ทันตชีววัสดุศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

4989710620 : MAJOR DENTAL BIOMATERIALS SCIENCE

KEYWORDS : CALCIUM HYDROXIDE / DYCAL / ANTI-INFLAMMATORY AGENT/ FLUOCINOLONE ACETONIDE / PULP CAPPING

PHUMISAK LOUWAKUL : DEVELOPMENT OF A PULP CAPPING MATERIAL CONTAINING FLUOCINOLONE ACETONIDE. ADVISOR : ASSOCIATE PROFESSOR VEERA LERTCHIRAKARN, Ph.D., 139 pp.

The aims of this study were to develop a pulp capping material containing fluocinolone acetonide (PCFA) and evaluate some of its physical, mechanical, and biological properties. The PCFA is hard setting calcium hydroxide cement containing 50 mmol/L of fluocinolone acetonide (FA). Conditioned medium from the setting material was collected for determination of FA release by high performance liquid chromatography and pH measurement by pH meter. Setting time, compressive strength, disintegration, and acid soluble arsenic content were measured according to ISO 3107:2004. Stability of the material was tested after 3 months storage at 45°C. Cytotoxicity and cell proliferation were evaluated by MTT assay. RT-PCR and Western blotting were used to study the effects of PCFA on RNA (dentin sialophosphoprotein: DSPP) and protein (dentin sialoprotein: DSP) synthesis. Anti-inflammatory effect of PCFA was determined by analysis of cyclooxygenase 2 (COX-2) expression. Dycal® was used as control. The results suggested that PCFA could release suitable concentrations of FA at the first 5 days. The pH, setting time, and arsenic content of PCFA were significantly higher than Dycal® ($p < 0.05$). The compressive strength and disintegration of the PCFA were comparable to the control. It had good stability after storage in the tested condition. The conditioned medium from PCFA could increase cell proliferation at 72 hours, increase DSPP expression and decrease COX-2 expression ($p < 0.05$). PCFA may be considered as alternative pulp capping material in the inflamed pulp tissue.

Field of Study : Dental Biomaterials Science Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

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I would like to express my sincere gratitude to Associate Professor Veera Lertchirakarn, Department of Microbiology, Faculty of Dentistry, Chulalongkorn University, who is my research advisor for helpful guidance, spending many hours to correct this thesis, suggestion and kind support throughout the course of doctoral degree program.

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Table of Contents

Page

List of Tables

List of Tables in Appendix

List of Figures

List of Figures in Appendix

List of Abbreviations

ALP	Alkaline phosphatase
cDNA	Complementary deoxyribonucleic acid
COX-2	Cyclooxygenase 2
DI	Deionized
DMEM	Dulbecco's Modified Eagle medium
DMP1	Dentin matrix protein 1
DMSO	Dimethylsulfoxide
DPP	Dentin phosphoprotein
DPSC	Dental pulp stem cell
DSPP	Dentin sialophosphoprotein
DSP	Dentin sialoprotein
FA	Fluocinolone acetonide
FBS	Fetal bovine serum
g	Gram
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GC	Glucocorticoid
GIC	Glass ionomer cement
GR	Glucocorticoid receptor
HDPC	Human dental pulp cell

HPLC	High performance liquid chromatography
HSP	Heat shock protein
IL	Interleukin
IRM	Intermediate restorative material
L	Litre
LPS	Lipopolysaccharides
LTB_4	Leukotriene B_4
mg	Milligram
mol	Molar
mRNA	Messenger RNA
MTA	Mineral trioxide aggregate
MTT	Thiazolyl blue tetrazolium bromide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PGE_2	Prostaglandins 2
PBS	Phosphate buffered saline
PCFA	Experimental pulp capping material containing fluocinolone acetonide
RER	Rough-surface endoplasmic reticulum
RNA	Ribonucleic acid
RT	Reverse transcription

RT-PCR	Reverse transcription polymerase chain reaction
S.D.	Standard deviation
TF	Transcription factor
TLC	Thin layer chromatography
TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Vital pulp therapy is a method of treatment in which the exposed dental pulp is covered with a material protecting the pulp from additional injury and permitting healing and repair. Theoretically, it is beneficial to preserve the vitality and health of the exposed pulp rather than to replace it with a root canal filling material when the pulp is mechanically exposed or superficially infected. The pulp tissue is a well vascularized and innervated connective tissue, so it has a good self-healing capacity. Reparative dentin formation is an important defense mechanism that protects the pulp from noxious elements in the oral cavity [1-6]. The major concern of endodontics has been the prevention or elimination of apical periodontitis. Therefore, the conservative treatment of vital pulp is the best way to ensure the prevention of periradicular pathology [7].

Traditionally, calcium hydroxide has been successfully used in capping the exposed pulps for many decades and remained the gold standard as pulp capping material primarily due to solid clinical documentations. Its high alkalinity provides the anti-bacterial property and encourages tissue repair [8-15]. Success of pulp capping procedure with calcium hydroxide depends on many factors especially microbial infection. Following proper disinfection, debridement and the absence of

microorganism, soft and hard tissue healing have been shown to occur at a very high rate [1, 2, 14, 16]. Therefore, the pulp capping procedure is recommended only for the sterile or mechanical pulp exposures in mature teeth. This treatment modality is remaining controversial for the caries-exposed pulp because of the unpredictable severity of the inflammatory condition of the underlying tissue and variable long-term clinical success [17-20]. Although dental pulp itself has a great potential of healing, severe and long-lasting inflammatory reactions always induce irreversible destruction of the tissue due to lack of collateral blood circulation and low compliance environment in unyielding hard tissue. Clinically, it is impossible to evaluate the extent of the exact inflammatory condition by current evaluation criteria such as sensitivity to electric pulp testing, spontaneous pain or pain characteristics, radiological signs of apical pathology. However, these criteria may not reflect the status of the pulp and dentin bridge formation [21]. Therefore, root canal therapy has been accepted as the treatment of choice for caries-exposed pulps.

To achieve a goal of dental pulp healing in the unknown inflammatory condition, further experiments are needed to discover an effective material that may provide clinicians with additional options for treatment of the caries-exposed pulps. Anti-inflammatory medicament mixed with a drug delivery vehicle would be an effective direct pulp-capping material [22]. Anti-inflammatory agents, such as corticosteroids, may be considered as candidates for reducing inflammation and stimulating healing.

The use of topical corticosteroids in vital pulp treatment was first reported more than 50 years ago by Rapoport and Abramson [23], with 80-93% success in pulp capping procedures. However, a commercial product (Ledermix®; Lederle Pharmaceuticals, Division of Cyanamid, Wolfratshausen, Germany) containing both 1% triamcinolone acetonide and 3.21% demethylchlortetracycline in a zinc oxide-eugenol base, leads to unpredictable and frequently unfavorable results [24-27]. Despite the unpredictable long-term success, the short-term application of a corticosteroid to inflamed dental pulp can be considered to resolve inflammation and consequently promote healing.

Fluocinolone acetonide is a synthetic corticosteroid commonly used for topical application in the management of dermatologic disorders and oral vesiculoerosive lesions [28-30]. The effects of fluocinolone acetonide on skin treatment are concentration-dependent. High concentrations of fluocinolone acetonide inhibit epidermal mitotic activity, but low concentrations slightly increase this activity [31]. Increased proliferation of cultured human skin fibroblasts is noted over a wide range of concentrations [32]. Some promising effects on human dental pulp cell proliferation and extracellular matrix formation have been shown [33].

The research interest has focused on the development of an experimental pulp capping material containing fluocinolone acetonide (PCFA) to promote healing of inflamed pulp tissue. The benefits of successful pulp capping procedures would reduce the need for complex treatments, endodontic and restorative procedures. The main

purposes of these studies are to develop a prototype of experimental pulp capping material and test some of its properties.

1.2 Research objectives

1. To develop a prototype of experimental pulp capping material containing fluocinolone acetonide (PCFA)

2. To investigate the release of fluocinolone acetonide and hydroxyl ions from PCFA

3. To test some of physical and mechanical properties of PCFA according to ISO 3107:2004

4. To assess the stability of PCFA by using accelerated test

5. To determine the effect of conditioned medium from PCFA on cultured human dental pulp cell proliferation, RNA and protein synthesis

6. To test the effect of conditioned medium from PCFA on artificially inflamed human dental pulp cells

1.3 Hypothesis

1) Null hypothesis H_0: Neither fluocinolone acetonide nor hydroxyl ions is released from PCFA at the designated time.

Alternative hypothesis H_1: Optimal concentrations of fluocinolone acetonide and hydroxyl ions can be released from PCFA at the appropriate time.

2) Null hypothesis H_0: The properties of PCFA are not different from the commercial material (Dycal®) according to ISO 3107:2004.

Alternative hypothesis H_1: The properties of PCFA are different from the commercial material (Dycal®) according to ISO 3107:2004.

3) Null hypothesis H_0: Releases of fluocinolone acetonide and hydroxyl ions from PCFA are not different between before and after the accelerated stability test.

Alternative hypothesis H_1: Releases of fluocinolone acetonide and hydroxyl ions from PCFA are different between before and after the accelerated stability test.

4) Null hypothesis H_0: Conditioned medium from PCFA has no effect on cultured human dental pulp cells with respect to cell proliferation, RNA and protein synthesis when compared with the commercial material (Dycal®).

Alternative hypothesis H_1: Conditioned medium from PCFA has some effect on cultured human dental pulp cells with respect to cell proliferation, RNA and protein synthesis when compared with the commercial material (Dycal®).

5) Null hypothesis H_0: The effect of conditioned medium from PCFA on induced inflamed human dental pulp cells is not different from the commercial material (Dycal®).

Alternative hypothesis H_1: The effect of conditioned medium from PCFA on induced inflamed human dental pulp cells is different from the commercial material (Dycal®).

1.4 Scope

1. The main objective of this thesis is to develop a prototype of pulp capping material containing fluocinolone acetonide (PCFA).

2. Some of the properties of PCFA are tested by following ISO 3107:2004 for cavity liners (type IV).

3. The *in vitro* studies are designed to test some effects of PCFA on cultured human dental pulp cells with regard to cell proliferation, RNA synthesis such as dentin sialophosphoprotein (DSPP) and cyclooxygenase 2 (COX-2), and protein synthesis such as dentin sialoprotein (DSP).

1.5 Limitations

1. The studies are designed to develop a prototype of pulp capping material included fluocinolone acetonide as an active ingredient for controlling the inflammatory process and stimulation of dental pulp tissue repair. The interaction between fluocinolone acetonide and other ingredients in setting calcium hydroxide cement is not known. The final ingredient of product may finally be adjusted or modified for appropriate clinical usage.

2. The actual ISO standard for calcium hydroxide base or liner material has not been yet established. The ISO standard number 3107:2004, designed for zinc oxide/eugenol and

zinc oxide/non-eugenol cements, is used in this study. Furthermore, the design of the *in vitro* studies followed ISO 3107:2004 may not be relevant to the clinical situation.

3. In the accelerated stability test, a product is stored at elevated stress conditions. Temperature is the most common acceleration factor used for chemicals, pharmaceuticals and biological products. Humidity and pH also have acceleration effects but the relationships are very complex. The product may be marketed based on accelerated stability data, but real-time stability test has to be done to confirm the shelf-life prediction.

4. Some *in vitro* experiments are designed to test the effects of the experimental pulp capping material on cultured human dental pulp cells. The effects of the interventions in these experiments cannot be used to explain the whole mechanism of *in vivo* pulp healing process and completely judged for human populations. Therefore, further experiments have to be investigated.

1.6 Benefits

Pulpitis is a disease caused by infection of dental pulp tissue within the enclosed environment. After the elimination of superficial infection and complete sealing of the cavity, the pulp tissue tends to be healed by itself. However, pre-existing inflammation may be persisted, uncontrolled and, later, cause tissue necrosis. Glucocorticoid is interested because of its anti-inflammatory action. The previous study reported that a

specific range of concentrations of fluocinolone acetonide had stimulatory effects on cultured human dental pulp cells. Therefore, it seemed to be beneficial in the application in vital pulp therapy. Calcium hydroxide has been widely used for several decades as direct pulp capping material because of its antibacterial and stimulatory effects, clinical long-term usage, practical use in clinical situation, low cost and availability. The incorporation of fluocinolone acetonide into the commercial-like product will be easily accepted by the practitioners. The anti-inflammatory and stimulatory effects are expected on inflamed dental pulp tissue healing. The benefits of successful pulp capping procedures would reduce the need for complex treatments, i.e. endodontic and restorative procedures, and loss of several permanent teeth.

1.7 Brief methodology

1. Development of a prototype of pulp capping material containing fluocinolone acetonide (PCFA)

2. *In vitro* release of fluocinolone acetonide and hydroxyl ions from PCFA

3. Test of some properties of developed pulp capping material according to ISO 3107:2004

   3.1) Determination of setting time

   3.2) Determination of compressive strength

   3.3) Determination of disintegration

3.4) Determination of acid-soluble arsenic content

4. Accelerated stability test

5. Cytotoxicity and cell proliferation assay, RNA and protein synthesis

5.1) Colorimetric (MTT) assay for cytotoxicity and cell proliferation

5.2) RNA and protein analysis

6. Test of anti-inflammatory effect

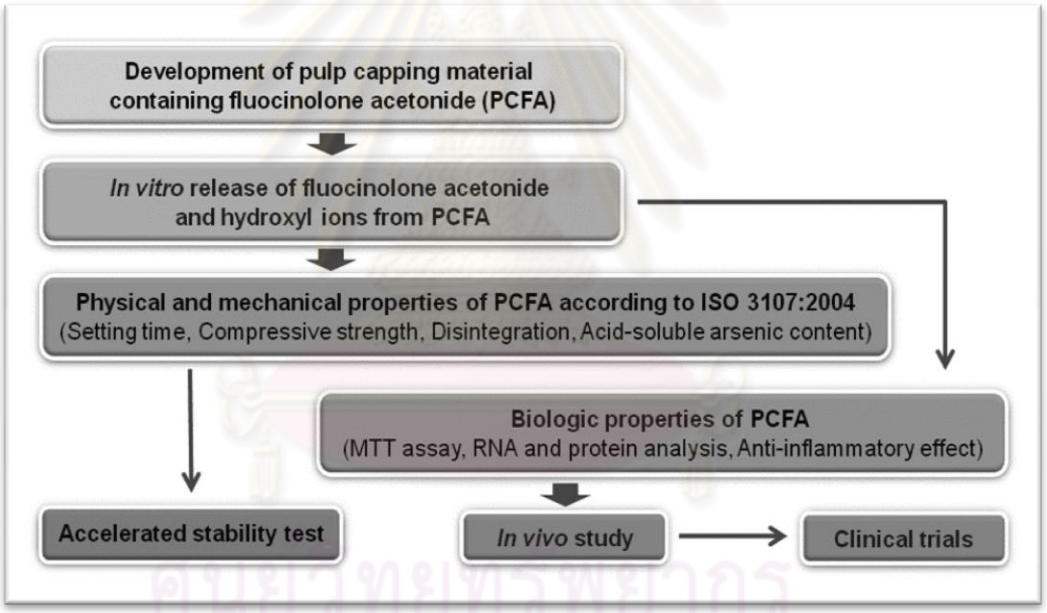

Figure 1 Diagram showing brief methodology of this study.

CHAPTER II

LITERATURE REVIEW

2.1 Vital pulp therapy and pulpal healing process

Vital pulp therapy may be broadly defined as any aspect of restorative dental treatment intended to minimize trauma to the dental pulp, including indirect and direct pulp capping, partial and full pulpotomy [34]. The objective of vital pulp therapy is to obtain healing of a pulpal wound in order to preserve a vital tooth with a healthy pulp [35]. The exposure site has to be sealed off by using an appropriate wound dressing to prevent bacterial contamination and promote pulp healing. An earliest attempt of vital pulp therapy was in 1756, when Phillip Pfaff applied a small piece of gold over an exposed pulp to promote healing. In 1826, Leonard Koeker cauterized the exposed portion of the pulp with a red hot iron wire, and then covered with a piece of lead foil. The pulp capping procedure has been performed with various types of materials since that period of time. However, calcium hydroxide has become a material of choice for direct pulp capping in human permanent teeth since it was introduced by Hermann in 1920 [9, 14, 36]. Zander was the first to report the use of calcium hydroxide in treatment of the exposed human dental pulps [8]. Other materials have also been tested and used as pulp capping materials such as adhesive materials [37-41], hydroxyapatite [42, 43], tricalcium phosphate [44], mineral trioxide aggregate (MTA) [45, 46], etc.

Pulpal healing after capping with calcium hydroxide has been studied in various animal and human experiments. When it is applied to the exposed pulp, the superficial necrotic zone is seen within an hour. The zone of necrosis composes of three layers: the compressed superficial layer, the liquefaction necrosis, and the zone of coagulation necrosis. Migration of inflammatory cells into the wound area occurs between six hours and a few days after the treatment and results in slight to moderate inflammation. Then, migration and proliferation of pulpal cells are observed adjacent to the necrotic zone [3, 9, 47].

Within the first four days, blood clot which forms at the time of injury is completely resolved [48]. The necrotic tissue is supposedly removed by phagocytes and replaced by granulation tissue, along with the proliferation and migration of pulpal cells (such as fibroblasts, mesenchymal, and endothelial cells) into the wound area during the following days. Matrix formation, i.e. collagen, is observed in association with the zone of firm necrosis after 4 days [3, 47].

After 7 days, the newly formed matrix has cellular inclusions and lining marginal cells. The spherical foci of mineralization in the deepest layer of the initial necrosis and adjacent pulp tissue are seen. In addition, the matrix vesicles which indicate initial mineralization are observed [3, 47]. About 2 weeks, the production of collagen matrix is continued. On the periphery of this tissue, cells resembling odontoblasts appear to be lining up. The pulp tissue below is normal and free of inflammation [9, 47].

After one month, the barrier is consisted of a coronal layer of irregular bone-like tissue with cellular inclusions. The pulpal part consists of predentin-like tissue and is lined with odontoblast-like cells. The one-month barrier shows mineralization of the collagen-rich tissues with cellular inclusions. Along the pulpal surface, cells are seen which have well developed rough-surface endoplasmic reticulum (RER). Adjacent parts of the barrier are predentin-like, they contain densely packed collagen fibrils and cellular extensions [3, 9, 47].

The pulp capped for eight weeks with calcium hydroxide shows an advanced stage of healing. The dentin barrier is thicker and the odontoblasts appear to be more regular aligned [3, 9]. After three months, the production of matrix appears to have stopped. The barrier is two-layered, consisting coronally of irregular tissue and pulpally of dentin-like tissue with irregular tubules, and lined with new odontoblasts. The tissue nearest the vital pulp is identical with predentin and contains densely packed collagen fibrils and cellular extensions [3, 9, 47]. The overall healing process of dental pulp is shown in figure 1.

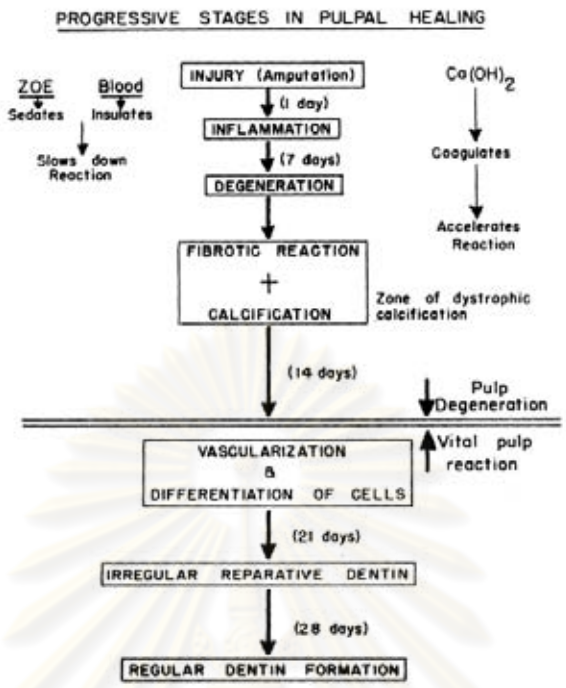

Figure 2 Diagram showing progressive stages in pulpal healing [49]

The origin of odontoblast-like cells which are responsible to dentinal bridge formation has been studied. Autoradiographic study indicates that they may be recruited from fibroblast-like cells located in the deeper pulp, migrated toward the site of exposure and differentiated into elongated and polarized odontoblast-like cells [50]. These cells locate adjacent to blood vessels and endothelial cells, which pericytes are especially implicated [48, 51]. It is also possible that pulp cells, endothelial cells, and pericytes become de-differentiate and then re-differentiate into odontoblast-like cells [4]. The pericytes and myofibroblast transitional cells appear and migrate to the site of pulp exposure and form reparative dentin. These cells can produce human dentin secretion in the cell culture experiments [5, 52]. Dental pulp stem cells (DPSC), found by Gronthos

and colleagues in 2000, are also candidates for repair and regeneration in dental pulp tissue [53-56].

In mixed populations of dental pulp cell culture, cells which can differentiate and produce hard tissue can express a number of markers for cell differentiation and mineralization. These cells can increase alkaline phosphatase activity prior to mineralization when they are grown in the presence of β-glycerophosphate. Other markers of differentiation including fibronectin, is also secreted in the initial stage of odontoblast differentiation and remains throughout odontogenesis. Type I collagen up-regulates as the cells differentiate, and osteonectin also expresses in young odontoblasts but not in pulp cells. Dentin sialophosphoprotein (DSPP) as well as dentin matrix protein 1 (DMP1), are expressed by differentiating odontoblasts. Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are cleaved products of DSPP. The DSP has been used as a late marker for odontoblast formation [57, 58].

The exposed pulp reveals typical features of primary mineralization consisting of young forming cells aligned with the calcifying matrix. The cells are characterized by large nuclei, abundant rough endoplasmic reticulum as well as Golgi and mitochondrial elements. Several long cellular processes form a network by intercellular communications and some processes can be detected penetrating vertically into the calcifying front. The cells are surrounded by a matrix, rich with collagen fibers. In many areas, an abundance of extracellular matrix vesicles is seen. Hydroxyapatite crystals

can be observed within the vesicles and dispersed into the matrix. Further calcification is characterized by arrangement of crystals beside collagen fibers and calcospheritic nodules to form calcifying front [3, 59-61].

The formation of a dentinal bridge may be related to the high pH of calcium hydroxide which is similar to bone formation, where increased tissue alkalinity stimulates calcification. The process may start with dystrophic calcification of the coagulation zone associated with blood components and tissue debris. The necrotic layer has a functional role as a surface where the pulpal cells attach and subsequently polarize. It may also act as a barrier, protecting the cells from the highly alkalinity and lethal environment of the capping material. It is also believed that tissue irritation or necrosis may be more important than calcium hydroxide in initiating dentin bridge formation or calcification [62]. The high pH causes chemical trauma to soft connective tissue and initiates a layer of coagulative necrosis or mummified zone which is caused by the initial arrest of cellular activity due to early suppression of pulpal enzymes. However, high alkalinity may not be an important factor in the bridge formation. As shown in a classical study, successful bridge formation in germ-free rats occurred without any medicament covering the exposure sites in the absence of bacterial contamination [1]. Furthermore, the proprietary cements with lower pH (e.g. Dycal®, Dentsply Caulk, Milford, DE, USA; Life®, Sybron Kerr, Orange, CA, USA) are effective in stimulation of dental pulp healing with no or only a thin layer of pulpal degeneration. The soft connective tissue of the

underlying pulp shows only with minimal inflammatory reactions followed by slow calcific bridge formation [11, 12, 48, 63-65].

The histologic feature of dentinal bridge has been studied in human teeth. After three months, the barrier consists of a coronal layer of irregular bone-like tissue with cellular inclusions and a pulpal layer of dentin-like tissue lined with odontoblast-like cells. From scanning electron microscopy, the dentin bridge is consisted of three layers which are a superficial amorphous debris-containing layer, an intermediate atubular layer (fibrodentinal core), and a tubular dentin-like structure, located adjacent to the pulp. The superficial and middle layers exhibit the lowest and highest mineral contents, while the last layer contains the mineral content which corresponds to the orthodentin of the pulpal wall [66].

Formation of incomplete dentinal bridge after pulp capping with calcium hydroxide have been reported. The morphology of the hard-tissue bridge is often irregular, with cellular inclusions and tunnel defects. Each tunnel is patent and communicates with the underlying pulp. Thus, a serious question arises as the long-term efficacy of the incomplete bridge to provide direct access for irritants or bacterial products to the underlying pulp tissue with leaked restoration [67]. Several researchers have reported failure associated with an incomplete dentinal bridge due to the presence of chronic inflammatory cell infiltration or necrotic pulp [68-73]. Even the dentin bridges

are completely formed, the inflammation or necrosis of underlying tissues has also been reported [63, 70].

In conclusion, pulpal healing process consists of early inflammatory response, cell proliferation and migration, extracellular matrix and hard tissue formation. The optimal end result is the reconstitution of dentinal defect with a bridge of reparative dentin bridge in direct continuum with reactionary dentin formed around the exposure area. The presence of calcified bridge after pulp capping is considered to be a sign of successful pulpal healing [10, 36, 64, 73, 74]. The formation of dentinal bridge may be considered as a two-part phenomenon. The initial stage is the formation of organic extracellular matrix by the progenitor cells that differentiate into matrix synthesizing odontoblast-like cells and deposit extracellular matrix. The later stage is the mineralization of newly formed tissue into calcific bridge.

2.2 Overview of pulp capping materials

Traditionally, calcium hydroxide has been the most common direct pulp-capping agent [36]. Calcium hydroxide, in dry powder, suspension or cement form, has been recommended for the treatment of exposed pulp due to its beneficial properties, such as induction of mineralization and inhibition of bacterial growth [75]. The actual mechanism of pulp repair using calcium hydroxide as a direct pulp capping agent is still not well understood. Calcium ions released from calcium hydroxide may act as signaling molecules to promote pulp tissue repair [76, 77]. High alkaline pH (about 12.5) causes

liquefaction necrosis of the superficial pulp tissue and disinfects the superficial pulp. Neutralization of the high pH in deeper layers of the pulp results in coagulation necrosis at the junction of necrotic and vital tissues, with only a mild irritation to the pulp tissue. This minor irritation stimulates an inflammatory response that, in the absence of bacteria, will heal with a hard tissue barrier [47, 78]. High pH of calcium hydroxide solutions can also solubilize and release some proteins and growth factors from dentin. These events may be responsible for the pulp repair and hard tissue barrier formation [79]. Moreover, calcium hydroxide may promote pulp tissue repair by directly inducing expression of bioactive molecules that involve in dentin formation [80, 81].

Hard-setting calcium hydroxide cements (e.g. Dycal®, Life®) are less caustic than pure calcium hydroxide and do not cause the superficial pulpal necrosis. These cements have been shown to initiate the same type of healing as calcium hydroxide powder [36]. A major disadvantage of calcium hydroxide materials is that they do not seal the exposed pulp from the external environment. The inability of calcium hydroxide to seal out bacteria may be a particular problem with composite resin restorations, which polymerization shrinkage can pull the pulp capping material out off the tissue surface [82]. Therefore, an additional base material, such as a resin-modified glass ionomer, must be placed to seal the pulp tissue against bacterial ingress.

However, dentin bridge beneath calcium hydroxide contains ‘tunnel defect’ which may be the cause of bacterial infection via microleakage again [67]. Other

investigators claim that calcium hydroxide tends to soften, disintegrate, and dissolve over time, leaving voids and other potential pathways for bacterial infiltration [83, 84]. Therefore, various other materials, including zinc oxide eugenol, glass ionomers, resin adhesives, and MTA, have been proposed as capping agents for vital pulp therapy.

Pulp capping procedure with resin adhesives has become increased in popularity over the last decade, and clinicians have reported relatively long-term evidence of success [85]. In recent years, scientific evidence from non-human primate studies suggests that resin adhesives can facilitate pulpal healing as long as bacteria cannot invade into pulpal cavity [39, 86-93]. Testing in human also supports the findings from animal studies [37, 94-96], but controlled clinical trials, and even retrospective analyses, are relatively few reports. Calcium hydroxide provides greater evidence of pulpal healing with dentinal bridging, and less inflammation, than the resin adhesives [96]. Furthermore, it must be noted that studies supporting resin pulp capping typically have involved exposure and capping of uninflamed pulps. Success rate with inflamed pulps is expected to be better for calcium hydroxide than for resin because resin is lack of the inherent hemostatic and bactericidal properties which calcium hydroxide is not [20].

MTA, firstly developed in 1993, is a modified form of Portland cement composed of calcium silicate, bismuth oxide, calcium carbonate and calcium aluminate. It is a biocompatible material and its sealing ability is better than amalgam and zinc oxide

eugenol. Although MTA was developed with the purpose of serving as root-end filling material, it has also proven to be successful in vital pulp therapy. It can stimulate thick dentinal bridging and appears to have particular promise as a pulp-capping material [97-105]. However, MTA has slow setting time and high cost. In addition, some authors have reported the similar result between MTA and calcium hydroxide when used in human teeth [18, 106, 107]. Further direct evidence by well-designed clinical trials and additional evidence of the long-term clinical success from observational studies are needed for this material [105].

2.3 Calcium hydroxide base/liner cement

Calcium hydroxide base/liner cement can be roughly classified into 2 types, non-setting and setting cements. Both calcium hydroxide/water slurry and proprietary setting calcium hydroxide base materials are capable of sterilizing human carious dentin beneath silver amalgam [108-111]. Dycal®, one of proprietary setting calcium hydroxide base materials, was developed into two paste system by Dougherty in 1962 (US patent no. 3047408). The components of Dycal® base and catalyst are shown in table 1 [112, 113].

The essential cement-forming reaction in Dycal® is obtained by the esterification of salicylic acid with glycols and other polyhydric compounds. Cement formation occurs as calcium ions replace the acidic phenolic protons to yield a chelate structure with the bidentate ester ligand. In the set cement, the divalent calcium ions are likely to be

chelated by two disalicylate molecules, act as bridging units between the difunctional ester molecules, and form a chain structure which can be regarded as a type of ionic polymer (Figure 2). The reaction continues after set and hardness develops as chelate formation. Although calcium ions may bridge disalicylate molecules to form a polymeric structure, the weakness and friability of the cement suggest that the chelates are bound together only by secondary attractions [11, 114].

Table 1 Components of Dycal® base and catalyst.

Base	Quantity (%)
1,3-Butylene glycol disalicylate	43.00
Zinc oxide	9.00
Titanium oxide-calcium phosphate	31.00*
Calcium tungstate	16.90
Iron oxide pigment	0.10
* A mixture of titanium dioxide and calcium phosphate in the approximate ratio of 1:2	
Catalyst	
Calcium hydroxide	51.00
Zinc oxide	9.23
Zinc stearate	0.29
Ethyl toluene sulfonamide	39.48

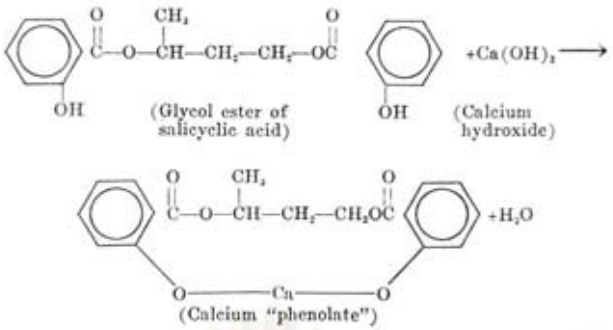

Figure 3 Proposed structure for a calcium-alkyl salicylate chelate [11]

When the mixtures are mixed together, they are reacted to form a hard, rigid mass of calcium phenolate, together with unreacted calcium hydroxide which is in large stoichiometric excess to that required for the reaction. Therefore, the amount of calcium hydroxide exists for ready dispersion throughout the body of the calcium salt when the material is set [11, 114]. The phenolic compound is liquid which makes the final product more homogenous and rigid. The liquid vehicle or plasticizer is a compound which is miscible with the appropriate esters of salicylic acid, and serves to dilute the ester without entering into the reaction between the calcium hydroxide and the salicylic ester. Such plasticizers are preferably liquid at room temperature and are typified by the compounds ethytoluene sulfonamide. The reaction can also be carried out in the presence of inert fillers which consists of titanium dioxide, zinc oxide, calcium sulfate, and pigment [11]. The hydrophilic nature of the plasticizer plays an essential role in controlling the rate of diffusion of water into the material and disintegration of the cement

producing an alkaline environment in the surrounding area [112, 114]. This is claimed by manufacturers to be responsible for their therapeutic action in clinical circumstances.

After capping procedure, calcium hydroxide is released from the cement and dissociates into calcium and hydroxyl ions. It has been shown that calcium ions from medicaments do not become a part of mineralized repair tissue. The calcium component of newly formed tertiary dentin comes from blood stream [115]. However, calcium from the cement may incorporate into the dentin bridge formation at the start of mineralization [116]. Therefore, the role of calcium ions released from the material is not well understood. It may be essential for cell proliferation, blood coagulation, initiation of mineralization, or other functions [3]. The hydroxyl ions are responsible for maintaining the high alkalinity which affects the healing process of pulp tissue. Neutralization of the acidity at the site of inflammation and antibacterial effect have a great influence on the repair of dental tissue [3, 66, 73, 117]. The release of hydroxyl ions can be measured indirectly by pH measurement. The antibacterial activity of the material results from the release of hydroxyl ions from the set material which generates a highly alkaline environment and effects the survival of microorganisms [109, 110, 112, 116]. At 24 hours, Dycal® creates environmental pH around 10.9-11.4 [118-120]. Calcium hydroxide can also suppress bacterial invasion of mechanically exposed pulp tissue which is intentionally opened to the oral environment for 24 hours, thus allowing a normal healing response [121].

Two main properties which appear to affect the failure of calcium hydroxide under the restorations are strength and solubility [118, 122, 123]. Compressive strength is most commonly used to test the strength of the cement because of the brittleness of the material. The compressive strength generally increases over the time particularly from initial set to 24 hours. The compressive strength of Dycal® is 1427 psi at initial set which seems to be sufficient for the condensation of amalgam restoration (greater than 170 psi) [113]. This value is increased to 1821-2424 psi at 24 hours, but decreased after long term period of storage [113, 122]. Decrease in compressive strength may be partly attributed to dissolution or water absorption during storage [124, 125]. The solubility of Dycal® is 2.7-6.76 % after submersion in distilled water for 24 hours and 2.65-4% after immersion in phosphoric acid for 60 seconds [118, 122, 123]. In the animal experiments, most of calcium hydroxide medicaments disintegrate and wash out after six months, leaving a void under the restoration and leading to bacterial infection [67]. Moreover, it allows long-term softening of the adjacent composite resin [85]. In laboratory studies, the strength and solubility of setting calcium hydroxide materials are significantly inferior to intermediate restorative material (IRM) and glass ionomer cement (GIC) [124-126]. Therefore, it should be used in small thin layer under other base materials.

Due to solid clinical documentation in a number of experimental and clinical situations, calcium hydroxide has remained the gold standard as a direct pulp capping material. Success rate of direct pulp capping in human teeth has been found to be

range between 75-90% [13, 14]. However, the experimental pulp exposures are mostly performed in healthy teeth. Pulp exposures in non-inflamed teeth may be found in conjunction with trauma, a condition which occurs relatively seldom. Most often the pulp is exposed due to caries, and the pulp is subsequently inflamed with various grades of severity. Different results can probably be expected when pulp capping is performed in teeth with inflamed pulps [21].

2.4 Inflammation of dental pulp tissues

Inflammation is one of the self-defense reactions against infection or irritation. These reactions are mediated by immune system, and characterized by inflammatory cell infiltration and synthesis of chemical mediators. The immune system is composed of innate and adaptive systems. In general, accumulation of neutrophils, macrophages and dendritic cells, which are typical components of innate immunity, is observed at the beginning of inflammation. The number of inflammatory cells and the area of infiltration are related to the virulent level of stimuli. Acute inflammation involves temporal host reactions which are characterized by severe infiltration of such inflammatory cells. These cells actively synthesize many kinds of chemical mediators. These mediators induce vasodilation of blood vessels and upregulation of capillary permeability, resulting in loss of blood plasma into tissues as well as the formation of edema and swelling. The four principal effects of acute inflammation, namely redness (*rubor*), heat (*calor*), swelling (*tumor*) and pain (*dolor*) can be attributed to vasodilation of small blood vessels. This

phenomenon increases blood flow, accumulation of fluid in the extravascular space, and synthesis of some specific mediators. Sustained exogenous stimuli which cannot be removed by phagocytes cause a shift from acute to chronic inflammation. The major cell component of chronic inflammation is lymphocytes, which belong to adaptive immunity [78, 127].

Dental pulp is encased by hard tissue which acts as physical and mechanical barriers against antigenic challenges from the oral cavity. Normal pulp posses an immunosurveillance system consisting of dendritic cells, macrophages and natural killer cells which are prepared to fight against antigenic challenges via dentinal tubules. When the pulpal surveillance system encounters excessive invasion of exogenous antigens, it evokes pulpal inflammation, which is characterized by the inflammatory cell infiltration and synthesis of various mediators. These initial reactions are mediated by innate immune reactions [128]. However, it is usually difficult for the innate immune system to cope with persistent and severe challenges. Thus, adaptive immune reactions predominantly mediated by lymphocytes are quickly initiated [129]. Although such defense mechanisms exist in the pulp, severe and long-lasting inflammatory reactions induce irreversible destruction of the dental pulp due to the scarcity of collateral circulation and low compliance environment [60].

The initiation of experimentally induced pulpal inflammation, which represents an acute inflammation model, is characterized by infiltration of neutrophils. The peak of their

accumulation is seen at 3 hours after application of Lipopolysaccharides (LPS) to pulp tissues, and then rapidly decreases. Following the neutrophil infiltration, macrophages become the majority of the pulpal immunocompetent cells, and their number peaks at 9 hours. Inflamed pulp shows rapid increase in the expression levels of several pro-inflammatory cytokines, including interleukin 1 alpha (IL-1α), IL-1β, IL-6, IL-12 and tumor necrosis factor alpha (TNF-α), which may contribute to further activation of macrophages and other immunocompetent cells [78, 127, 130]. Nitric oxide (NO) also plays a role in the progression of pulpal inflammation by accelerating the expression of chemokines, which would further induce the inflammatory cell infiltration and expression of pro-inflammatory cytokines and cyclooxygenase 2 (COX-2) [131-133].

Upregulation of arachidonic acid metabolites, such as prostaglandins E_2 (PGE_2) and leukotriene B_4 (LTB_4), has been reported in experimentally induced rat dental pulp and human dental pulp cells. LPS and inflammatory cytokines are able to induce COX-2, a key enzyme responsible for PGE_2 production. PGs produced by pulpal fibroblasts and infiltrating macrophages through COX-2 expression are important modulators of pulpal inflammation. They are related to the activation of the inflammatory response, as well as pain sensation in pulpal pathosis [134-142]. LTB_4 may be involved in neutrophil infiltration in LPS-induced pulpitis. Once circulating neutrophils are activated, they start to produce LTB_4 which causes the activation of other neutrophils and their extravasation.

It is possible that LTB_4 also acts as a modulator of the actions of other chemotactic factors [143].

The two key components in pulpal inflammation are the microcirculation and the sensory nerve activity. The response of sensory nerves to stimuli depends upon the severity of the pulp injury and the stages of inflammation. Several neuropeptides released from sensory or sympathetic nerves are important regulators of pathophysiological condition, immunological defense system, and repair of pulp tissues [144-147].

In caries-exposed pulp, the underlying pulp becomes inflamed to a varying and unknown degree, depending on the extent of the dental caries [148, 149]. Unfortunately, there is no reliable mean available to guide clinicians in determining how advanced the inflammation is in caries-exposed vital pulp. Thus, the coronal pulp tissue is usually infected by dental caries, while the apical pulp tissue remains vital with a varying degree of inflammation [149]. Each type of irritant or injury will have a different effect on the pulp. Short-term irritants will usually cause acute inflammation which will then be followed by resolution of the inflammation and repair of the tissue since the irritant does not persist or is no longer occurring. In contrast, long-term irritants will cause chronic inflammation of the pulp or pulp necrosis which will then be followed by infection of the pulp space [150].

2.5 Corticosteroids

Corticosteroids are a class of steroid hormones produced in the adrenal cortex. They are involved in a wide range of physiologic systems such as stress response, immune response and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behavior. They can be classified into 2 major groups, mineralocorticoids (such as aldosterone) and glucocorticoids (such as cortisol). Both of them are synthesized from cholesterol which is converted into pregnenolone, and then to the end products of adrenal biosynthesis (aldosterone, dehydroepiandrostenedione and cortisol). Most steroidogenic reactions are catalysed by enzymes of the cytochrome P450 family. They are located within the mitochondria and require adrenodoxin as a cofactor (except 21-hydroxylase and 17α-hydroxylase). Aldosterone and corticosterone share the first part of their biosynthetic pathway. The last part is either mediated by the aldosterone synthase (for aldosterone) or by the 11β-hydroxylase (for corticosterone) [151].

Mineralocorticoids control electrolyte and water levels, mainly by promoting sodium retention in the kidney. Glucocorticoids control carbohydrate, fat and protein metabolisms. They also have regulatory roles in development, metabolism, neurobiology, cardiovascular system, immune system, programmed cell death, and many other functions aimed at restoring homeostasis during stress. Once within the blood, cortisol is transported to target organs where it elicits numerous metabolic effects

including increased blood glucose levels, stimulation of gluconeogenesis in the liver, and the mobilization of both amino and fatty acids [151, 152]. In addition to these physiological roles, glucocorticoids are among the most widely prescribed class of drugs in the world. The pharmacological benefits of glucocorticoids are primarily anti-inflammatory and immunosuppressive. Glucocorticoids reduce pain and inflammation by a variety of mechanisms including disruption of the arachidonic acid pathway and blocking the production of inflammatory mediators [153]. Inflammatory diseases are characterized at the molecular level by chronically increased expression of multiple cytokines, chemokines, kinins and their receptors, adhesion molecules, and inflammatory enzymes such as inducible NOS and COX-2. At the cellular level, inflamed regions show a substantial influx of various inflammatory cells, arterial dilation, increased blood flow, plasma protein leakage, and edema. These parameters of inflammation are effectively reduced by treatment with glucocorticoids by both direct and indirect mechanisms. Therefore, they are the most potent anti-inflammatory agents currently available for the treatment of chronic inflammatory diseases [152, 154]. Glucocorticoids are also potent endogenous immunological suppressors. However, whilst the anti-inflammatory power of synthetic glucocorticoids derives from endogenous anti-inflammatory mechanisms, the clinical usefulness of these drugs is limited by hypothalamic-pituitary-adrenal insufficiency [155].

Corticosteroid medicaments can be divided into 4 types according to their usages; topical steroids, inhaled steroids, oral forms, and systemic forms. Topical steroids are the topical forms of corticosteroids which have been successfully used in treatment of various dermatological diseases such as rash, eczema, dermatitis [156, 157]. Some advantages of topical corticosteroids have been reported i.e. anti-inflammatory action [23, 27, 158], pain reduction or pain relief [159, 160]. However, side effects of topical steroids are reported such as allergic contact dermatitis, atrophy, addiction or rebound syndrome, perioral dermatitis, ocular effects, tachyphylaxis, vehicle-related adverse effects etc. Although some disadvantages have been considered to be anti-proliferative and immunosuppressive effects which are believed to retard tissue healing, the stimulatory effects have been reported. Steroids which possess high glucocorticoids activity (such as triamcinolone acetonide, dexamethasone, cortisol, corticosterone, and aldosterone) stimulate both DNA synthesis and cell division. The relative potency of these active steroids is related to their relative glucocorticoid potency [161].

The biological actions of glucocorticoids are mediated through the cytoplasmic glucocorticoid receptor (GR), a 777 amino acid protein, which is a member of the superfamily of ligand regulated nuclear receptors. In the absence of ligand, GR is predominantly maintained in the cytoplasm as an inactive multi-protein complex. This consists of two heat shock protein 90 molecules plus a number of other proteins

including the immunophilins p59 and calreticulin. Entry of glucocorticoids into the cell and subsequent binding to the ligand binding domain of GR lead to a conformational change in the receptor. This causes dissociation of the multi-protein complex and allows nuclear translocation of GR by virtue of the nuclear localization sequence within the DNA binding domain. Once within the nucleus, GR binds DNA sequences known as glucocorticoid response elements within the promoter region of target genes to activate transcription of responsive genes (referred to as transactivation). The GR can positively or negatively regulate gene expression, depending on the response element sequence and promoter context. The GR also modulates gene expression, independent of glucocorticoid response elements, by physically interacting with other transcription factors (e.g., activating protein AP-1and nuclear factor NF-**K**B). It is often stated that the metabolic effects of glucocorticoids result from increased transcription of genes such as tyrosine aminotransferase and phoshoenolpyruvate carboxykinase, whereas the anti-inflammatory properties are attributed to negative transcriptional effects on inflammatory gene expression [152, 162-164].

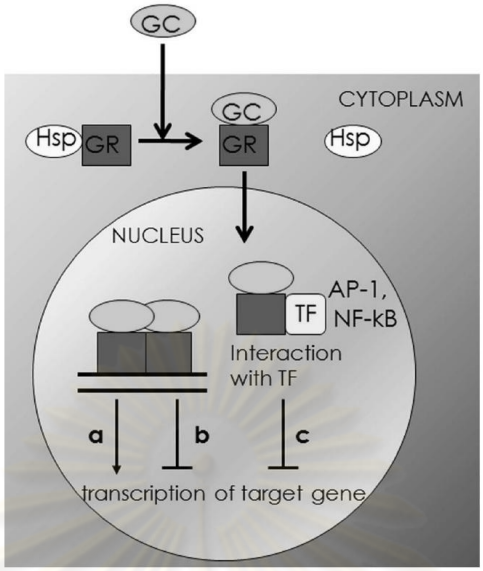

Figure 4 Action modes of the glucocorticoid receptor. (GC= Glucocorticoid; GR = Glucocorticoid receptor; Hsp = heat shock protein; TF = transcription factors) [165]

2.6 Use of topical corticosteroids in dental pulp

The rationale of treatment with corticosteroids depends upon an ability to inhibit inflammatory processes such as inhibition of hyperemia and edema, pain reduction, and presumably induction of pulpal healing [27, 166]. Inflammatory change within the pulp is the same as that elsewhere in the body but is different only by anatomic confines of rigid dentinal walls. Thus, inflammatory processes cause venous collapse, increase pressure on nerve ending and elicit pain.

Some topical steroids have been used for reduction of the inflammation within the pulp tissue. Betamethasone applied on the dentin of rat teeth can reduce the vascular phase of pulpal inflammation regarding vessel diameter and number of blood

vessels [167]. Topical steroids show superior inflammatory effect than non-steroidal anti-inflammatory agent. Dexamethasone antagonizes the effects of endotoxin and capsaicin, while NSAID affects mainly the endotoxin-induced inflammation [168]. As pulp capping agent, the corticosteroids can control the inflammation directly by reducing the inflammatory processes, decreasing pain from pressure and enhancing venous drainage in conjunction with the removal of the source of irritation. Moreover, favorable conditions for pulpal wound repair require an environment free of bacteria, absence of severe haemodynamic change, and absence of severe inflammatory cell infiltration. Thereafter, the dentinogenic potential of pulpal cells can be expressed [169].

Many reports have presented the influence of glucocorticoids on the healing of dental pulp. The use of topical corticosteroids in the treatment of human vital pulp was first reported by Rapoport and Abramson [23]. They applied hydrocortisone acetate (saline suspension) 25 mg/mL administered either by liquid or powder form to the exposed pulp. The results showed 80-93% of success in the pulp capping operations. Recently, several immunosuppressive drugs have been tested in inflamed dog teeth. Only oral corticosteroid could reduce inflammatory cell infiltration and tissue necrosis with comparable hard tissue formation to the control group [170].

Schroeder and Triadan were first proposed, and used successfully, a combination of triamcinolone, chloramphenicol, 4% xylocaine solution and ointment base in treatment of pulpitis [160]. In the same year, Schroeder developed the first

proprietary products named “Ledermix®”, which is a combined preparation of 1% triamcinolone and 3.21% demethylchlortetracycline. The Ledermix cement, formulated for application to the exposed dental pulp, reveals disappointing results in rat molar pulps [74] but shows different results in human teeth. When applied to normal pulp tissue, it does not impair pulpal vitality and evokes a moderate calcific response after prolonged period of contact. When applied to minimally inflamed pulps, resolution occurs in a proportion of cases but the outcome of treatment is unpredictable. Relief of symptom and continued positive response to vitality test are found in chronically inflamed pulps [24]. It is successful in pulpotomy of normal human teeth [27], but fails to induce healing in inflamed pulp cases [26]. However, successful treatment of carious exposure with Ledermix® has been reported [25]. In rat model, Ledermix® used in cariously exposed teeth results in pulpal necrosis limited to the coronal pulp, and bridge formation occurs in most of the cases [171]. In contrast, failure to induce reparative dentin formation has also been reported when Ledermix® cement is used as pulp capping agent in rats [172]. Otic suspensions, combinations of antibiotics and hydrocortisone, are effective in treating bacterial infected pulps and stimulate the production of a hard tissue bridge in contaminated dental pulps [173].

There have been some evidences regarding the effect of glucocorticoids on extracellular matrix synthesis. Cortisol and related glucocorticoids have two different effects on bone collagen synthesis *in vitro*. Collagen synthesis is stimulated in short term

cultures but inhibited in long term cultures. The low physiologic concentrations of glucocorticoids may be essential for maintenance of the differentiated function of osteoblasts, the cells responsible for collagen synthesis [174]. After short term treatment, low concentrations of cortisol, corticosterone and dexamethasone increase the incorporation of [^{3}H]proline into type I collagen in cultured rat calvariae [175]. In vascular smooth muscle cells, 10^{-7} M dexamethasone shows an increase in the synthesis and secretion of collagen [176]. Therefore, specific concentrations of some corticosteroids may have stimulatory effect on collagen synthesis. Dexamethasone is able to promote fibronectin synthesis and suppress NGF secretion, suggesting that it may be used to reduce pain and promote dental pulp tissue healing [177].

However, the inhibitory effect of corticosteroids on collagen synthesis has been reported. In rabbit dental pulps, collagen synthesis is inhibited by some corticosteroids except prednisolone. Ledermix® also inhibits collagen synthesis in human teeth. The different glucocorticoids may exert different effects on collagen synthesis and this effect is dose-dependent [178]. In rabbit dental pulps, collagen synthesis is also inhibited selectively by prednisolone treatment. It may disturb normal development and metabolism of teeth. The corticosteroid-induced inhibition of collagen biosynthesis seems to be dose-dependent [179]. Moreover, dexamethasone strongly inhibits collagen synthesis, but enhances alkaline phosphatase activity which is a marker for hard tissue biomineralization [180-183].

Glucocorticoid therapy may induce excessive dentin formation. Pulp obliterations are observed in patients treated with long-term systemic corticosteroids [184]. Dexamethasone may be able to stimulate osteogenic differentiation in human dental pulp cultures. Although dexamethasone inhibits cell proliferation and markedly reduces the proportion of SMA-positive cells, but it still strongly stimulates alkaline phosphatase (ALP) activity and induces the expression of the transcript encoding the major odontoblastic marker, which is DSPP [185]. Physiologic concentrations of dexamethasone and hydrocortisone induce differentiation of osteoblastic cells and formation of bone nodules in rat calvaria cell culture [186]. Thus, some topical corticosteroids may be able to induce mineralization process and hard tissue formation.

2.7 Fluocinolone acetonide

Fluocinolone acetonide was first discovered by Syntex and marketed under the name of “Synalar” by Imperial Chemical Industries. The full chemical name is 6 alpha, 9 alpha-difluoro-16 alpha-hydroxyprednisolone-16, 17-acetonide. It contains two fluorine atoms compared to the one atom of triamcinolone acetonide [187]. It is a synthetic corticosteroid which is commonly used as topical medication in treatment of various dermatologic disorders and also oral vesibuloerosive lesions [28-30, 188]. The efficacy is superior to triamcinolone acetonide in treatment of oral lichen planus [189]. Topical 0.1% fluocinolone acetonide gel is safe, effective and easy to apply [28]. It can reduce TNF-α which is associated with the immunopathogenesis of oral lichen planus [190].

When topically applied on the skin, 0.2% fluocinolone acetonide inhibits mitotic activity of the epidermis but 0.025% fluocinolone acetonide does not. Slightly increase mitotic activity is surprisingly found when low concentration of fluocinolone acetonide is used [31]. In cultured human skin fibroblasts, a wide range of concentrations of fluocinolone acetonide has no inhibitory effect but additional produces a slightly increase in growth rate. It has a transient stimulatory effect on fibroblasts by promoting an earlier entry into period of DNA synthesis (S phase), which is also accompanied by a substantial increase in the length of S phase [32].

In human skin collagen synthesis, formation of radioactive hydroxyproline is inhibited by fluocinolone acetonide and other glucocorticoids. The effect is dose-dependent. The 10 μg/mL concentration of fluocinolone acetonide has no significant effect on hydroxyproline formation, but higher, non-physiologic, concentrations of fluocinolone acetonide inhibit the rate of collagen formation [191]. Recently, the stimulatory effects of fluocinolone acetonide on human dental pulp cell proliferation and extracellular matrix formation were reported [33]. A wide range of concentrations of fluocinolone acetonide (0.1 to 10 μmol/L) was not toxic to the cells, but able to enhance cell proliferation, fibronectin and type I collagen synthesis. Thus, fluocinolone acetonide may be beneficial to stimulate the early phase of pulp tissue healing [33].

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The chemical agents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The media and supplements used in cell culture were purchased from Gibco BRL (Carlsbad, CA, USA).

3.2 Methods

3.2.1 Preparation of pulp capping material containing fluocinolone acetonide (PCFA)

The PCFA was experimental developed according to US patent no. 3047408 and literatures [112, 113]. The components of PCFA, a two-paste calcium hydroxide cement containing base and catalyst, were shown in table 2. The 1,3 butylene glycol disalicylate was prepared from 1,3 butylene glycol (Sigma Chemical Co.) mixed with methyl salicylate (Sigma Chemical Co.) in a boiling flask which was fixed with reflux condenser. Then, the base component was developed by trans-esterification of 20 parts of 1,3 butylene glycol disalicylate and 20 parts of titanium dioxide-calcium sulfate fillers. The reaction was set at 110 to 210^{o}C using sodium methoxide (Sigma Chemical Co.) as catalyst. The mixture was heated slowly with stirring until gentle reflux conditions were reached and maintained for approximately 48 hours. After the reaction was completed, the substance was purified by multiple washings with deionized (DI) water to remove the water soluble materials. The condensed material remained as light brown viscous liquid.

After the synthesis, the reaction was preliminary tested by thin layer chromatography (TLC). The final product was then separated from the condensed material by column chromatography and tested by nuclear magnetic resonance (NMR) technique to confirm the structure of the required substance.

Table 2 Components of PCFA base and catalyst.

Base	Quantity (%)
1,3-Butylene glycol disalicylate	43.00
Zinc oxide	9.00
Titanium oxide-calcium phosphate	31.00*
Calcium tungstate	16.90
Iron oxide pigment	0.10
* A mixture of titanium dioxide and calcium phosphate in the approximate ratio of 1:2	
Catalyst	
Calcium hydroxide	51.00
Zinc oxide	9.23
Zinc stearate	0.29
Ethyl toluene sulfonamide + 50 mmol/L Fluocinolone acetonide	39.48

Fluocinolone acetonide powder (FARMABIOS S.R.I., Stabilimento e Direzione, Gropello Cairoli, Italy) was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co.) before mixing with ethyl toluene sulfonamide (Sigma Chemical Co.). Then, mixture of ethyltoluene sulfonamide and fluocinolone acetonide was incorporated into catalyst which was developed by mixing calcium hydroxide with zinc oxide, zinc stearate at room temperature. The prepared catalyst appeared as white cream paste. Freshly prepared cements were used for each experiment. Equal amounts of the base and catalyst were mixed and allowed to set at room temperature to form hard setting calcium hydroxide cement.

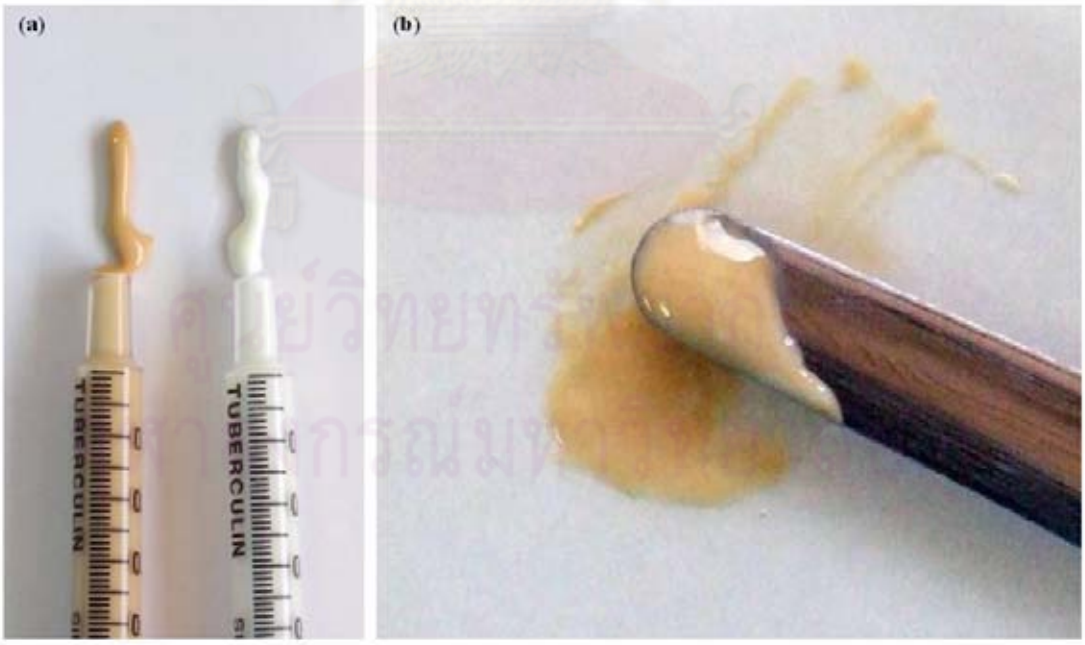

Figure 5 An experimental pulp capping material containing fluocinolone acetonide (PCFA).

(a) PCFA base [Left] and catalyst [Right] (b) Mixing of the PCFA

The *in vitro* release of fluocinolone acetonide assay was modified from Haesslein and co-workers [192]. The samples were mixed and immediately put into cylindrical plastic molds (5 mm height, 8 mm diameter). Before complete setting, 1 mL of DI water was poured onto the surface of each specimen. All specimens were kept at 37^{o}C, 100% humidity for 24 hours. The amount of released fluocinolone acetonide was determined by high performance liquid chromatography (HPLC) system equipped with a UV-VIS detector model SPD-10A (Shimadzu Class LC 10A; Shimadzu Corp., Kyoto, Japan). In a typical run, 50 µL of samples were analyzed on a reverse phase Inertsil™ C18, 5 µm column (250 mm × 4.6 mm) (GL Sciences Inc., Tokyo, Japan). A water-methanol mixture (30:70 by volume) delivered at a flow rate of 0.7 mL/minute was used as mobile phase. Fluocinolone acetonide was detected at a wavelength of 238 nm and an approximate retention time of 11 minutes. Fluocinolone acetonide with certain concentration was prepared as the standard concentrations from a stock solution of fluocinolone acetonide in DMSO (4.52 mg/mL) by dilution with DI water. The concentration of standard was proportional to the integrated area of the corresponding peak in UV absorbance. The calibrated standard curve was linear (R^2 > 0.99) over the recorded concentration range of fluocinolone acetonide between 1 to 100 µmol/L.

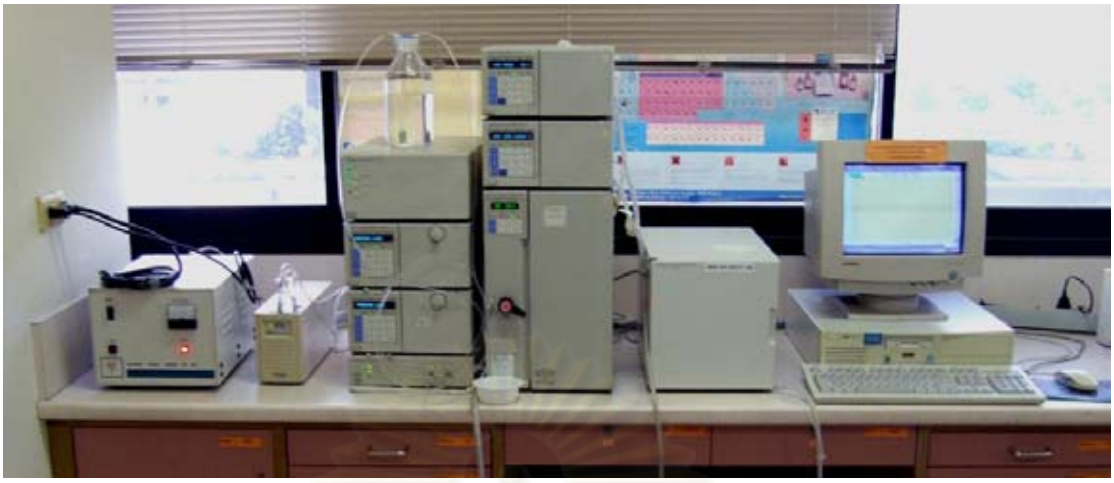

Figure 6 High performance liquid chromatography system with a UV-VIS detector model SPD-10A.

Then, various concentrations of fluocinolone acetonide (10, 20, 30, 40, 50, 75, 100, and 150 mmol/L concentrations) were loaded into the experimental cements to determine the optimal concentration of fluocinolone acetonide added into PCFA. The amount of fluocinolone acetonide release was recorded, and a suitable concentration of fluocinolone acetonide loading was selected for further investigation.

3.2.2 *In vitro* release of fluocinolone acetonide

The *in vitro* release of fluocinolone acetonide assay was modified from Haesslein and co-workers [192]. The samples with the incorporation of fluocinolone acetonide at selected concentration from the former experiment were mixed and immediately put into cylindrical plastic molds (5 mm height, 8 mm diameter). Before complete setting, 1 mL of deionized (DI) water was poured onto the surface of each specimen. All specimens were kept at 37°C, 100% humidity. The conditioned medium was collected at specified

time intervals (4 hours, 8 hours, and 1, 2, 3, 5, 7, 14, 21, and 28 days, and monthly afterwards) and replaced with the same amount of fresh DI water. The media samples were collected until the results showed a constant rate of release. The collected media was stored at 2-8 °C until analysis. Ten experimental samples were prepared for each group.

The *in vitro* release of fluocinolone acetonide was detected by HPLC system equipped with a UV-VIS detector model SPD-10A (Shimadzu Class LC 10A; Shimadzu Corp.) as described above. The released fluocinolone acetonide was calculated and recorded in μmol/L. The cement without fluocinolone acetonide was used as a control. The fluocinolone acetonide release rate was determined from the amount of fluocinolone acetonide released between five consecutive data points divided by the corresponding release time in days, and expressed as μmol/L/day.

3.2.3 pH measurement

The method of pH measurement was modified from Gencay and colleagues [193]. Each specimen was mixed and placed in a cylindrical stainless steel mold with 2 mm height and 4 mm diameter. Then, the mold and cement was pressed between two microscopic glass slides. The samples were allowed to set in room temperature. Each sample was taken out of the mold and placed into a separate vial, containing 10 mL of DI water. The samples were stored at 37°C, and pH measurement was done at 1, 3, 24, 48, 72, and 168 hours (7 days) after the incubation. The pH value was measured by a

digital pH meter (Orion Research 420A; Orion Research Inc., Boston, MA, USA), which connected to a glass electrode to the pH meter (Orion Ag/AgCl 91 Series electrode, Orion Research Inc.). The pH meter was calibrated with buffer solutions (Orion buffer solution, perfect buffer 10, Orion Research Inc. [pH < 7,00; pH < 11,00]) before each experiment. Ten samples were prepared for each group. The pH values were shown as mean ± standard deviation compared with the control.

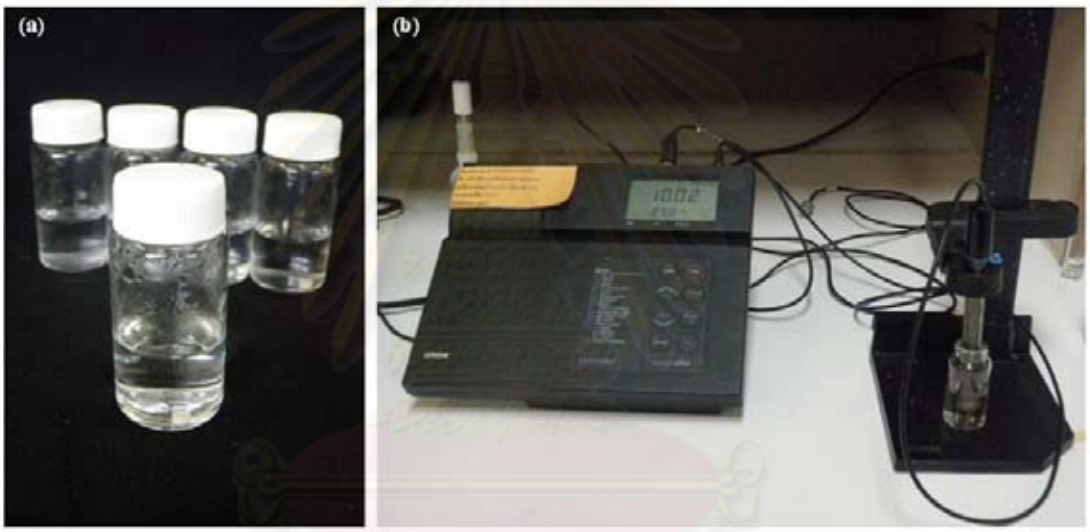

Figure 7 Model for pH measurement.

(a) Preparation of tested specimens (b) pH meter model Orion Research 420A.

3.2.4 Determination of setting time

According to International Standard ISO 3107:2004, the cylindrical specimens were prepared from stainless steel molds with 2 mm height and 10 mm diameter. The mold was placed on a flat glass plate and filled with the mixed material. The molds and tested materials were placed on a metal block in a controlled chamber (37^0C and 95%

relative humidity) within 60 second from started mixing. A flat-ended indenter needle (100 g) was lowered vertically onto the surface of the cement. The indentation was repeated at 15 second intervals in different area. The needle tip was cleaned before each indentation. The setting time was recorded as the period of time from the start of mixing to the time when the needle failed to completely penetrate the whole depth of material. Ten samples were tested for each group.

Figure 8 Model for determination of setting time.

3.2.5 Determination of compressive strength

The method used in this study was carried out according to ISO 3107:2004 standard. Specimens were prepared from stainless molds with 4 mm height and 6 mm diameter. The cement was put in the mold and placed on a metal plate within 1 minute

after mixing. Another metal plate was placed on top and both plates were squeezed together. The whole assembly was transferred to the cabinet (37^0C and 95% relative humidity) within 2 minutes after the end of mixing. One hour after the completion of mixing, flat surfaces of the specimen were prepared at 90^0 to the long axis. The specimen was removed from the mold immediately and examined for voids or chipped edges. Any defective specimens were discarded. Ten specimens were used for the experiment in each group. The diameter of the cylinders was measured and recorded. The specimens were immersed in distilled water and maintained at 37^0C for 24 hours. Then, the samples were put in distilled water at 23^0C for 15 minutes and blot-dried prior to test. The flat ends of each specimen were placed between the platens of Instron universal testing machine (Model 8872; Instron corp., Canton, MA, USA). The compressive load was applied along the long axis of the specimen using cross-head speed of 1.00 mm/minute until fracture occurred. The maximum force was recorded. The compressive strength was calculated from the relationship between surface area and load.

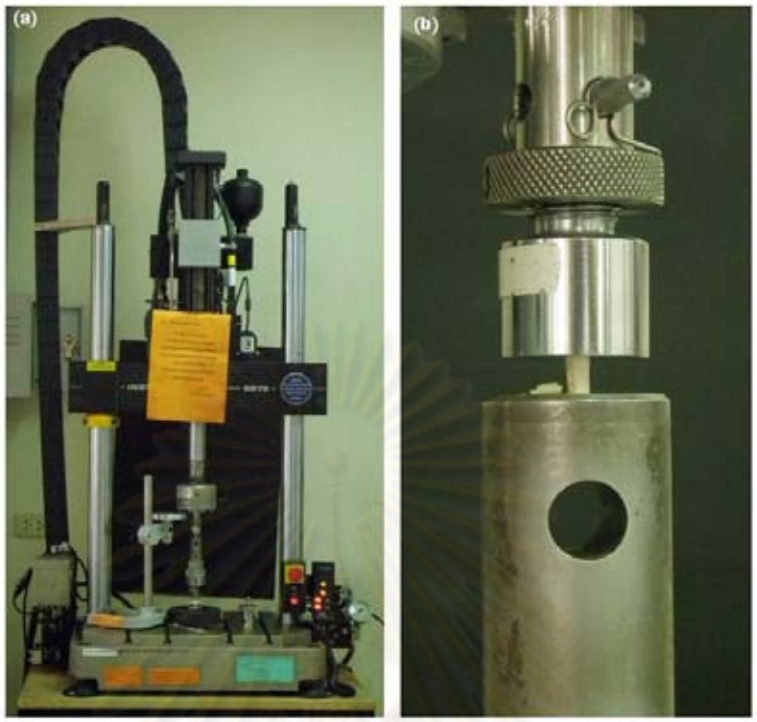

Figure 9 Model for determination of compressive strength. (a) Instron universal testing machine model 8872 (b) Fracture of specimen under compressive loading.

3.2.6 Determination of disintegration

The disintegration of testing materials was examined according to ISO 3107:2004. Specimens were prepared from splited stainless steel rings with 1.5 mm height and 20 mm internal diameter. The mold was placed on a thin cellulose acetate sheet backed by a flat glass plate. A stainless steel wire with 0.25 mm diameter was inserted through the split ring at least 10 mm into the ring. The PCFA or Dycal® as a control group was mixed and filled in the ring, then, covered with another flat glass plate faced with a sheet of cellulose acetate, and firmly pressed together. After the complete

set which was approximately 3 minutes after the start of mixing, the molds and plates were transferred into the cabinet and maintained at 37^0C and 95% relative humidity for 1 hour. Then, the specimen and attached wire were carefully removed from the ring. The excess cement was removed from the edge of the specimen disc, and lightly brushed to remove any loose material from the surface. The specimen was weighed and net weight of cement was evaluated by total weight minus with stainless steel wire. Two of the sample discs were placed in a 100 mL beaker. The discs were submerged immediately by pouring 50 mL of distilled water into the beaker. The specimen was suspended by the wire without contact to each other and to the beaker. Then, the beaker was sealed with plastic wrap and aluminum foil. After immersing the discs for 24 hours at 37^0C, the specimens were removed from the water. Their surfaces were rinsed with 2 mL of distilled water. The surfaces were gently dried with clean absorbent paper. The specimens were then stored in a desiccator for 24 hours and reweighed. The final net mass of cement was then recorded by Pharmacy line model AB104-s (Mettler-Toledo Inc., Columbus, OH, USA). The disintegration was calculated as percentage of the original weight. Ten samples were prepared from each group.

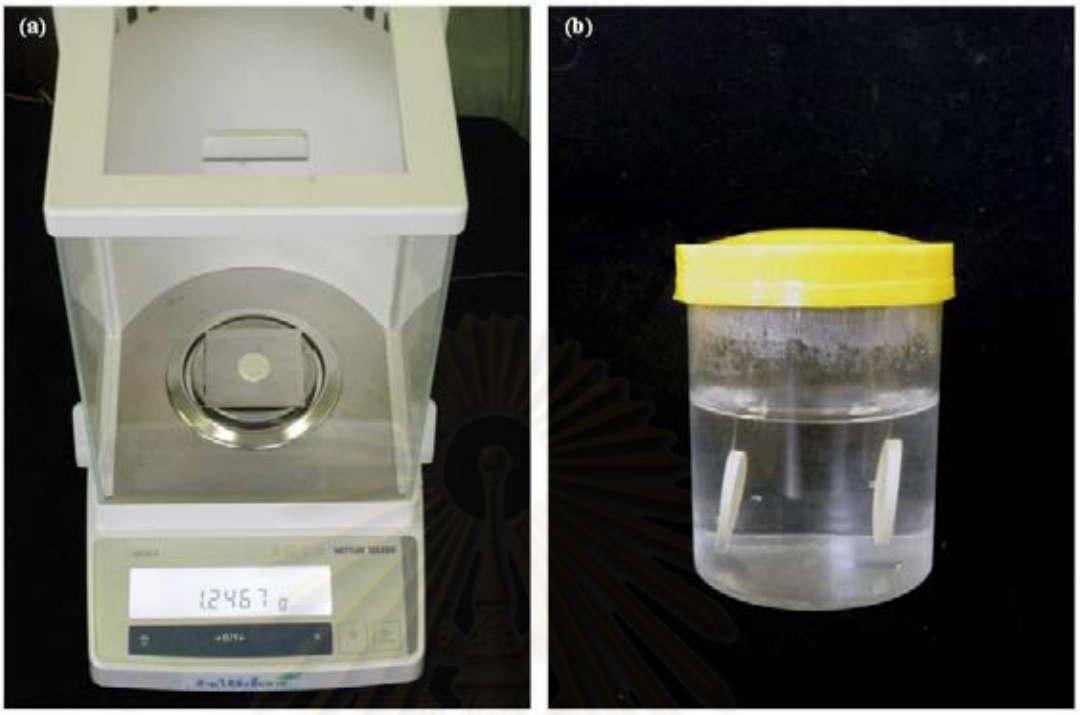

Figure 10 Model for determination of disintegration.

(a) Weighing of samples before and after immersion (b) Immersion of specimens in 50 mL DI water.

3.2.7 Determination of acid-soluble arsenic content

The method used in this study was according to ISO 3107:2004. The complete set experimental cement or Dycal® as a control group was pulverized and passed through a 75 μm (200 mesh) sieve. Two grams of the sieved powder was dispersed in 30 mL of water with 10 mL of 37% hydrochloric acid (Merck KGaA, Darmstadt, Germany). The mixture was maintained at 37^0C for 1 hour, and filtered. The total arsenic content of the sample was determined with the atomic absorption microscopy (AAnalyst 800, PerkinElmer Inc., Shelton, CT, USA). Ten samples were prepared from each group.

Figure 11 Model for determination of acid-soluble arsenic content. (a) Material was pulverized and passed through 75-micron sieve (b) Atomic absorption microscopy model AAnalyst 800.

3.2.8 Accelerated stability test

PCFA was freshly prepared according to the method in 2.1. Five batches of the experimental pulp capping agent were then stored at 45°C for 3 months [194]. Then, the *in vitro* releases of fluocinolone acetonide and pH measurement were done as in 2.2 and 2.3 for 7 days. The amounts of fluocinolone acetonide and pH of the conditioned medium were plotted and calculated as percentage of freshly prepared materials. The result was acceptable when the physical (e.g., color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation) and chemical properties (loss of active ingredients did not exceed 10%) were not changed.

3.2.9 Cytotoxicity and cell proliferation assay

1) Cell culture

Human dental pulp cells were obtained from caries-free lower third molars extracted for orthodontic reason at the department of oral surgery, Faculty of Dentistry, Chulalongkorn University with patient's informed consent. The study protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The teeth were extensively washed with sterile phosphate-buffered saline solution (PBS) and cracked open along the longitudinal axis. The pulp was gently removed by forceps, minced into small pieces (1x1x1 mm^3) and seeded in 35-mm plastic tissue culture dishes (Nunc, Naperville, IL, USA). The explants were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B and incubated at the condition of 5% CO_2, 37 ºC. The medium and supplements were purchased from Gibco BRL (Carlsbad). After the outgrowth of human cultured dental pulp cells reached confluence, they were subcultured into new culture dishes. The 3rd to 5th passages of three different donors were used in this study.

2) Preparation of test materials

The PCFA or Dycal® was mixed and immediately put into cylindrical plastic molds (5 mm height, 8 mm diameter). Dycal® was mixed according to the

manufacturers' recommendation. Before complete setting, 1 mL of culture media was poured on the surface of each specimen. All specimens were kept at 37°C, 100% humidity for 24 hours. Then, the specimen was removed and the conditioned medium was filtered by sterile 0.2 µm pore diameter filters. The pilot study revealed that full concentrations of conditioned medium from both PCFA and Dycal® were toxic to HDPCs. This might be due to high pH value of these materials. In addition, the result from the pilot study of *in vitro* assay suggested that PCFA could release 50 µmol/L of fluocinolone acetonide, so the conditioned medium from PCFA was diluted 50 times in order to obtain the conditioned medium containing 1 µmol/L of fluocinolone acetonide. This concentration was the result from the calculation of fluocinolone acetonide from approximately amount of PCFA used in pulp capping procedure. The conditioned medium was also diluted 100 times to simulate in case that released fluocinolone acetonide was diluted by fluid in the pulp tissue. The 50 and 100 time dilutions of conditioned medium form PCFA was assigned as F50 and F100, respectively. The same dilutions of conditioned medium form Dycal® were used as controls and were assigned as D50 and D100, respectively.

3) Colorimetric (MTT) assay for cell proliferation

The 5 x 10^4 primary human dental pulp cells were seeded in 24-well plates with DMEM containing 10% FBS and incubated for 24 hours. Then, the media was replaced twice by serum free medium at 3 hours intervals in order to wash out the

serum. The cells were then treated with the prepared conditioned medium from PCFA or controls (F50, F100, D50, and D100) for 24, 48, and 72 hours. Serum free medium and DMEM containing 10% FBS was used as negative and positive controls, respectively. The viable cells were detected by using the MTT dye, which was reduced by the mitochondrial dehydrogenase presented in living cells and formed blue formazan crystals. Two hundred microlitres of MTT solution [3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co.] was added to each well, and incubated for 4 hours. Subsequently, 900 µL of dimethyl sulfoxide and 125 µL of glycine buffer (0.1 M NaCl + 0.1 M Glycine, pH 10.5) were added into each well to dissolve the formazan crystal. The survival or proliferation rates of the cells were calculated from spectrophotometer measurement at 570 nm wavelength. Data obtained from the MTT assay was shown as relative optical density by comparing with the negative control.

3.2.10 RNA and protein synthesis

1) Analysis of dentin sialophosphoprotein (DSPP) synthesis by RT-PCR technique [195]

The 1 x 10^5 primary human dental pulp cells were seeded in DMEM with 10% FBS in 6-well tissue culture plates, and incubated for 24 hours. Then, the cells were treated with the conditioned medium (F50, F100, D50, and D100) and supplemented with 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate. The medium with and without 10 nM of dexamethasone were used as positive and negative controls. The

medium was changed every 48 hours for 14 days in every group. The medium was removed, and total RNA was extracted using the Trizol reagent (Gibco BRL) according to the manufacturer's instructions. One microlitre of each RNA sample was converted to cDNA by reverse transcription using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) for 90 minutes at 42°C. Subsequent to the reverse transcription, polymerase-chain reaction (PCR) was performed for detection of DSPP cDNA. The primers specific to DSPP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared following the reported sequences from GenBank. The oligonucleotide sequences of DSPP and GAPDH primers were:

DSPP	sense	5' AATGGGACTAAGGAAGCTG 3'
	antisense	5' AAGAAGCATCTCCTCGGC 3'
GAPDH	sense	5' TGAAGGTCGGAGTCAACGGAT 3'
	antisense	5' TCACACCCATGACGAACATGG 3'

The PCR was performed using Tag polymerase (Qiagen, Hilden, Germany) with 25 µL PCR volume. The reaction mixtures contained 25 pM of primers and 1 µL of RT reaction. The amplication profiles for DSPP (38 cycles) and GAPDH (22 cycles) were set at denaturation for 1 minute at 94°C, primer annealing for 1 minute at 60°C, and chain elongation for 2 minutes at 72°C in DNA thermal cycler (Biometra, Gottingen, Germany). The amplified DNA was then electrophoresed on a 1% agarose gel and visualized by ethidium bromide fluorostaining. The relative intensities of the gel bands were measured

by imaging software analysis (Scion Image version Alpha 4.0.3.2; Scion, Frederick, MD, USA). The experiments were performed from three different donors.

2) Analysis of dentin sialoprotein (DSP) synthesis by Western blot technique [57]

The 1 x 10^5 primary human dental pulp cells were seeded in DMEM with 10% FBS in 6-well tissue culture plates, and incubated for 24 hours. Then, the cells were treated with the conditioned medium and supplemented with 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate. The medium with and without 10 nM of dexamethasone were used as positive and negative controls. The medium in each well was changed every 48 hours for 14 days. Then, the cells were washed three times with PBS. The cells were lysed with RIPA buffer (50 mmol/L TrisHCL, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 0.25% Na-deoxycholate). The total amount of protein from each extract was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of protein from each sample was mixed with running buffer, loaded to a 10% polyacrylamide gel, and separated by electrophoresis along with pre-stained high molecular weight standards (Bio-Rad, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) using a trans-blot cell (Gibco BRL) with a 180 mA current for 1.5 hours. Then, the nitrocellulose membrane was stained for DSP by using goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted to 1/100, or β-actin by using mouse monoclonal antibody (Chemicon International, Temecula, CA) diluted to 1/1000. After

washing in PBS, the membrane was incubated with biotinylated-secondary antibody (Sigma Chemical Co.) for 50 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 50 minutes, respectively. The protein bands were detected using a SuperSignal® West Pigo Trial Kit (Pierce, Rockford, IL, USA) and were exposed on CL-X Posture film (Pierce, Rockford, IL, USA). The relative intensity of DSP-specific bands was digitalized and compared with the protein marker to indicate the type of protein. The band intensity was determined by scion image analysis software (Scion Corporation) and optical density was adjusted to percentage of expression, by comparing with control. The intensity of protein bands indicated the relative amounts of protein in the samples. The experiments were performed from three different donors.

3.2.11 Test of anti-inflammatory effect

1) Induction of COX-2 mRNA expression

The 1.5 x 10^5 primary human dental pulp cells were seeded in DMEM with 10% FBS in 6-well tissue culture plates and incubated for 24 hours. Then, the medium was replaced twice by serum free medium at 3 hours intervals in order to wash out the serum before exposure of the conditioned medium. The cells were then exposed to 20 µg/mL of LPS of *Pseudomonas aeruginosa* (Sigma Chemical Co.) for 3 hours to induce inflammation in the cell culture [136]. RNA isolation and RT-PCR were performed

to confirm the expression of COX-2 mRNA which is a gene encoding the key enzyme in the inflammatory process.

2) Determination of anti-inflammatory effect

After treatment with LPS for 3 hours, the cells were washed 2 times with serum free medium. The cells were then treated with conditioned medium (F50, F100, D50, and D100) for 24 hours. Serum free medium with and without LPS treatment were used as controls. At the end of culture, the medium was removed, and total RNA was extracted using the Trizol reagent (Gibco BRL) according to the manufacturer's instructions. One microlitre of each RNA sample was converted to cDNA by reverse transcription using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) for 90 minutes at 42°C. Subsequent to the reverse transcription, polymerase-chain reaction (PCR) was performed for detection of COX-2 cDNA. The primers specific to COX-2 and GAPDH were prepared following the reported sequences from GenBank. The oligonucleotide sequences of COX-2 and GAPDH primers were:

COX-2	sense	5' TTCAAATGAGATTGTGGGAAAATTGCT 3'
	antisense	5' AGATCATCTCTGCCTGAGTATCTTT 3'
GAPDH	sense	5' TGAAGGTCGGAGTCAACGGAT 3'
	antisense	5' TCACACCCATGACGAACATGG 3'

The PCR was performed using Tag polymerase (Qiagen, Hilden, Germany) with 25 µL PCR volume. The reaction mixtures contained 25 pM of primers and 1 µL of RT

reaction. The amplication profiles for COX-2 (27 cycles) and GAPDH (22 cycles) were set at denaturation for 1 minute at 94°C, primer annealing for 1 minute at 60°C, and chain elongation for 2 minutes at 72°C in DNA thermal cycler (Biometra, Gottingen, Germany). The amplified DNA was then electrophoresed on a 1% agarose gel and visualized by ethidium bromide fluorostaining. The relative intensities of the gel bands were measured by imaging software analysis (Scion Image version Alpha 4.0.3.2; Scion, Frederick, MD, USA). The experiments were performed from three different donors.

3.3 Statistics

All experiments were conducted at least in triplicate. The results were reported as mean ± standard deviations. Data were analyzed by using SPSS program (SPSS 17.0; SPSS Inc., Chicago, IL) and Stats Direct software (StatsDirect Ltd, Altrincham Cheshire, UK). Several types of statistical tests were selected to determine statistically significant difference from the data. The data from pH, setting time, compressive strength, disintegration, and acid soluble arsenic content were normal distributions. Therefore, two independent-samples t tests were used to determine the significant difference between the data from the experimental (PCFA) and control groups (Dycal®). For MTT assay, all data were normal distributed, but the equal variances of 72-hour group was not assumed. Therefore, one way ANOVA with Post hoc multiple comparisons was used for the 24- and 48-hour groups, but Kruskal Wallis test with

pairwise comparisons (Conover-Inman method) was used for 72-hour group. The Kruskal Wallis tests with pairwise comparisons (Conover-Inman method) were also used for the RNA and protein analysis, and test of anti-inflammatory effect because the data was not normal distributions and sample sizes was limited. All statistical significance was determined at $p < 0.05$.

CHAPTER IV

RESULTS

4.1 *In vitro* release of fluocinolone acetonide

The amount of released fluocinolone acetonide was determined by HPLC and UV detection directly from the conditioned medium. The HPLC method allowed for the detection of fluocinolone acetonide at a retention time of 11.0 minutes (Fig. 11a). The amounts of released fluocinolone acetonide from various concentrations of the experimental materials were presented in Fig. 11b. The raw data was shown in appendix, table 3. Cement with 50 mmol/L fluocinolone acetonide could release fluocinolone acetonide at a concentration of 50 µmol/L from 50 mm^2 surface area which was equal to 1 µmol/L/mm^2. Therefore, 50 mmol/L of fluocinolone acetonide loading was selected for further investigation.

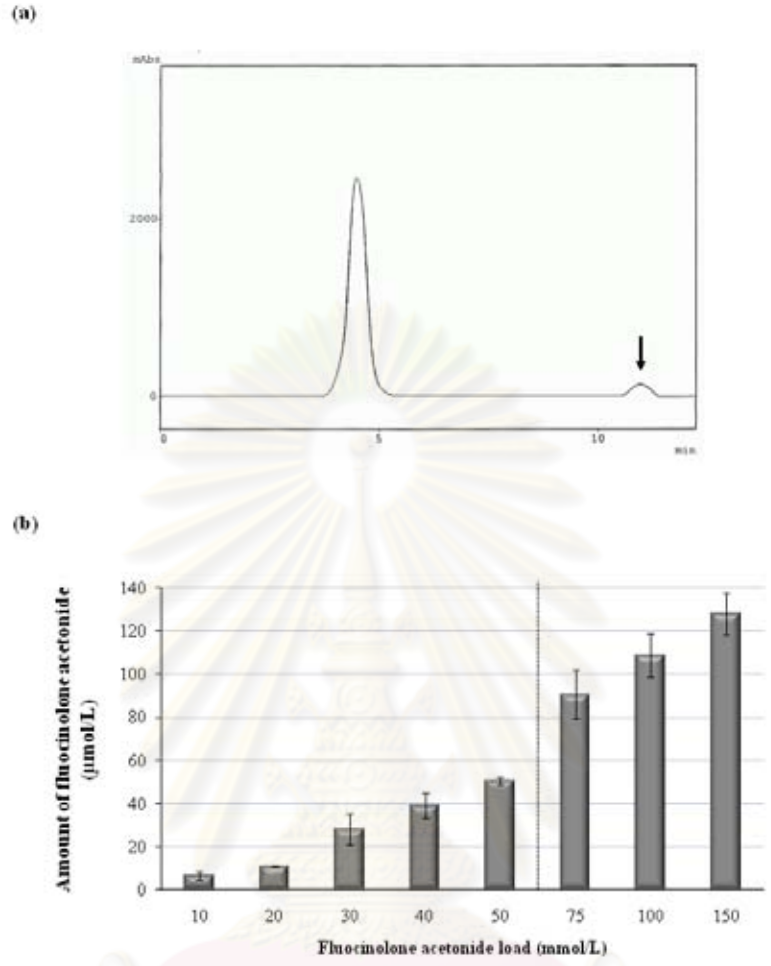

Figure 12 Release of fluocinolone acetonide from experimental cements. (a) Chromatogram obtained from HPLC and peak of fluocinolone acetonide (arrow); (b) Selection of 50 mmol/L fluocinolone acetonide loading from various doses of experimental pulp capping materials. The result was represented as mean ± standard deviation.

The cumulative release of fluocinolone acetonide from the PCFA was investigated in the *in vitro* release study over 6 months. The result was shown in Fig. 12a as the mean ± SD of the released fluocinolone acetonide. The raw data was shown in

appendix, table 4. Fluocinolone acetonide was dramatically released over the first 5 days (Fig. 12b). This early rapid release also affected the cumulative release of fluocinolone acetonide, especially in the first 5 days as shown in Fig. 12a. After 1 month, fluocinolone acetonide was released from the PCFA at an almost constant rate of 0.029–0.021 μmol/L/day.

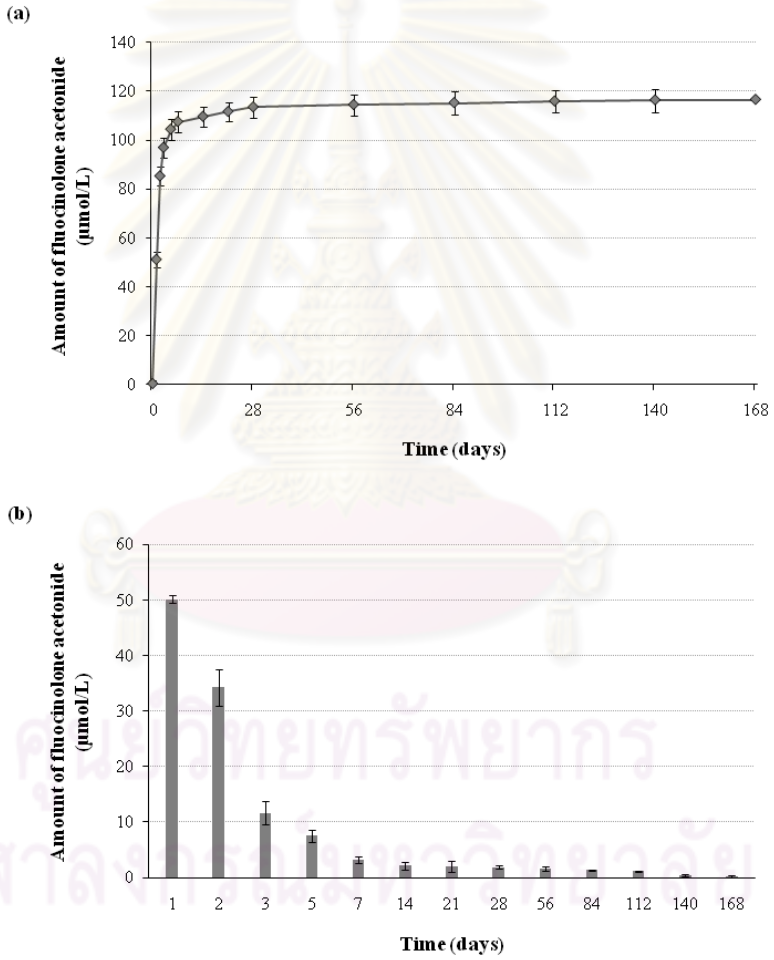

Figure 13 Release of fluocinolone acetonide from experimental cement over 6 months. (a) Cumulative release of fluocinolone acetonide; (b) Fluocinolone acetonide concentration released over each different periods of time. The result was represented as mean ± standard deviation.

4.2 pH measurement

The pH of the PCFA and Dycal® became increased from neutral after 1 hour. The pH of PCFA was significantly higher than that of Dycal® at every time interval ($p < 0.05$). The pH of Dycal® and PCFA ranged between 9.80 to 10.86 and 10.57 to 11.72, respectively. The pH of both materials remained stable after 72 hours as shown in Fig. 13. The raw data was shown in table 5 in the appendix.

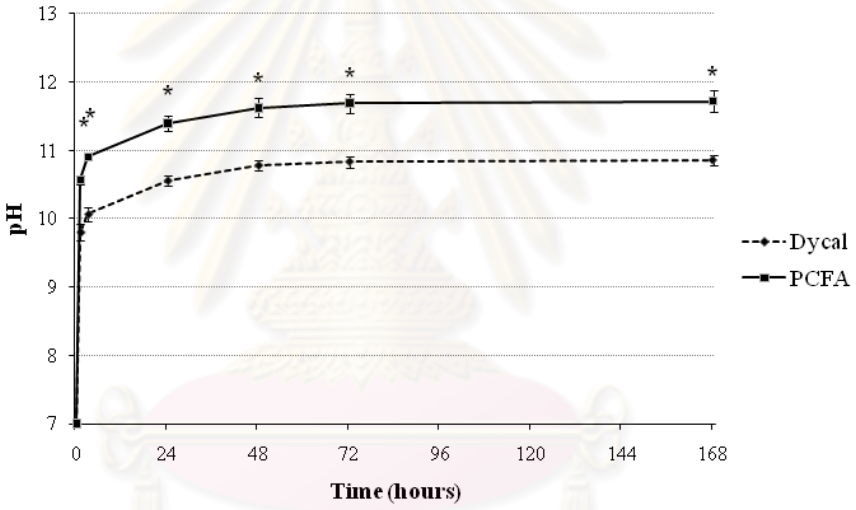

Figure 14 pH values of Dycal® and PCFA at various time periods. Data was represented as mean ± standard deviation at each time interval. *Statistically significant difference between materials.

4.3 Determination of setting time, compressive strength, disintegration, and amount of acid-soluble arsenic content

4.3.1) Setting time

The setting time of Dycal® and PCFA ranged from 60 to 90 seconds and 115 to 165 seconds, respectively. The mean setting times of Dycal® and PCFA were 75 and 150 seconds, respectively (Fig. 14). The setting time of PCFA was about 2-fold longer than Dycal®. The setting time of PCFA was significantly higher than Dycal® ($p < 0.05$). The raw data was shown in appendix (table 7).

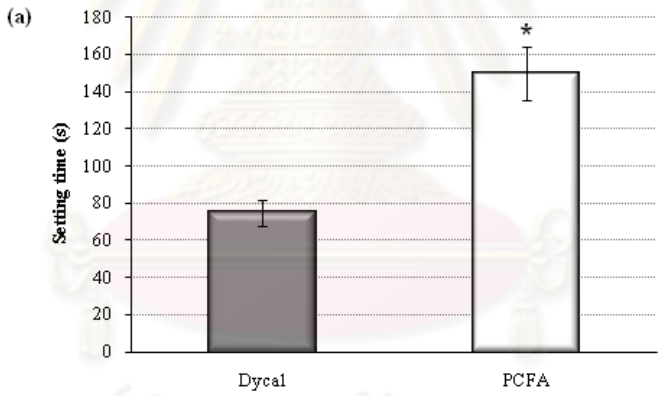

Figure 15 Setting time of Dycal® and PCFA. The result was represented as mean ± standard deviation. *Statistically significant difference between materials ($p < 0.05$).

4.3.2) Compressive strength

The compressive strength of Dycal® and PCFA were 17.09 ± 2.91 and 15.11 ± 1.25 MPa, respectively (Fig. 15). The compressive strength between Dycal® and PCFA was not significantly different ($p < 0.05$). The raw data was shown in table 7 in the appendix.

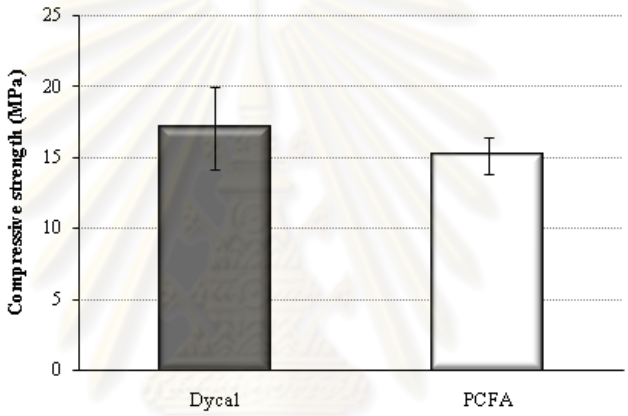

Figure 16 Compressive strength of experimental cement (PCFA) and control (Dycal®). The result was represented as mean ± standard deviation.

4.3.3) Disintegration

The disintegration of Dycal® and PCFA were 3.13 ± 0.43% and 3.15 ± 0.22% respectively (Fig. 16). The disintegration between Dycal® and PCFA was not significantly different ($p < 0.05$). The raw data was also shown in table 8 in the appendix.

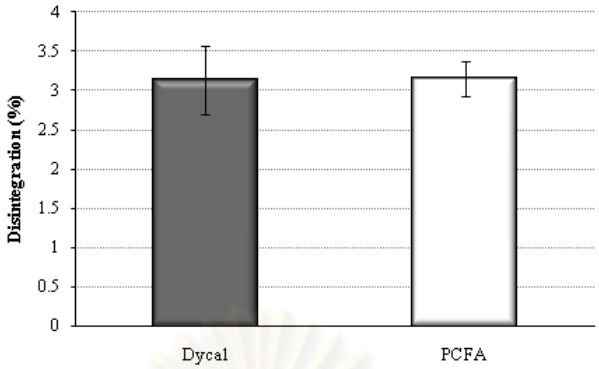

Figure 17 Disintegration of experimental cement (PCFA) and control (Dycal®). The result was represented as mean ± standard deviation.

4.3.4) Acid-soluble arsenic content

The acid-soluble arsenic content of Dycal® was less than < 0.05 mg/Kg, whereas of PCFA was 0.21 ± 0.02 mg/Kg (Fig. 17). The acid-soluble arsenic content of PCFA was approximately 4-fold higher than Dycal®. The result from PCFA was significantly higher than Dycal® ($p < 0.05$). The raw data was shown in appendix, (table 7).

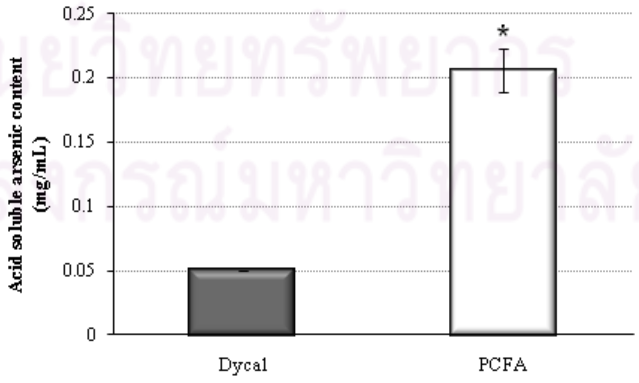

Figure 18 Acid-soluble arsenic content of experimental cement (PCFA) and control (Dycal®). Data was represented as mean ± standard deviation. *Data show statistically significant difference between materials ($p < 0.05$).

4.4 Accelerated stability test

The *in vitro* release of fluocinolone acetonide and pH measurement were done after three-month storage of PCFA. No change in physical properties was observed. However, minor decrease in the amount of fluocinolone acetonide and pH of the conditioned medium over the period of 7 days was noted. At 24 hours, cumulative release of fluocinolone acetonide was 46.85 μmol/L, which decreased 7.99% from the freshly prepared material. The differences of released fluocinolone acetonide and pH between the freshly-prepared and stored materials were within 10% at all time periods, except the release of fluocinolone acetonide at 48 hours which was decrease to 55.46%. The release of fluocinolone acetonide was significantly different at 4, 8, 24, 48, and 168 hours (Fig. 18a). The pH change was significantly different ($p < 0.05$) at all time periods (Fig. 18b). However, the pH after three-month storage was still in the high pH range (9.69-11.65). All data was shown in the appendix, table 10.

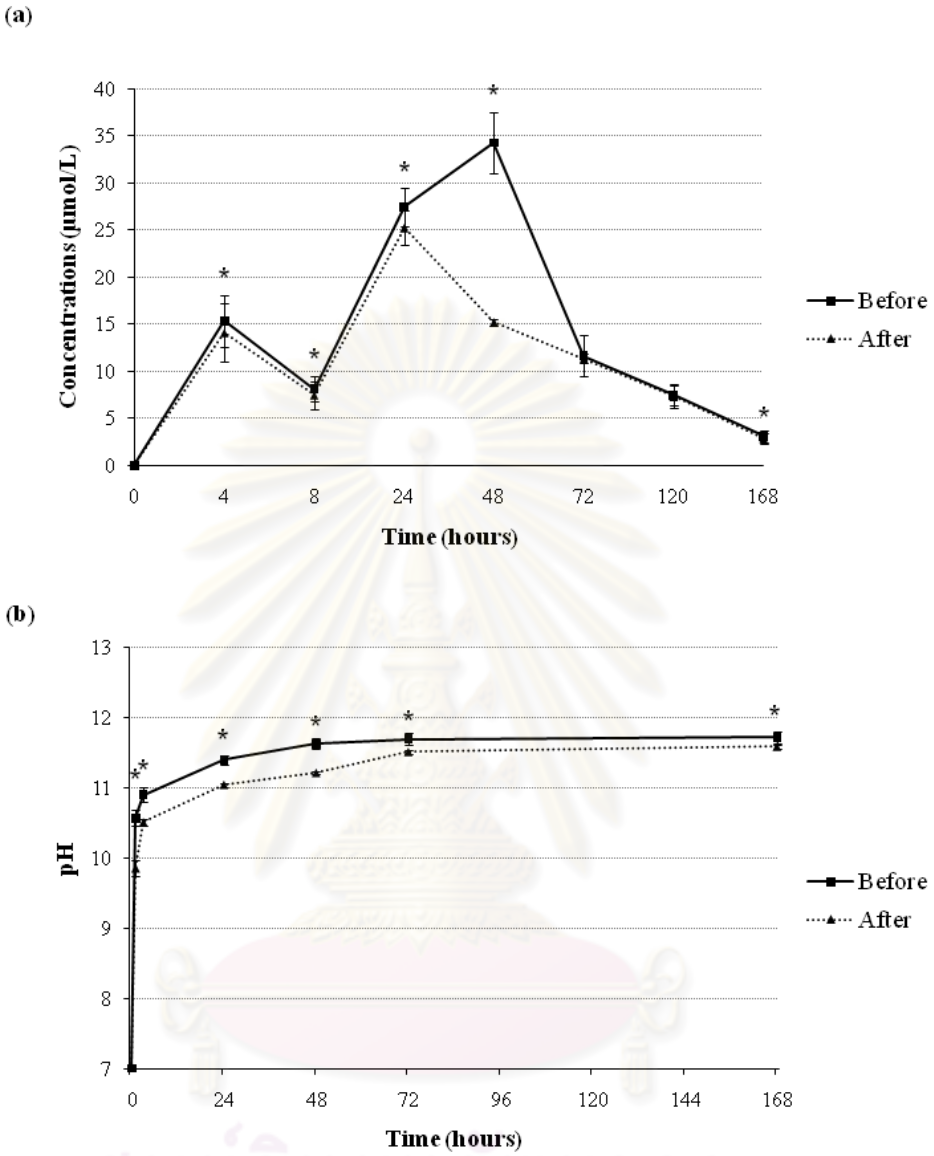

Figure 19 Accelerated stability tests of PCFA. The material was kept at 45°C for 3 months. (a) Released fluocinolone acetonide concentration over 7-day period before and after storage; (b) pH values of PCFA before and after storage. Data was represented as mean ± standard deviation at each time interval. *Data show statistically significant difference between before and after storage at each time interval ($p < 0.05$).

4.5 Cytotoxicity and cell proliferation assay

Full concentration of the conditioned medium from both Dycal® and PCFA had toxicity to the HDPCs. The conditioned medium was, therefore, diluted before evaluation of cytotoxicity and cell proliferation. The diluted conditioned medium from PCFA (F50 and F100) slightly increased the cell proliferation with time. At 48 hours, the culture media with 10% FBS significantly ($p < 0.05$) increased cell proliferation when compared with the negative control (SFM). At 72 hours, the culture media with 10% FBS, F50, and F100 significantly increased cell proliferation when compared with the media obtained from the other groups. The effect of culture media with 10% FBS and F50 was not significantly different ($p < 0.05$) (Fig. 19). The raw data was shown in table 12 in the appendix.

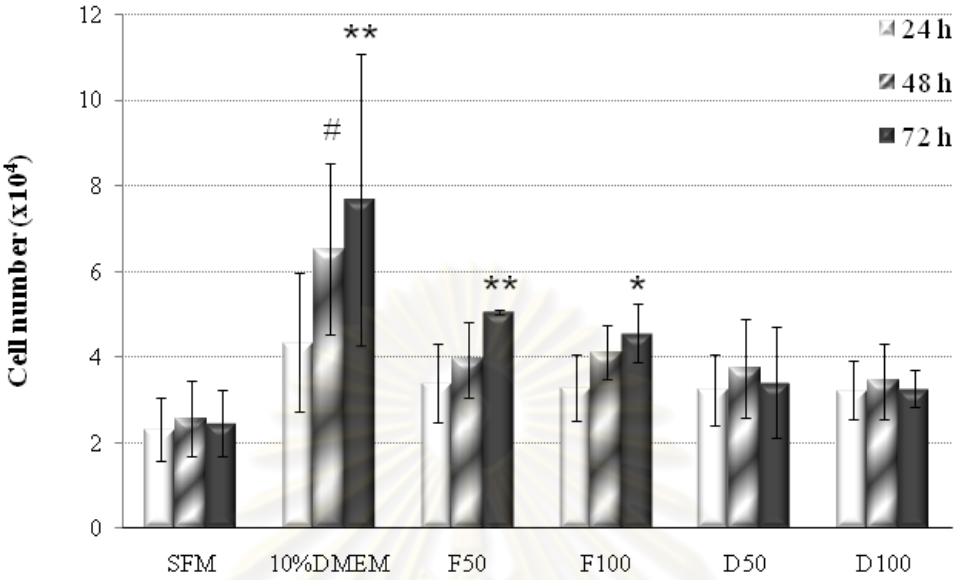

Figure 20 Effects of conditioned medium from Dycal® and PCFA on cytotoxicity and cell proliferation of HDPCs at 24, 48, and 72 hours. SFM was used as negative control. F50 and F100 were the conditioned medium from PCFA diluted to 50 and 100 times, respectively. D50 and D100 were the conditioned medium from Dycal® diluted to 50 and 100 times, respectively. The results presented as cell numbers (mean ± SD). #Statistically significant difference ($p < 0.05$) compared with the negative control group at 48 hours. *,**Statistically significant difference ($p < 0.05$) compared with the other groups at 72 hours.

4.6 RNA and protein synthesis

The effects of diluted condition medium from Dycal® and PCFA on DSPP expression and DSP synthesis were shown in figure 20a and 20b, respectively. The gene expression resulted from RT-PCR revealed that all experimental and positive control (dexamethasone) groups enhanced DSPP expression compared to the negative

control. The conditioned medium form the PCFA increased the expression of DSPP approximately 2.5 folds when compared with the negative control. The positive control and F50 groups significantly ($p < 0.05$) increased the gene expression when compared to the negative control and D50 (Fig. 20c). For protein analysis, the results from Western blotting revealed the conditioned medium form the PCFA increased the synthesis of DSP approximately 1.7 to 1.8 folds when compared with the negative control. However, the effect was not significantly different ($p < 0.05$) among the experimental and control groups (Fig. 20c). The raw data was shown in table 14 in appendix.

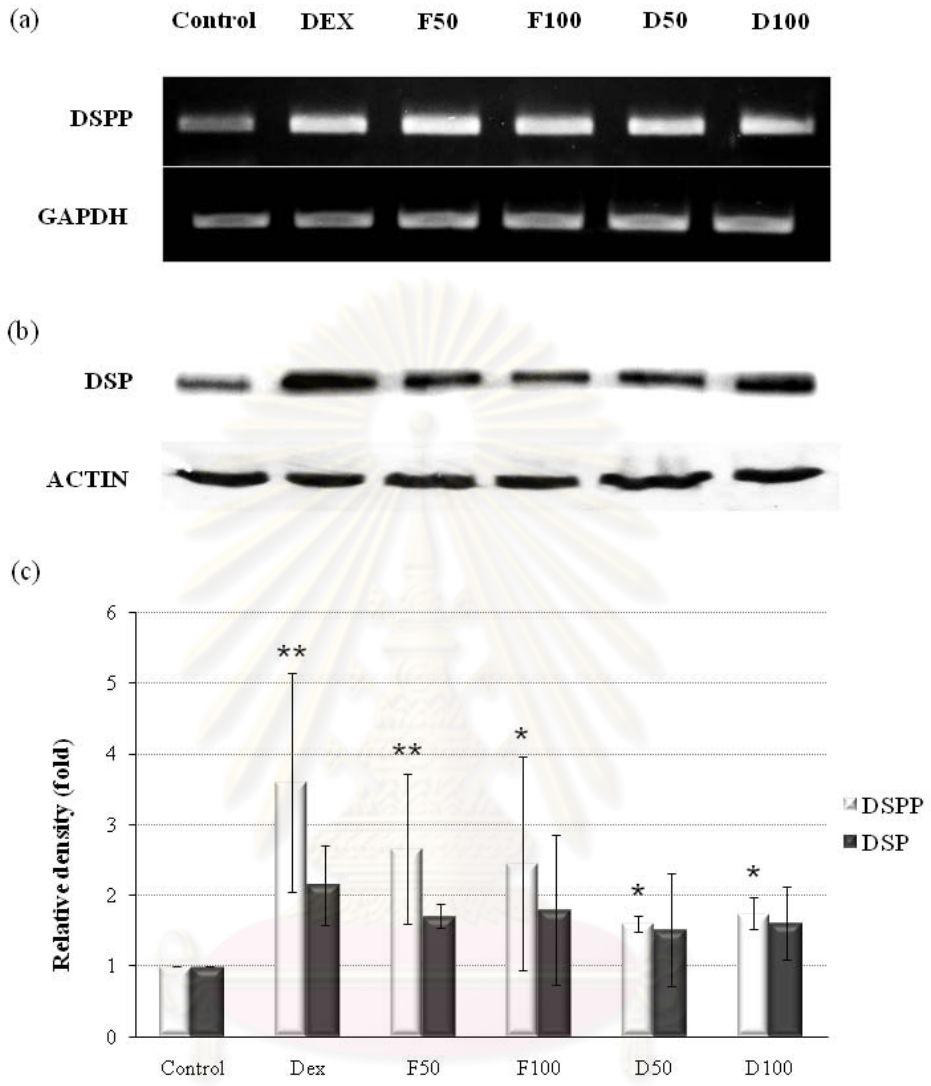

Figure 21 Effects of conditioned medium from PCFA and Dycal® on DSPP expression and DSP synthesis of HDPCs. The results from RT-PCR and Western blotting showed the effects of the conditioned medium on (a) DSPP and (b) DSP in HDPCs, respectively. (c) The graph showed mean ± standard deviation of band density from three separate experiments. *,**Statistically significant difference was found in DSPP expression compared with the other groups ($p < 0.05$). No significant difference was found in DSP synthesis.

4.7 Test of anti-inflammatory effect

The results from pilot study showed that HDPCs were induced to express the COX-2 gene expression after treatment with LPS at 3, 6 and 24 hours. Therefore, three-hour treatment of LPS was done before the experiments. The conditioned medium from F50 and F100 of PCFA could approximately reduce the expression of COX-2 to 43% and 59%, respectively when compared with the control (LPS-untreated) group as well as 63% and 74%, respectively when compared with the LPS-treated group. All concentrations of conditioned medium form PCFA significantly decreased COX-2 expression, but conditioned medium form Dycal® did not affect ($p < 0.05$) this expression (Fig. 21a, b). The raw data was shown in table 16 in appendix.

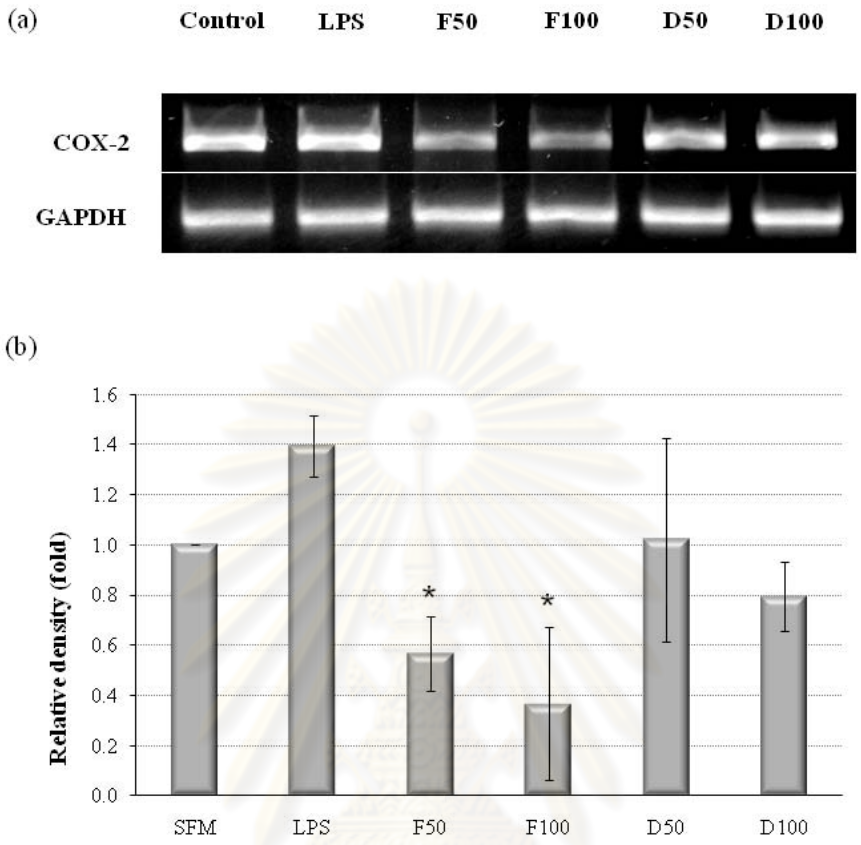

Figure 22 Effects of conditioned medium from PCFA and Dycal® on COX-2 expression from HDPCs. (a) The results from RT-PCR showed the effects of the conditioned medium on COX-2 mRNA expression in HDPCs. (b) The graph showed mean ± standard deviation of band density from three separate experiments. *Statistically significant difference was found in COX-2 expression compared with the other groups ($p < 0.05$).

CHAPTER V

DISCUSSION

Direct pulp capping or partial pulpotomy is treatment of an exposed vital pulp with a dental material to facilitate the formation of reparative dentin and maintenance of vital and healthy pulp tissue. It has been studied as an alternate way to avoid vital pulp extirpation. However, long term success rate of direct pulp capping is much lower than that of vital pulp extirpation [17-20]. Therefore, the pulp capping procedure is currently considered controversial by many clinicians. To increase the success rate, a critical need exists to develop new biologically based therapeutics that reduce pulp inflammation, promote the continued formation of new dentin-pulp complex, and restore vitality by stimulating the regrowth of pulpal tissue [22]. Currently, MTA has been proved to have particular promise as a pulp capping material. It has good sealing ability and can stimulate thick dentinal bridge [98, 103, 104], even in cases of irreversible pulpitis [97, 99, 196]. However, MTA has slow setting time and high cost. Some authors have reported the similar result between MTA and calcium hydroxide when used in human teeth [18, 106, 107]. Recently, a wide range of concentration of fluocinolone acetonide (0.1-10 μmol/L) demonstrated positive effects on human dental pulp cell proliferation, and both fibronectin and type I collagen synthesis [33]. With the known anti-inflammatory effects, it may have some potential in stimulation of early phase of healing of dental pulp tissue. Therefore, the new experimental calcium hydroxide base pulp

capping material containing fluocinolone acetonide (PCFA) was developed. The expected benefits of PCFA may be combination of therapeutic effect of calcium hydroxide, control of pre-existing inflammation and stimulatory effect of fluocinolone acetonide. The specific concentration of fluocinolone acetonide has been demonstrated to be crucial for biological response of dental pulp cells [33]. Thus, the control of corticosteroid release is important for successful use of PCFA in vital pulp treatment. The appropriate concentrations of fluocinolone acetonide in promotion of early healing response of pulp cells were 0.1-10 μmol/L [33]. The result of this study revealed that a suitable concentration (0.1-10 μmol/L/mm^2) was released from the experimental cement with 50 mmol/L of fluocinolone acetonide. This concentration was then chosen for further experiments. The *in vitro* release study showed that appropriate concentrations of fluocinolone acetonide (0.1-1 μmol/L) could release from the material within the first 5 days. Therefore, the initial burst release of fluocinolone acetonide would be beneficial to early phase of dental pulp healing. Over a period of 6 months, the constant release of physiologic concentrations (0.0001 to 10 μg/mL) of fluocinolone acetonide was found [197]. The minimal amount of released fluocinolone acetonide from the material was preferred to prevent the adverse effects of the corticosteroid.

The success of pulp capping procedures with calcium hydroxide was related to its high alkalinity and it was suggested the rise in pH was the most important factor conductive to pulp healing [8, 106, 119, 120, 198]. The conditioned medium obtained

from Dycal® had pH values of 9.80-10.86 over the 168-h (7 days) duration of this assay. This finding was in agreement with previous reports [114, 118, 124, 193, 199-201]. The conditioned medium of PCFA also maintained high alkaline levels, with pH values of 10.57-11.72, which were significantly higher than those of Dycal® during the 168-hour (7 days) duration. This difference may be due to the different amount of hydroxyl ions released from each material. The higher pH value may have some advantages in neutralization of the acid at the site of inflammation, antibacterial effect, and stimulation of the repair and dentin bridge formation in the exposed pulp [120, 193, 201, 202].

The ISO standard for a pulp capping material has not been developed. Therefore, ISO 3107:2004 was selected as a guideline for evaluation of the material properties. The setting time of the PCFA (150 s) was longer than that of Dycal® (75 s). However, some earlier studies reported a longer setting time of Dycal® (145 s). This may be due to the different methods used in these studies [118, 203]. The compressive strength of PCFA (15.11 ± 1.25 MPa) was comparable to Dycal® which was 17.09 ± 2.91 MPa. Previous studies reported the compressive strength of Dycal® in a range of 14.5 to 36 MPa [124-126] which was in the same range of this study. However, the compressive strength of both cements was much higher than the minimal strength required by the ISO 3107:2004. They were strong enough to resist the average stress of 10.5 N/mm^2, which was applied through an amalgam condensation cycle [204]. The disintegration of PCFA (3.16%) was equal to that of Dycal® (3.13%) and was also comparable to the

previous reports of Dycal® (2.7 to 6.76%) [118, 122]. The solubility of this calcium hydroxide base cement may be beneficial for the release of hydroxyl ions and fluocinolone acetonide. However, this property may cause the dissolution of the material and leave space under a restoration [122]. Due to the low compressive strength, disintegration and lack of adhesion to dentin, these type of cement should be used in thin layer and covered by other base or liner material [205].

The acid-soluble arsenic content of PCFA was 0.21 mg/Kg which was higher than that of Dycal® (0.05 mg/Kg). However, the arsenic content in PCFA was approximately ten-fold below the maximal acceptable dose according to the ISO 3107:2004 standard. This implies that this new experimental material should be safe for clinical use, according to the arsenic content. After the incorporation of fluocinolone acetonide into the new experimental pulp capping material, fluocinolone acetonide could release in the desirable dose and some physical and mechanical properties were not different from Dycal®.

The accelerated stability test is designed to increase the rate of chemical degradation or physical change of an active substance or pharmaceutical product by using exaggerated storage conditions as part of the formal stability studies [206, 207]. In this study, the material was tested in a standard condition of temperature at 45°C at ambient humidity [194]. Within this limited condition, most of the active ingredients were decreased in a range of 10%. The shelf life of this material may be acceptable at about

1 year, when stored at below 30°C. However, the results from these accelerated tests do not always predict the physical and chemical changes. A wide range of temperature and relative humidity must be included to increase the reliability of the stability test. Furthermore, the intermediate and long-term stability studies also should be done [194].

Calcium hydroxide materials are normally caustic to the cells because of its high alkalinity [208, 209]. The result from this study showed that conditioned medium from both PCFA and Dycal® had cytotoxic effect to the HDPCs, which was different from the earlier study [75]. This may be because of different method and period of experiment to obtain the conditioned medium. The conditioned medium was carried out for 1 hour in the earlier study, but it was collected at 24 hours in this study. The high alkaline pH of the conditioned medium may have deleterious effect to the cells. However, the conditioned medium was achieved from the plastic mold with 50 mm^2 surface area. Therefore, the diluted concentrations of conditioned medium of PCFA and Dycal® were used in this experiment. After the treatment of conditioned medium, the stimulatory healing effects were observed in the positive control and PCFA groups. At 72 hours, the diluted conditioned medium from PCFA groups significantly increased the cell proliferation which was not significantly different to the positive control. The result confirmed the stimulatory healing effects of released fluocinolone acetonide which corresponded to the earlier study [33]. This implies that PCFA may cause superficial

necrosis in the superficial dental pulp, but fluocinolone acetonide may have some stimulatory healing effects in the underlying pulp tissue.

Dentin bridge formation is one of the criteria for successful treatment of exposed dental pulp. In the absence of inflammation, the pulp tissue can repair and form hard tissue to seal off the underlying pulp tissue [11, 12, 48, 63-65]. The DSPP and DSP were selected to examine as mineralization markers in this study because they have been demonstrated as the important markers of dentin formation [58, 210-214]. Increase of DSPP and DSP activities were demonstrated in all experimental groups. These results may be related with the high pH of calcium hydroxide [11, 12, 48, 63-65]. Although, the inhibitory effects of corticosteroids on tissue healing have been shown [215-221], the conditioned medium from PCFA in this study significantly increased the DSPP expression which corresponded to the earlier studies [185]. HDPCs also exhibited slightly increased in DSP synthesis, but the results were not statistically significance. The results from these experiments revealed that hard tissue formation was not inhibited by PCFA.

Induced inflamed human dental pulp cells can be induced by many substances such as bacteria, lipopolysaccharide, or proinflammatory cytokines [139]. LPS of *P. aeruginosa* was selected to use in this study because it has been shown to be effective in stimulation of COX-2 gene expression. The result showed that PCFA decreased COX-2 gene expression which confirmed the availability and anti-inflammatory effect of

fluocinolone acetonide. The overall results from these *in vitro* studies imply that PCFA may cause superficial pulp necrosis, stimulate early phase of healing in the underlying tissue, decrease pre-existing inflammation, and not interfere with the hard tissue formation.

However, the cell culture has several limitations including the finite doubling potential of most normal cells. Some cultured cells have tendency to change their morphology, functions, or the range of gene expressions. Furthermore, the mechanisms of dental pulp healing *in vivo* are more complex, involving both cellular and extracellular events which cannot be completely simulated in the cell culture system. Finally, the methods of application and time used in these experiments were totally different to the real clinical situation. Therefore, an *in vivo* study is essential to confirm the effects of PCFA on inflamed dental pulp tissue.

PCFA also has some disadvantages. Since the disintegration and leakage of the calcium hydroxide base/liner material has been concerned, further studies are also required to improve its mechanical properties. Addition of fillers, micro- or nano-fillers, may improve the strength and disintegration of this material. The adhesive property of the material to dentin was important to prevent microleakage and increase the retention of the material. Some studies have attempted to add poly(acylic acid) or adhesive monomers to increase this property [200, 222-225].

This study model was also developed as an example of drug delivery system used in pulp capping procedures. It may be applied to other chemicals or biomolecules, or other types of pulp capping agent such as MTA. A series of experiments were designed after the incorporation of the active ingredients into the experimental pulp capping agents. Some of its physical, mechanical, and biological properties were studied. Similar experiments have been reported in other experimental pulp capping agents such as a resinous direct pulp capping agent containing calcium hydroxide (MTYA1-Ca), calcium phosphate/calcium silicate/bismutite cement, calcium phosphate cements [200, 203, 226]. Therefore, this model may be useful in the development of another experimental pulp capping agent which is modified as a drug delivery system.

CHAPTER VI

SUMMARY

Based on the results from this study,

1) The alternative hypothesis: Optimal concentrations of fluocinolone acetonide and hydroxyl ions can be released from PCFA at the appropriate time was accepted.

2) The null hypothesis: The properties of PCFA are not different from the commercial material (Dycal®) according to ISO 3107:2004 was accepted for compressive strength and disintegration. The alternative hypothesis: The properties of PCFA are different from the commercial material (Dycal®) according to ISO 3107:2004 was accepted for setting time and acid-soluble arsenic content.

3) The alternative hypothesis: Releases of fluocinolone acetonide and hydroxyl ions from PCFA are different between before and after the accelerated stability test was accepted, except for the release of fluocinolone acetonide at 72 and 120 hours.

4) The null hypothesis: Conditioned medium from PCFA has no effect on cultured human dental pulp cells with respect to cell proliferation, RNA and protein synthesis when compared with the commercial material (Dycal®) was accepted for cell proliferation at 24 hours, and protein synthesis (DSP); but the alternative hypothesis was accepted for cell proliferation at 48 and 72 hours, and for RNA expression (DSPP).

5) The alternative hypothesis: The effect of conditioned medium from PCFA on induced inflamed human dental pulp cells is different from the commercial material (Dycal®) was accepted.

An experimental pulp capping containing fluocinolone acetonide (PCFA) was developed as a two-paste hard setting calcium hydroxide cement. Fluocinolone acetonide could release at a range of suitable concentrations from PCFA in the first 5 days. The pH, setting time, and acid soluble arsenic content of PCFA were significantly higher than those of Dycal®. The compressive strength and disintegration of PCFA were comparable to control. The stability of PCFA was acceptable at approximately 1 year. Most of biologic effect of PCFA is comparable to Dycal, but it could stimulate DSPP and reduce COX-2 gene expression in human dental pulp cells. PCFA may be considered as an alternative in pulp capping of inflamed dental pulp tissue.

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APPENDIX

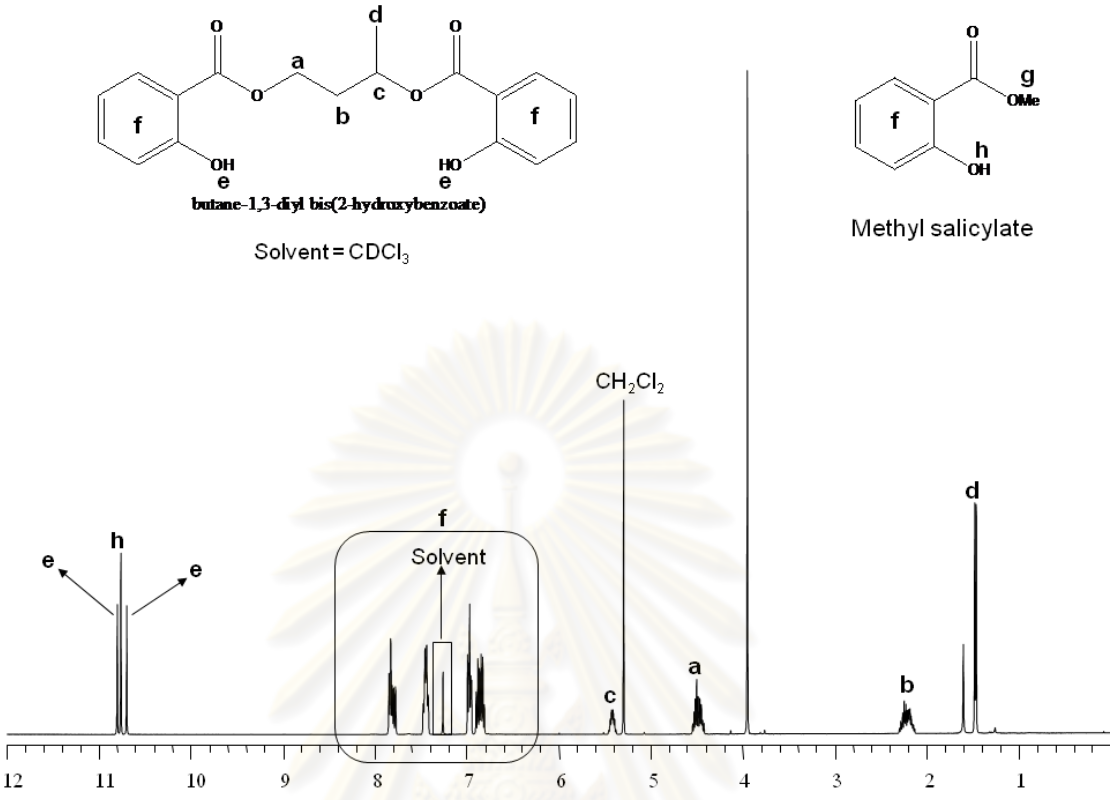

Figure 1 NMR spectra of synthesized 1,3-butylene glycol disalicylate.

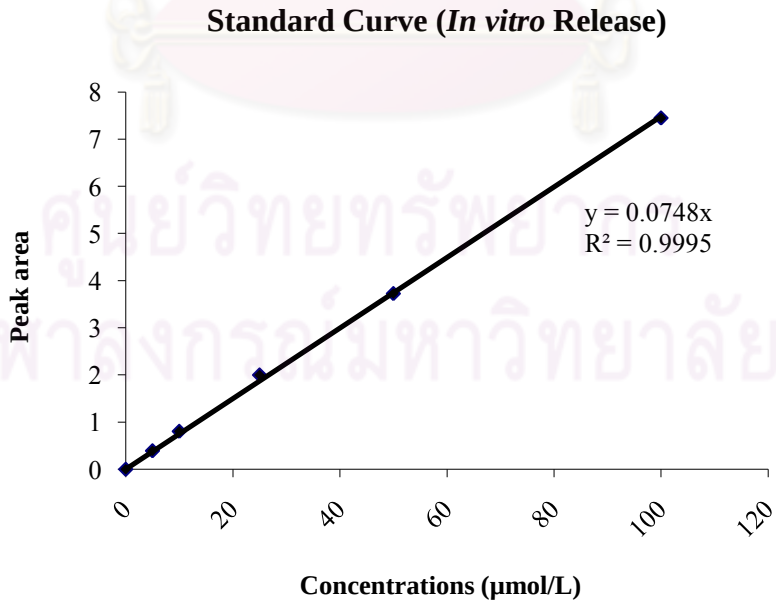

Figure 2 A standard curve of known concentrations of fluocinolone acetonide and peak area obtained from HPLC.

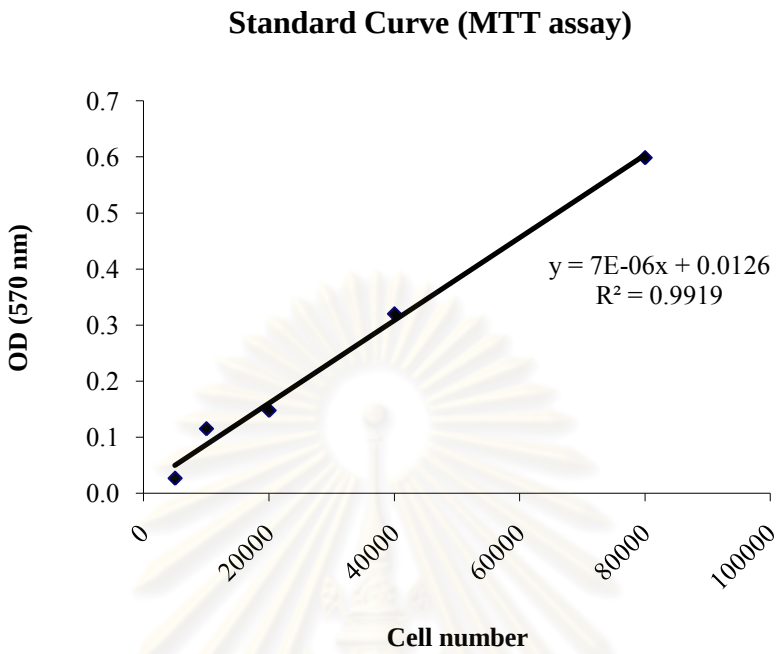

Figure 3 A standard curve of number of viable HDPC examined by MTT assay.

Table 1 Release of fluocinolone acetonide form various doses of experimental pulp capping materials: Selection of 50 mmol/L concentration (raw data).

Release	C	10	20	30	40	50	75	100	150
1	0	6.552139	11.18449	20.16444	32.58422	49.86497	78.91043	97.57888	123.9679
2	0	4.696524	10.93717	29.90374	42.75535	48.58824	92.26471	116.9144	121.2714
3	0	8.501337	11.0361	34.1123	42.05882	53.1016	101.4198	111.877	139.3516
						49.0615			
						51.99599			
Mean	0	6.583333	11.05258	28.06016	39.1328	50.52246	90.86497	108.7901	128.197
SD	0	1.902598	0.124485	7.154356	5.681914	1.944942	11.31977	10.03059	9.753831

Table 2 Cumulative release of fluocinolone acetonide at various time periods (raw data).

Release (hours)	0	4	8	24	48	72	120	168
1	0	14.7807	24.3155	49.8556	87.0521	103.4331	111.3168	115.2713
2	0	13.6524	23.8168	50.2393	85.7527	98.7246	106.1858	108.7139
3	0	18.0348	27.1364	52.3583	88.0615	98.9358	105.8409	109.861
4	0	12.1056	19.2874	49.3489	87.1243	96.6069	104.274	107.0133
5	0	17.1818	23.6791	51.7459	82.6991	92.5159	99.6095	103.0039
6	0	20.2941	29.3569	54.6109	86.3248	96.0654	103.3542	107.0694
7	0	17.8636	24.8395	50.7272	86.5079	96.5788	107.1256	110.4692
8	0	13.4278	19.7807	49.8743	78.9665	91.2018	97.8261	100.3622
9	0	12.5227	21.4037	49.766	87.5414	100.6751	107.3208	109.9919
10	0	13.5201	21.2447	50.7153	81.5201	93.5067	100.2981	102.7941
Mean	0	15.33836	23.48607	50.92417	85.15504	96.82441	104.3151	107.4550
SD	0	2.790732	3.18288	1.596604	3.031634	3.777700	4.124393	4.424393

Release (hr)	336	504	672	1344	2016	2688	3360	4032
1	116.7627	117.7981	119.8719	121.9111	123.139	124.2135	124.9208	125.2151
2	110.4398	112.1177	114.2218	115.3261	116.6468	117.7919	118.276	118.2993
3	111.9721	113.6222	114.9524	116.1864	117.3939	118.6601	119.3125	119.6603
4	108.2714	109.9811	111.9794	114.0705	115.6491	116.8925	117.2679	117.5484
5	105.5615	106.6613	108.7044	110.1571	111.3108	112.4036	112.8287	113.0832
6	108.3662	110.5829	112.4422	114.241	115.6337	116.5536	116.7504	117.067
7	112.6576	113.7638	115.6632	115.6632	115.6632	115.6632	116.1835	116.4094
8	102.6842	105.5915	107.8351	107.8351	107.8351	107.8351	108.0401	108.1105
9	113.5457	116.8561	118.3575	118.3575	118.3575	118.3575	118.58	118.6939
10	105.4301	108.9696	110.4337	110.4337	110.4337	110.4337	111.0508	111.1654
Mean	109.5691	111.5944	113.4461	114.4181	115.2062	115.8804	116.3210	116.5252
SD	4.312225	4.029739	3.935586	4.14575	4.379157	4.654309	4.731397	4.783418

Table 3 pH values of Dycal® and PCFA at various time periods (raw data).

Dycal	0	1	3	24	48	72	168
1	7	9.87	10.05	10.27	10.46	10.49	10.64
2	7	9.81	10.06	10.53	10.77	10.8	10.91
3	7	9.89	10.17	10.64	10.87	10.94	10.89
4	7	9.89	10.1	10.59	10.81	10.84	10.93
5	7	9.69	10.03	10.62	10.86	10.91	10.94
6	7	9.78	10.05	10.63	10.84	10.92	10.98
7	7	9.7	9.98	10.49	10.74	10.82	10.77
8	7	9.8	10.09	10.58	10.71	10.81	10.83
9	7	9.76	10.06	10.62	10.77	10.82	10.59
10	7	9.82	10.06	10.59	10.97	11.01	11.1
Mean	7	9.801	10.065	10.556	10.78	10.836	10.858
SD	0	0.071251	0.049272	0.110775	0.135072	0.139778	0.155263

PCFA	0	1	3	24	48	72	168
1	7	10.46	10.66	11.3	11.53	11.6	11.64
2	7	10.73	10.98	11.45	11.68	11.77	11.78
3	7	10.61	10.93	11.39	11.61	11.69	11.71
4	7	10.62	10.94	11.48	11.72	11.78	11.81
5	7	10.48	10.89	11.34	11.56	11.64	11.68
6	7	10.36	10.87	11.4	11.62	11.68	11.71
7	7	10.68	11.03	11.49	11.71	11.78	11.8
8	7	10.68	11	11.47	11.71	11.79	11.81
9	7	10.58	10.9	11.37	11.6	11.64	11.68
10	7	10.49	10.84	11.32	11.51	11.56	11.58
Mean	7	10.569	10.904	11.401	11.625	11.693	11.72
SD	0	0.117705	0.104265	0.069033	0.077352	0.083407	0.078599

Table 4 Statistical analysis of data from pH measurement. The data was analyzed by t test to perform pair wise comparison between 2 different groups. The results showed statistically significant difference between pH of condition medium from Dycal® and PCFA.

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2-tailed)	Std. Error Difference	95% Confidence Interval of the Difference	
								Lower	Upper
1h	A	3.511	.077	-17.651	18	.000	.04351	-.85941	-.67659
	N			-17.651	14.815	.000	.04351	-.86084	-.67516
3h	A	2.403	.138	-23.007	18	.000	.03647	-.91562	-.76238
	N			-23.007	12.829	.000	.03647	-.91789	-.76011
24h	A	.480	.497	-20.472	18	.000	.04128	-.93172	-.75828
	N			-20.472	15.074	.000	.04128	-.93294	-.75706
48h	A	.633	.437	-17.167	18	.000	.04922	-.94841	-.74159
	N			-17.167	14.330	.000	.04922	-.95034	-.73966
72h	A	.271	.609	-16.650	18	.000	.05147	-.96514	-.74886
	N			-16.650	14.688	.000	.05147	-.96691	-.74709
168h	A	3.304	.086	-15.664	18	.000	.05503	-.97762	-.74638
	N			-15.664	13.329	.000	.05503	-.98059	-.74341

A = Equal variances assumed, N = Equal variances not assumed

Table 5 Determination of setting time, compressive strength, disintegration, and acid-soluble arsenic content according to ISO 3107:2004 (raw data).

Dycal	Setting time	Compressive strength	Disintegration	Acid-soluble arsenic content
1	90	19.19	2.452003339	0.05
2	75	10.89	3.196868374	0.05
3	60	16.46	3.161794977	0.05
4	75	19.28	2.799114343	0.05
5	75	19.26	3.226880497	0.05
6	75	14.25	3.876611681	0.05
7	75	20.43	3.305695551	0.05
8	75	15.42	2.851700052	0.05
9	75	18.05	3.687973765	0.05
10	75	17.71	2.777565231	0.05
Mean	**75**	**17.094**	**3.13362078**	**0.05**
SD	7.071068	2.907229	0.432716946	0

PCFA	Setting time	Compressive strength	Disintegration	Acid-soluble arsenic content
1	150	14.33	2.919641223	0.235
2	165	14.51	3.324435817	0.22
3	150	17.36	2.788366945	0.22
4	150	14.75	3.014705882	0.215
5	115	14.65	3.095261615	0.19
6	150	14.67	3.122034231	0.21
7	150	16.59	3.453936568	0.185
8	165	16.53	3.315588854	0.2
9	165	13.5	3.431989924	0.2
10	150	14.24	3.086178862	0.185
Mean	**150**	**15.113**	**3.15521399**	**0.206**
SD	14.49138	1.252615	0.220975379	0.016799471

Table 6 Disintegration of Dycal® and PCFA (raw data).

Dycal	Before				After				Diff	m1-m2
	1	2	m1-m2	Mean	1	2	3	Mean		m1*100
1	1.2855	1.2861	1.262	1.277867	1.2465	1.2466	1.2465	1.246533	0.031333	2.452003339
2	1.2227	1.2233	1.2326	1.2262	1.1868	1.1871	1.1871	1.187	0.0392	3.196868374
3	1.2134	1.2132	1.2169	1.2145	1.1762	1.176	1.1761	1.1761	0.0384	3.161794977
4	1.2195	1.2192	1.2196	1.219433	1.1851	1.1852	1.1856	1.1853	0.034133	2.799114343
5	1.2062	1.1988	1.1991	1.201367	1.1626	1.1624	1.1628	1.1626	0.038767	3.226880497
6	1.1662	1.1661	1.1656	1.165967	1.1208	1.1208	1.1207	1.120767	0.0452	3.876611681
7	1.2303	1.2303	1.23	1.2302	1.1896	1.1894	1.1896	1.189533	0.040667	3.305695551
8	1.155	1.1548	1.1548	1.154867	1.1219	1.1218	1.1221	1.121933	0.032933	2.851700052
9	1.2401	1.2399	1.2402	1.240067	1.194	1.1945	1.1945	1.194333	0.045733	3.687973765
10	1.3105	1.2953	1.3149	1.3069	1.2705	1.2705	1.2708	1.2706	0.0363	2.777565231
Mean			m1	1.223737				1.18547	0.038267	**3.13362078**
S.D.										0.432716946

PCFA	Before				After				Diff	m1-m2
	1	2	3	Mean	1	2	3	Mean	m1-m2	m1*100
1	1.0932	1.0926	1.092	1.0926	1.0607	1.0605	1.0609	1.0607	0.0319	2.919641223
2	1.0985	1.0988	1.0995	1.098933	1.0625	1.0622	1.0625	1.0624	0.036533	3.324435817
3	1.0855	1.084	1.0833	1.084267	1.0539	1.054	1.0542	1.054033	0.030233	2.788366945
4	1.0888	1.0881	1.0871	1.088	1.0552	1.0554	1.055	1.0552	0.0328	3.014705882
5	1.0123	1.0128	1.0118	1.0123	0.9811	0.9806	0.9812	0.980967	0.031333	3.095261615
6	1.0198	1.0177	1.0182	1.018567	0.9865	0.9868	0.987	0.986767	0.0318	3.122034231
7	1.0154	1.0162	1.0142	1.015267	0.9804	0.9799	0.9803	0.9802	0.035067	3.453936568
8	1.0161	1.0155	1.0116	1.0144	0.9809	0.9808	0.9806	0.980767	0.033633	3.315588854
9	1.0579	1.0592	1.0589	1.058667	1.0226	1.0223	1.0221	1.022333	0.036333	3.431989924
10	1.0211	1.0276	1.0263	1.025	0.9933	0.9932	0.9936	0.993367	0.031633	3.086178862
Mean			m1	1.0508				1.017673	0.033127	**3.15521399**
S.D.										0.220975379

Table 7 Statistical analysis of data from determination of setting time, compressive strength, disintegration, and acid-soluble arsenic content. One-Sample Kolmogorov-Smirnov Test was used for determination of normal distribution for all data. Setting time, compressive strength, and disintegration were then analyzed by t test to perform pair wise comparison between 2 different groups. Acid-soluble arsenic content was analyzed by Mann-Whitney U test to perform comparison between 2 different groups (continue).

GROUP		SETTING	STRENGTH	DISINTEG	AS
Dycal	N	10	10	10	10
	Kolmogorov-Smirnov Z	1.265	.582	.460	1.581
	Asymp. Sig. (2-tailed)	.082	.888	.984	.013
FA	N	10	10	10	10
	Kolmogorov-Smirnov Z	1.178	.993	.525	.441
	Asymp. Sig. (2-tailed)	.125	.278	.946	.990

		Levene's Test for Equality of Variances		t-test for Equality of Means					
				t	df	Sig. (2-tailed)	Std. Error Difference	95% Confidence Interval of the Difference	
		F	Sig.					Lower	Upper
SETTING TIME	A	1.699	.209	-14.905	18	.000	5.099	-86.713	-65.287
	N			-14.905	13.056	.000	5.099	-87.011	-64.989
COMPRESSIVE STRENGTH	A	4.960	.039	1.979	18	.063	1.00105	-.12213	4.08413
	N			1.979	12.230	.071	1.00105	-.19556	4.15756
DISINTEGRATION	A	2.875	.107	-.141	18	.890	.153647	-.344394	.301207
	N			-.141	13.395	.890	.153647	-.352535	.309348

A = Equal variances assumed, N = Equal variances not assumed

Table 7 Statistical analysis of data from determination of setting time, compressive strength, disintegration, and acid-soluble arsenic content. Acid-soluble arsenic content was analyzed by Mann-Whitney U test to perform comparison between 2 different groups – continued.

Acid-soluble arsenic content	
Mann-Whitney U	.000
Wilcoxon W	55.000
Z	-4.044
Asymp. Sig. (2-tailed)	.000
Exact Sig. [2*(1-tailed Sig.)]	.000(a)

a Not corrected for ties.

b Grouping Variable: GROUP

Table 8 Accelerated stability tests of PCFA. Release of fluocinolone acetonide and pH measurement were reported after three-month storage (raw data).

Release of FA	0	4	8	24	48	72	120	168
1	0	13.598244	8.772016	23.496892	15.17678	12.31052	7.253004	3.63814
2	0	12.560208	9.351248	24.3087	15.192328	11.934148	6.864304	2.325852
3	0	17.512016	9.293472	23.204148	15.366944	10.924356	8.192692	3.698492
4	0	10.217152	6.607256	27.65658	15.433368	11.483992	7.053732	2.520156
5	0	15.807256	5.977516	25.821456	14.676944	10.871456	6.526112	3.122848
6	0	19.590572	8.337776	23.23368	15.376788	10.801352	6.705696	3.417984
7	0	16.434512	5.497828	23.816684	15.438244	11.105228	9.703056	3.076112
8	0	11.433576	5.844668	27.686112	15.724824	11.256476	6.094356	2.333212
9	0	11.520884	8.17052	26.093316	14.605368	12.083004	8.874044	2.457412
10	0	12.438492	7.106632	27.112952	15.460416	11.027672	6.248088	2.29632
Mean	0	14.111291	7.4958932	25.243052	15.2452	11.37982	7.3515084	2.8886528
SD	0	3.0618281	1.469372	1.8408635	0.3529155	0.5471066	1.1919892	0.5662377

pH	0	1	3	24	48	72	168
1	7	9.8	10.58	11.03	11.19	11.51	11.59
2	7	9.69	10.51	11.07	11.21	11.5	11.62
3	7	9.89	10.52	11.06	11.22	11.52	11.58
4	7	9.88	10.43	11.01	11.23	11.54	11.58
5	7	10	10.5	11.04	11.22	11.48	11.56
6	7	9.83	10.56	10.99	11.18	11.53	11.56
7	7	9.71	10.5	11.08	11.2	11.48	11.65
8	7	9.86	10.52	11.1	11.22	11.52	11.55
9	7	9.88	10.45	11	11.23	11.53	11.58
10	7	9.98	10.51	11.04	11.24	11.49	11.59
Mean	7	9.852	10.508	11.042	11.214	11.51	11.586
SD	0	0.100532	0.044422	0.035839	0.018974	0.021602	0.029889

Table 9 Statistical analysis of data from accelerated stability tests of PCFA. The data was analyzed by paired t-test to perform pair wise comparison between 2 dependent groups. The results showed statistically significant difference in the release of fluocinolone acetonide and pH measurement after three-month storage.

Released fluocinolone acetonide	Paired Differences					t	df	Sig. (2-tailed)
	Mean	S.D.	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
4	1.2271	.46556	.14722	.8940	1.5601	8.335	9	.000
8	.6518	.40645	.12853	.3611	.9426	5.071	9	.001
24	2.1950	.16008	.05062	2.0805	2.3096	43.363	9	.000
48	18.9857	3.37652	1.06775	16.5703	21.4011	17.781	9	.000
72	.2895	1.73539	.54878	-.9519	1.5310	.528	9	.611
120	.1393	1.02793	.32506	-.5961	.8746	.428	9	.678
168	.2512	.04924	.01557	.2160	.2864	16.132	9	.000
pH								
1	.7170	.18768	.05935	.5827	.8513	12.081	9	.000
3	.3960	.13260	.04193	.3011	.4909	9.444	9	.000
24	.3590	.06385	.02019	.3133	.4047	17.780	9	.000
48	.4110	.08075	.02554	.3532	.4688	16.095	9	.000
72	.1830	.08206	.02595	.1243	.2417	7.052	9	.000
168	.1340	.07849	.02482	.0779	.1901	5.399	9	.000

Table 10 Effects of conditioned medium from Dycal® and PCFA on cytotoxicity and cell proliferation of HDPCs at 24, 48, and 72 hours (raw data).

24 h	SFM	10%DMEM	F50	F100	D50	D100
1	2.00571429	5.37714286	4.20571429	3.934286	4.062857	3.72
2	1.80571429	2.49142857	2.42	2.42	2.391429	2.434286
3	3.14857143	5.17714286	3.56285714	3.462857	3.262857	3.534286
Mean	**2.32**	**4.34857**	**3.39619**	**3.2724**	**3.239**	**3.2295**
SD	0.72449842	1.6114387	0.90444857	0.774904	0.835969	0.694928
48 h	SFM	10%DMEM	F50	F100	D50	D100
1	2.20571429	6.16285714	4.27714286	4.134286	3.877143	2.605714
2	1.92	4.72	2.94857143	3.477143	2.52	3.334286
3	3.57714286	8.66285714	4.62	4.72	4.805714	4.362857
Mean	**2.56762**	**6.51524**	**3.94857**	**4.1105**	**3.7343**	**3.4343**
SD	0.88586788	1.99490849	0.8828294	0.621771	1.149534	0.882829
72 h	SFM	10%DMEM	F50	F100	D50	D100
1	2.29142857	5.10571429	5.12	3.791429	3.662857	2.777143
2	1.79142857	6.43428571	5.03428571	5.077143	2.02	3.591429
3	3.30571429	11.5485714	4.99142857	4.82	4.548571	3.405714
Mean	**2.46286**	**7.69619**	**5.04857**	**4.5629**	**3.4105**	**3.2581**
SD	0.77156084	3.40175025	0.06546537	0.680336	1.28304	0.426742

Table 11 The data from MTT assay was tested by Levene statistic to test of homogeneity of variances between groups for further statistical analysis. The variance of data was different when the significant level was more than 0.05.

Test of Homogeneity of Variances

TIME	Levene Statistic	df1	df2	Sig.
24 h	1.435	5	12	.281
48 h	1.217	5	12	.359
72 h	6.513	5	12	.004

Table 12 The data from MTT assay was analyzed by One-way ANOVA to compare means in 24- and 48-hour groups. The data was statistically significant difference at the confidence level of 95%.

TIME		Sum of Squares	df	Mean Square	F	Sig.
24 h	Between Groups	6.236	5	1.247	1.308	.324
	Within Groups	11.444	12	.954		
	Total	17.680	17			
48 h	Between Groups	26.303	5	5.261	3.930	.024
	Within Groups	16.062	12	1.339		
	Total	42.365	17			

Table 13 The multiple comparisons of MTT assay in 24- and 48-hour groups were analyzed by Bonferroni test. The data was statistically significant difference when the significant level was less than 0.05.

TIME		24 h			48 h		
TYPE	TYPE	Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.
SFM	FA50	-1.0762	.79735	1.000	-1.3810	.94465	1.000
	FA100	-.9524	.79735	1.000	-1.5429	.94465	1.000
	D50	-.9190	.79735	1.000	-1.1667	.94465	1.000
	D100	-.9095	.79735	1.000	-.8667	.94465	1.000
	10%	-2.0286	.79735	.386	-3.9476(*)	.94465	.019
FA50	SFM	1.0762	.79735	1.000	1.3810	.94465	1.000
	FA100	.1238	.79735	1.000	-.1619	.94465	1.000
	D50	.1571	.79735	1.000	.2143	.94465	1.000
	D100	.1667	.79735	1.000	.5143	.94465	1.000
	10%	-.9524	.79735	1.000	-2.5667	.94465	.281
FA100	SFM	.9524	.79735	1.000	1.5429	.94465	1.000
	FA50	-.1238	.79735	1.000	.1619	.94465	1.000
	D50	.0333	.79735	1.000	.3762	.94465	1.000
	D100	.0429	.79735	1.000	.6762	.94465	1.000
	10%	-1.0762	.79735	1.000	-2.4048	.94465	.385
D50	SFM	.9190	.79735	1.000	1.1667	.94465	1.000
	FA50	-.1571	.79735	1.000	-.2143	.94465	1.000
	FA100	-.0333	.79735	1.000	-.3762	.94465	1.000
	D100	.0095	.79735	1.000	.3000	.94465	1.000
	10%	-1.1095	.79735	1.000	-2.7810	.94465	.184
D100	SFM	.9095	.79735	1.000	.8667	.94465	1.000
	FA50	-.1667	.79735	1.000	-.5143	.94465	1.000
	FA100	-.0429	.79735	1.000	-.6762	.94465	1.000
	D50	-.0095	.79735	1.000	-.3000	.94465	1.000
	10%	-1.1190	.79735	1.000	-3.0810	.94465	.102
10%	SFM	2.0286	.79735	.386	3.9476(*)	.94465	.019
DMEM	FA50	.9524	.79735	1.000	2.5667	.94465	.281
	FA100	1.0762	.79735	1.000	2.4048	.94465	.385
	D50	1.1095	.79735	1.000	2.7810	.94465	.184
	D100	1.1190	.79735	1.000	3.0810	.94465	.102

Table 14 The data from MTT assay was analyzed by Kruskal-Wallis test to compare means in 72-hour group. The data was statistically significant difference at the confidence level of 95%.

TIME	TYPE	N	Mean Rank
72 h	SFM	3	3.00
	FA50	3	13.67
	FA100	3	11.33
	D50	3	6.67
	D100	3	5.67
	10%	3	16.67

TIME		MTT
72 h	Chi-Square	14.427
	df	5
	Asymp. Sig.	.013

Table 15 The multiple comparisons of MTT assay in 72-hour group were analyzed by Conover-Inman method. The data was statistically significant difference when the significant level was less than 0.05.

Groups	Rank difference	Sig.
10% DMEM and SFM	13.666667 > 4.397789	P < 0.0001
10% DMEM and F50	3 > 4.397789	P = 0.163
10% DMEM and F100	5.333333 > 4.397789	P = 0.0215
10% DMEM and D50	10 > 4.397789	P = 0.0003
10% DMEM and D100	11 > 4.397789	P = 0.0001
SFM and F50	10.666667 > 4.397789	P = 0.0002
SFM and F100	8.333333 > 4.397789	P = 0.0014
SFM and D50	3.666667 > 4.397789	P = 0.0943
SFM and D100	2.666667 > 4.397789	P = 0.2111
F50 and F100	2.333333 > 4.397789	P = 0.2702
F50 and D50	7 > 4.397789	P = 0.0046
F50 and D100	8 > 4.397789	P = 0.0019
F100 and D50	4.666667 > 4.397789	P = 0.0393
F100 and D100	5.666667 > 4.397789	P = 0.0158
D50 and D100	1 > 4.397789	P = 0.6292

Table 16 Effects of conditioned medium from PCFA and Dycal® on relative amount of DSPP expression and DSP synthesis of HDPCs (raw data).

DSPP	Control	Dex	F50	F100	D50	D100
	1.000	1.963	1.761	1.613	1.542	1.610
	1.000	3.770	2.390	1.554	1.738	1.634
	1.000	5.050	3.838	4.193	1.533	2.005
Mean	**1.000**	**3.594**	**2.663**	**2.453**	**1.604**	**1.750**
SD	0	1.551126	1.064841	1.506804	0.115948	0.221482

DSP	Control	Dex	F50	F100	D50	D100
1	1.000	2.280	1.861	1.112	1.047	1.024
2	1.000	1.525	1.735	3.024	2.433	2.015
3	1.000	2.637	1.531	1.277	1.072	1.774
Mean	**1.000**	**2.147**	**1.709**	**1.804**	**1.517**	**1.604**
SD	0	0.567747	0.166529	1.059479	0.793089	0.516827

Table 17 The data from DSPP expression and DSP synthesis were analyzed by Kruskal-Wallis test to compare mean rank among the different groups. The data was statistically significant difference at the confidence level of 95%.

	GROUP	N	Mean Rank
DSPP	Negative control	3	2.00
	Positive control (Dex)	3	15.00
	F50	3	13.67
	F100	3	10.33
	D50	3	6.33
	D100	3	9.67
	Total	18	
DSP	Negative control	3	2.00
	Positive control	3	13.67
	F50	3	11.33
	F100	3	11.00
	D50	3	9.00
	D100	3	10.00
	Total	18	

	DSPP	DSP
Chi-Square	12.114	8.427
df	5	5
Asymp. Sig.	.033	.134

a Kruskal Wallis Test

b Grouping Variable: TYPE

Table 18 The multiple comparisons of DSPP expression were analyzed by Conover-Inman method. The data was statistically significant difference when the significant level was less than 0.05.

Groups	Rank difference	Sig.
Control and DEX	13 > 6.090869	P = 0.0006
Control and F50	11.666667 > 6.090869	P = 0.0013
Control and F100	8.333333 > 6.090869	P = 0.0115
Control and D50	4.333333 > 6.090869	P = 0.1471
Control and D100	7.666667 > 6.090869	P = 0.0178
DEX and F50	1.333333 > 6.090869	P = 0.642
DEX and F100	4.666667 > 6.090869	P = 0.1209
DEX and D50	8.666667 > 6.090869	P = 0.0092
DEX and D100	5.333333 > 6.090869	P = 0.0806
F50 and F100	3.333333 > 6.090869	P = 0.2562
F50 and D50	7.333333 > 6.090869	P = 0.0223
F50 and D100	4 > 6.090869	P = 0.178
F100 and D50	4 > 6.090869	P = 0.178
F100 and D100	0.666667 > 6.090869	P = 0.8155
D50 and D100	3.333333 > 6.090869	P = 0.2562

Table 19 Effects of conditioned medium from PCFA and Dycal® on relative amount of COX-2 expression from HDPCs (raw data).

COX-2	SFM	LPS	F50	F100	D50	D100
1	1.000	1.427	0.677	0.711	1.188	0.871
2	1.000	1.496	0.625	0.255	1.318	0.877
3	1.000	1.258	0.397	0.133	0.556	0.634
Mean	**1.000**	**1.394**	**0.566**	**0.366**	**1.021**	**0.794**
SD	0	0.122451	0.148934	0.304659	0.407629	0.138597

Table 20 The data from COX-2 expression was analyzed by Kruskal-Wallis test to compare means among the different groups. The data was statistically significant difference at the confidence level of 95%.

TIME	TYPE	N	Mean Rank
72 h	SFM	3	12.00
	FA50	3	16.67
	FA100	3	5.00
	D50	3	3.67
	D100	3	11.33
	10%	3	8.33
	Total	18	

TIME		MTT
72 h	Chi-Square	12.326
	df	5
	Asymp. Sig.	.031

a Kruskal Wallis Test

b Grouping Variable: TYPE

Table 21 The multiple comparisons of COX-2 expression were analyzed by Conover-Inman method. The data was statistically significant difference when the significant level was less than 0.05.

Groups	Rank difference	Sig.
CONTROL and LPS	4.666667 > 5.959554	P = 0.1137
CONTROL and F50	7 > 5.959554	P = 0.025
CONTROL and F100	8.333333 > 5.959554	P = 0.0101
CONTROL and D50	0.666667 > 5.959554	P = 0.8116
CONTROL and D100	3.666667 > 5.959554	P = 0.2049
LPS and F50	11.666667 > 5.959554	P = 0.0011
LPS and F100	13 > 5.959554	P = 0.0005
LPS and D50	5.333333 > 5.959554	P = 0.0749
LPS and D100	8.333333 > 5.959554	P = 0.0101
F50 and F100	1.333333 > 5.959554	P = 0.6347
F50 and D50	6.333333 > 5.959554	P = 0.0391
F50 and D100	3.333333 > 5.959554	P = 0.2464
F100 and D50	7.666667 > 5.959554	P = 0.016
F100 and D100	4.666667 > 5.959554	P = 0.1137
D50 and D100	3 > 5.959554	P = 0.2943

Biography

Mr. Phumisak Louwakul was born in August 25, 1976 in Bangkok, THAILAND. He graduated from Faculty of Dentistry, Chiang Mai University in 2000. He worked at Sirindhorn College of Public Health, Trang from 2000 to 2001. He has been working at the Department of Restorative Dentistry and Periodontics, Faculty of Dentistry, Chiang Mai University, THAILAND from 2002 until now. By the grant of Co-operative Research Network (CRN), he started his postgraduate study for the Master Degree of Science in Endodontology, Faculty of Dentistry, Chulalongkorn University, THAILAND. He graduated the master degree in 2006, and applied for the doctoral degree in Dental Biomaterials Science in the same year. From his Master thesis, he published one article named “Effect of Fluocinolone Acetonide on Human Dental Pulp Cells: Cytotoxicity, Proliferation, and Extracellular Matrix Formation” in Journal of Endodontics, volume 37, 2011, 181-184.