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รายงานผลการวิจัย

ผลของฟลูโอซิโนโลน อะซีโทไนด์ต่อเซลล์เพาะเลี้ยงจากเนื้อเยื่อในโพรงฟันมนุษย์

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โครงการวิจัย **ผลของฟลูโอซิโนโลน อะซีโทไนด์ต่อเซลล์เพาะเลี้ยงจากเนื้อเยื่อในโพรงฟันมนุษย์**

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บทคัดย่อ

การรักษาการเผยผึ่งของเนื้อเยื่อในโพรงฟัน มีวัตถุประสงค์เพื่อที่จะคงสภาพความมีชีวิตและสุขภาพที่ดีของเนื้อเยื่อในโพรงฟันไว้ สารเคมีบางชนิด เช่น สารสเตียรอยด์ อาจสามารถช่วยการกระตุ้นการหายและการสร้างเนื้อเยื่อแข็งจากเนื้อเยื่อในโพรงฟันได้ การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของฟลูโอซิโนโลน อะซีโทไนด์ต่อเซลล์เพาะเลี้ยงจากเนื้อเยื่อในโพรงฟันมนุษย์ ในการศึกษาผลของความเป็นพิษต่อเซลล์และการเพิ่มจำนวนเซลล์เป็นการวัดด้วยสารเอ็มทีที ที่ระยะเวลา 24, 48 และ 72 ชั่วโมง พบว่าที่ระยะเวลา 24 ชั่วโมงทุกกลุ่มความเข้มข้นของฟลูโอซิโนโลน อะซีโทไนด์ ไม่มีความเป็นพิษต่อเซลล์ ผลการศึกษาการเพิ่มจำนวนเซลล์ที่ระยะเวลา 48 ชั่วโมง พบว่าในความเข้มข้น 0.1และ 1 ไมโครโมลของฟลูโอซิโนโลน อะซีโทไนด์ มีผลทำให้จำนวนเซลล์เพิ่มสูงขึ้นอย่างมีนัยสำคัญ ($p< 0.05$) และที่ระยะเวลา 72 ชั่วโมงทุกกลุ่มความเข้มข้นของฟลูโอซิโนโลน อะซีโทไนด์ มีผลทำให้จำนวนเซลล์เพิ่มสูงขึ้นอย่างมีนัยสำคัญ ($p<0.05$) ในการศึกษาปริมาณการสร้างไฟโบรเนกตินวัดด้วยวิธีเวสเทิร์น (Western blot analysis) ที่ระยะเวลา 48 ชั่วโมง พบว่าปริมาณการสร้างไฟโบรเนกตินที่เพิ่มขึ้นแปรผกผันกับความเข้มข้นของฟลูโอซิโนโลน อะซีโทไนด์ ที่เพิ่มขึ้น การศึกษาปริมาณการสร้างคอลลาเจนชนิดที่ 1 วัดด้วยวิธีเวสเทิร์น (Western blot analysis) ที่ระยะเวลา 5 วัน พบว่าฟลูโอซิโนโลน อะซีโทไนด์สามารถกระตุ้นให้เกิดการสร้างคอลลาเจนชนิดที่ 1 ได้อย่างมีนัยสำคัญทางสถิติ ประมาณ 2 เท่าโดยได้ทำการยืนยันผลการทดลองด้วยวิธี รีเวิร์ส ทรานสคริบชัน โพลิเมอเรสเชนรีเอคชัน (Reverse transcription polymerase

chain reaction; RT-PCR) โดยศึกษาผลของฟลูโอซิโนโลน อะซีโทไนด์ที่ความเข้มข้น 1 ไมโครโมล พบว่าสามารถกระตุ้นให้เกิดการสร้างเอ็มอาร์เอ็นเอ (mRNA) ของคอลลาเจนชนิดที่ 1 ได้เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ประมาณ 2.8 เท่า ส่วนผลการวัดค่าการทำงานของเอนไซม์ อัลคาไลน์ฟอสฟาเตส วัดที่ระยะเวลา 24 และ 72 ชั่วโมง พบว่าที่ระยะเวลา 24 ชั่วโมงทุกกลุ่มความเข้มข้นของ ฟลูโอซิโนโลน อะซีโทไนด์ ไม่มีผลต่อค่าการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตส อย่างไรก็ตามที่ระยะเวลา 72 ชั่วโมง พบว่าค่าการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเตสลดลงอย่างมีนัยสำคัญ ($p<0.05$) โดยแปรผันตรงกับความเข้มข้นของฟลูโอซิโนโลน อะซีโทไนด์ ที่เพิ่มสูงขึ้น ส่วนผลการสร้างตะกอนแคลเซียมนั้น เมื่อทำการเพาะเลี้ยงเซลล์ที่ระยะเวลานาน 28 วัน พบว่าไม่มีความแตกต่างกันระหว่างกลุ่มทดลองกับกลุ่มควบคุมห้องปฏิบัติการ โดยสรุปพบว่า ฟลูโอซิโนโลน อะซีโทไนด์ สามารถส่งเสริมการเพิ่มจำนวนเซลล์และปริมาณการสร้างไฟโบรเนกตินและสร้างคอลลาเจนชนิดที่ 1 แต่ไม่กระตุ้นให้เกิดการสร้างตะกอนแคลเซียม ผลการการวิจัยนี้ชี้แนะว่าฟลูโอซิโนโลน อะซีโทไนด์อาจจะสามารถนำมาพัฒนาเป็นวัสดุปิดโพรงฟันต่อไปในอนาคตได้

Project Title : The effects of fluocinolone acetonide on human cultured dental pulp cell *in vitro*

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ABSTRACT

The purpose of treatment of pulpal exposure is to preserve vitality, healthy and promote healing of exposed pulp tissue. Fluocinolone acetonide (FA) may have a potential to promote tissue healing. The aim of this study was therefore to investigate the effects of FA on human cultured dental pulp cell *in vitro*. The MTT assay was performed to examine both cytotoxicity and prolifration of FA at 24, 48 and 72 hours. The results revealed that FA (0.1 to 50 µM) had no cytotoxicity effect. In addition, these doses also stimulated cell proliferation. There was a significant increase of cell proliferation at low concentrations (0.1 and 1 µM) after 48 hours ($p<0.05$). However, all doses of FA showed statistically significant difference in cell proliferation at 72 hours ($p<0.05$). The effect of FA on fibronectin synthesis was examined by Western blot analysis from cell extracted for 48 hours. The results showed that FA stimulated the synthesis of fibronectin in reverse dose-dependent manner. The Western blot analysis was performed to examine the effect of FA on type I collagen synthesis at 5 days. The result showed that 1 and 10 µM of FA significantly stimulated the synthesis of collagen for about 2-fold ($p<0.05$). The result was confirmed by reverse transcription polymerase chain reaction (RT-PCR) which indicated that 1 µM FA could significantly induce the expression of type I collagen mRNAs for about 2.8 times ($p<0.05$). The activity of alkaline phosphatase was detected at 24 and 72 hours and showed

no statistically significant difference in any group at 24 hours ($p>0.05$). However, after 72 hours all doses of FA decreased alkaline phosphatase activity in a dose-dependent manner. Long term cultures were done to examine the *in vitro* calcification. After 28 days, the result showed no difference between FA-treated groups and the controls. These results demonstrated that FA enhances the effect on proliferation, fibronectin and type I collagen synthesis but not in calcification process. The results suggested that FA may be the potential substance as a pulp capping material.

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ABBREVIATIONS

FA	Fluocinolone acetonide
ALP	alkaline phosphatase
CDC	cell division cycle
DMEM	Dulbecco's Modified Eagle medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
HDC	human cultured dental pulp cells
PBS	phosphate buffered saline
RIPA	radioimmunoprecipitation

CHAPTER I

INTRODUCTION

The dental pulp may become exposed to the oral environment by many ways such as carious lesions that extend through the enamel or cementum and dentin, tooth fracture, or dental operative procedures (Mjör, 2002). When the pulp is mechanically exposed or superficially infected, it is beneficial to preserve the vitality and health of the exposed pulp rather than replacing it with a root canal filling material. The major reason is because the pulp is a well vascularized and innervated connective tissue that has self-healing capacity unless properly treated. Reparative dentin formation is an important defense mechanism that protects the pulp from noxious elements in the oral cavity (Kakehashi *et al*., 1965; Schröder, 1985; Yamamura, 1985; Murray *et al*., 2002; Goldberg *et al*., 2003). Until now, the major concern of endodontics has been the prevention or elimination of apical periodontitis. The conservative treatment of vital pulp is, therefore, the best way to ensure the prevention of periradicular pathology (Trope, 2003).

Vital pulp therapy may be broadly defined as any aspect of restorative dental treatment intended to minimize trauma to the dental pulp, including direct and indirect pulp capping, partial and complete pulpotomy (Rutherford and Fitzgerald, 1995). 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 (Ward, 2002). The opened exposure has to be sealed off by using an appropriate wound dressing to prevent bacterial contamination and promote pulpal healing. Following proper disinfection, debridement and the absence of microorganism, soft and hard-tissue healing have been shown to occur at a very high rate (Kakehashi *et al*., 1965; Baume and Holz, 1981; Mejàre and Cvek, 1993).

Calcium hydroxide has been introduced in capping the exposed pulps for many decades (Zander, 1939; Glass and Zander, 1949; Kozlov and Massler, 1960; Stanley and Lundy, 1972; Tronstad, 1974). Its high alkalinity provides the anti-bacterial property and encourages tissue repair. When it is applied to the exposed pulp, it cauterizes superficial tissue and causes the zone of coagulative necrosis. This firm necrotic layer irritates the underlying tissue and stimulates hard-tissue repair (Schröder and Granath, 1971). The formation of dentinal bridge has been believed to be the principal biological result for a successful treatment of an exposed or amputated pulp (Kozlov and Massler, 1960; Rowe, 1967; Stanley, 1989; Mjör *et al*., 1991).

Although pulp capping with calcium hydroxide has been accepted to be highly successful, many disadvantages have been reported. The morphology of the hard-tissue bridge is often irregular, with cellular inclusions and tunnel defects. Thus, it often becomes highly permeable to bacteria and bacterial elements, and increases the risk of pulpal infection from possible surface seal breakdown (Cox *et al*., 1996). The softening and disintegration phenomenon of calcium hydroxide has been demonstrated. Its unstable physical properties may allow material particles to migrate into pulp tissue and cause inflammatory response (McComb, 1983; Hwas and Sandrik, 1984). Most calcium hydroxide medicaments have been reported to disintegrate and wash out after 6 months, leaving a void underneath the restoration and thereby a pathway for bacterial infection (Cox *et al*., 1996). It is also disintegrated by phosphoric acid-etching agents (Phillips *et al*., 1984) and allows long-term softening of the adjacent composite resin (Cox *et al*., 1996).

In order to overcome the previously described problems of direct pulp capping with calcium hydroxide, there have been various attempts to use other materials such as adhesive materials (Heitmann and Unterbrink, 1995; Ölmez *et al*., 1998; Tarim *et al*., 1998; Kitasako *et al*., 2002; Scarano *et al*., 2003), hydroxyapatite (Jaber *et al*., 1991; Sübay and Asci, 1993),

tricalcium phosphate (Chohayeb *et al*., 1991) and mineral trioxide aggregate (Ford *et al*., 1996; Tziafas *et al*., 2002). The recent literatures have been interested in a number of biologic molecules as an alternative way to stimulate pulpal regeneration (Rutherford *et al*., 1993; Rutherford and Fitzgerald, 1995; Goldberg *et al*., 2003; Tziafas, 2004). Although the new effective capping materials have been introduced, calcium hydroxide has remained the gold standard as pulp capping material primarily due to solid clinical documentation (Haskell *et al*., 1978; Baume and Holz, 1981; Fitzgerald and Heys, 1991).

Further experiments are needed to discover more effective materials that may provide clinicians with additional options for treatment of exposed vital pulps. The agent used in vital pulp therapy should ideally be non-toxic, possess anti-microbial and anti-inflammatory activities in order to control pre-existing inflammatory states of the exposed pulp and operative-induced inflammation (Ward, 2002). The principal objective of capping procedure is to obtain pulp tissue healing. Therefore, an anti-inflammatory agent, such as corticosteroids, might be considered as a candidate for this purpose.

Topical corticosteroid is glucocorticoid hormone that uses topically in treatment of various dermatologic disorders such as itching, redness, dryness, crusting, scaling, inflammation, and discomfort of many skin conditions (Maibach and Stoughton, 1973; Sneddon, 1976; Pariser, 1991). They are also being used in various types of oral diseases: minor and major aphthous ulceration, herpes simplex, lichen planus, erythema multiforme, mucous membrane pemphigoid, epidermolysis bullosa, pemphigus vulgaris, bullous pemphigoid, dermatitis herpetiformis, lupus erythematosus (Kay, 1976).

Some advantages of topical corticosteroids have been reported such as anti-inflammatory action (Rapoport and Abramson, 1958; Ulmansky *et al*., 1971; Fachin and Zaki, 1991), pain reduction or pain relief (Fry *et al*., 1960; Schröeder and Triadan, 1962), do not inhibit dentin bridge formation (Schneider, 1968; Barker and Ehrmann, 1969; Barker *et*

al., 1972). However, possible adverse effects are also reported such as adrenal suppression, Cushing's syndrome, striae, allergic contact dermatitis (Maibach and Stoughton, 1973), inhibition of the cellular response to an irritant that may increase risk of infection and bacteremia (Sinkford and Harris, 1964; Klotz *et al*., 1965), suppression of fibroblastic mitosis (Taylor *et al*., 1989), and collagen biosynthesis (Uitto and Mustakallio, 1972).

Topical glucocorticoids have been interested by many researchers for pulpal treatment. Hydrocortisone acetate was the first glucocorticoid which successfully used in treatment of human vital pulp (Rapoport and Abramson, 1958). Triamcinolone acetonide is a major constituent in Ledermix®, which is the first corticosteroid-antibiotic agent used in vital pulp and endodontic therapy (Schröeder and Triadan, 1962; Barker and Ehrmann, 1969; Ulmansky *et al*., 1971; Barker *et al*., 1972; Paterson, 1981).

However, there was a case report that pulpal obliterations are observed in patients treated with long-term systemic corticosteroids. It was proposed that glucocorticoid therapy may induce excessive dentin formation (Symons and Symons, 1994). From a recent laboratory study; dexamethasone was able to stimulate osteogenic differentiation in human dental pulp cell cultures, strongly stimulate alkaline phosphatase activity, and induce the expression of dentin sialophosphoprotein (Alliot-Licht *et al*., 2005).

Fluocinolone acetonide has provided distinct advantages in topical therapy. It has been frequently used as topical medicament in treatment of various oral vesibuloerosive lesions and available at the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. There is an evidence supported that it has transient stimulatory effect on cell proliferation and multiplication in cultured human skin fibroblasts (Kirk and Mittwoch, 1977). However, the effect on pulpal healing or repair process is unknown. The research interest is focused on fluocinolone acetonide whether it could promote pulpal healing and could be developed as a new effective pulp capping agent. The benefits of successful pulp

capping procedures would reduce the need for complex treatments, endodontic and restorative procedures. The basic knowledge from this study would encourage further research programs to develop and, finally, industrialize to a domestic commercial product.

CHAPTER II

SURVEY OF RELATED LITERATURES

2.1 Healing and repair process: An overview

The inflammatory response is closely interwined with healing and repair process. Inflammation serves to destroy, dilute, or wall off the injurious agent and possible heal and reconstitute the damage tissue. Repair begins during the early phase of inflammation but reaches completion usually after the injurious influence has been neutralized. During repair process the injured tissue is replaced by regeneration of parenchymal cells, by filling of the defect with fibroblastic tissue (scarring) or most common by a combination of these two processes (Cotran, Kumar and Robbins, 1989). The inflammatory response occurs in the vascularized connective tissue, including plasma, circulating cells, blood vessels, and cellular and extracellular constituents of connective tissue. Inflammation is divided into acute and chronic patterns. Acute inflammation is of relatively short duration, lasting for minutes, several hours, or a few days, and its main characteristics are the exudation of fluid and palsma protein and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation is of longer duration and is associated histologically with the presence of mononuclear inflammatory cells such as lymphocytes, plasma cells and macrophages, the proliferation of blood vessels, fibrosis, and tissue necrosis (Cotran, Kumar and Robbins, 1989). Therefore, mononuclear inflammatory cells, fibroblasts, collagen, and small blood vessels are the primary components of chronic inflammatory tissue (Trowbridge, 1990).

The body's ability to replace injured or dead cells and to repair tissue after inflammation is critical for survival. Repair of tissue involves two distinct processes that regeneration, denoting the replacement of injured cells by cells of the same type, and

replacement by connective tissue, called fibroplasia or fibrosis, which leaves a permanent scar. In most instances, both processes contribute to repair. In addition, both regeneration and fibroplasia are determined by essentially similar mechanism involving cell migration, proliferation and differentiation as well as cell matrix interactions. In adult tissue, the size of population of cells is determined by the rates of proliferation, differentiation, and death by apoptosis (McCarthy, 1992). The relationships of increased cell numbers may result from either increased proliferation or decreased cell death.

2.2 Vital pulp therapy and pulpal healing process

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. Since then, the pulp capping procedure has been performed with various types of materials. 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 (Glass and Zander, 1949; Baume and Holz, 1981; Stanley, 1989). Zander was among the first to report on the use of calcium hydroxide in treatment of the exposed human dental pulps (Zander, 1939).

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 (Glass and Zander, 1949). It is described to be consisted of three layers: the compressed superficial layer, the liquefaction necrosis, and the zone of coagulation necrosis (Schröder and Granath, 1971). Migration of inflammatory cells into the wound area occurs after 6 hours which results in slight to moderate inflammation (Glass and Zander, 1949; Schröder and Granath, 1971; Schröder, 1985).

Within the first four days, blood clot which formed at the time of injury is completely resolved (Fitzgerald, 1979). 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 (Schröder and Granath, 1971; Schröder, 1985).

After 7 days, newly formed matrix with marginal fibroblasts is seen below the demarcation (Schröder and Granath, 1971). 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 (Schröder, 1985). The radiographic examination may show the evidence of mineralization (Schröder and Granath, 1971). Nine days after exposure, organization and differentiation of functioning odontoblasts is observed at the exposure site directly adjacent to the capping material. Well-organized odontoblast-like cells can be identified at the wound surface 10-11 days after capping procedure (Mjör *et al*., 1991).

About 2 weeks, collagen matrix intervening between the capping material and odontoblast-like cells is observed (Glass and Zander, 1949; Schröder and Granath, 1971; Mjör *et al*., 1991). On the periphery of this tissue, cells resembling odontoblasts appear to be lining up and seem to be the earliest sign of differentiation of odontoblast-like cells. The pulp tissue below is normal and free of inflammation (Glass and Zander, 1949). Four weeks later, the superficial necrotized tissue is fallen out. Against the fibrillar structure formed, a new thin dentin bridge is deposited with a well-organized layer of new odontoblastoid cells. The tissue below is normal and the pulp is healed (Glass and Zander, 1949; Schröder and Granath, 1971; Schröder, 1985). 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 (Glass and Zander, 1949; Schröder, 1985). After three months, the production of matrix appears to have stopped. Some tubules resembled to dentin are seen in the last-formed tissue (Schröder and Granath, 1971; Schröder, 1985).

The origin of odontoblast-like cells which are responsible to dentinal bridge formation has been studied. Autoradiographic study indicated 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 (Fitzgerald *et al*., 1990). Some evidence demonstrated that they located adjacent to blood vessels and endothelial cells, which pericytes were especially implicated (Sveen and Hawes, 1968; Fitzgerald, 1979). It is also possible that pulp cells, endothelial cells, and pericytes become de-differentiate and then re-differentiate into odontoblast-like cells (Yamamura, 1985). The pericytes and myofibroblast transitional cells were observed to migrate to the site of pulp exposure and formed reparative dentin. Isolation of these cells in laboratory culture was also found to produce human dentin secretion (Alliot-Licht *et al*., 2001; Murray *et al*., 2002).

The process of mineralization of dentinal bridge starts with dystrophic calcification of the coagulation zone, leading to deposition of mineral in the vital tissue containing the newly formed collagen. 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 in the matrix. Further calcification is

characterized by arrangement of crystals beside collagen fibers and calcospheritic nodules to form calcifying front (Hirschfeld *et al*., 1982; Schröder, 1985).

The structure of dentinal bridge has been studied in human teeth. After three months, the barrier was histologically found to consist of a coronal layer of irregular bone-like tissue with cellular inclusions. The pulpal part consisted of dentin-like tissues and was lined with odontoblast-like cells. From scanning electron microscopic evaluation, the dentin bridge was consisted of three layers which were a superficial amorphous debris-containing layer, an intermediate atubular layer (fibrodentinal core), and a tubular dentin-like structure, located adjacent to the pulp. In addition, densitometric measurement revealed that the superficial and middle layer exhibited the lowest and the highest mineral content, respectively. The last layer contained the mineral content which corresponded to the orthodentin of the pulpal walls (Franz *et al*., 1985).

In conclusion, pulpal healing process conclusively 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 dentine in direct continuum with reactionary dentine formed around the pulpal exposure area. The presence of calcified bridge after pulp capping is considered to be a sign of successful pulpal healing (Kozlov and Massler, 1960; Rowe, 1967; Stanley, 1989; Mjör *et al*., 1991; Mjör, 2002). Basically, the formation of dentinal bridge may be considered as a two-part phenomenon. The first stage is the formation of organic extracellular matrix by the progenitor cells that differentiate into matrix synthesizing odontoblast-like cells and deposit a collagenous extracellular matrix, mainly composed of type I collagen. Finally, the newly formed tissue is subsequently calcified to form a hard tissue barrier (Mjör *et al*., 1991; Linde and Goldberg, 1993).

2.2.1 Extracellular matrix and cell–matrix interactions

The extracelluar matrix is secreated locally and assembles into a network in the spaces surrounding cells. It forms a significant proportion of volume of any tissue and consists of the macromolecules outside cells. The extracelluar matrix subserves many functions (Hay, 1991). Extracelluar matrix also provides a substratum for cells to adhere, migration and proliferation and can direct influence the form and function of cells. Three groups of macromolecules are physically associated to form the extracelluar matrix. This first is fibrous structural proteins, such as the collagen and elastin. The second is a diverse group of adhesive glycoproteins, including fibronectin and laminin and the third is a gel of proteoglycans and hyaluronan. These macromolecules assemble into two general organizations, which are interstitial matrix and basement membrane (Yurchenco and O' Rear, 1994).

2.2.2 Fibronectin

Fibronectin is a multifunctional adhesive protein whose primary role is to attach cells to variety of matrices. It is a large glycoprotein consisting of two chains held together by disulfide bonds (Potts and Campbell, 1994). It has been suggested that fibronectin acts as a mediator of cell adhesion, both to other cells and to extracellular components. Linde *et al*. (1982) first showed that fibronectin was present in connective tissue of dental pulp. Fibronectin can be seen in a reticular pattern throughout the dental pulpal tissue, with increased concentration being present in the blood vessell walls (Linde *et al*., 1982). In the human pulp, a higher amount of fibronectin was found to be extracable from the apical part than from the coronal and middle parts (Van Amerongen *et al*., 1984). Fibronectin is only present in the organic matrix from which mental dentine forms. Fibronectin can not be demonstrated in a later stage of dentinogenesis in either predentine or dentine (Linde *et al*., 1982).

Fibronectin, a class of high molecular weight extracellular glycoprotein found in connective tissues and basement membranes, has an important role in the regulation during development and repair of dental pulp. It is implicated in the cytological modifications during odontoblast differentiation (Hynes, 1985). In addition, fibronectin has been involved in a variety of cell functions including acts as mediator of cell adhesin, both to the other cells and to extracellular components, migration, growth and differentiation (Ruostlahti, 1981).

2.2.3 Type I collagen and mineralization process

A collagen molecule consists of three alpha chains which coiled around each other into right-handed triple helix. Type I collagen is a heterotrimer 297 nm in length that consists of two α1(I) chains and one α2(I) chain. Each α chain consists of about 1000 amino acid residues, with glycine occurring in every third position of the sequence. Another characteristic is the presence of regularly spaced amino acids, e.g. proline and hydroxyproline. The specific amino acid compositions are prerequisite for the arrangement of the three α chains into a triple helical structure that is stabilized by hydrogen bonds (Linde, 1985; Gage *et al.*, 1989).

Collagen is synthesized in connective tissue by mechanisms similar to those of other proteins. The α chains are synthesized on ribosomes as procollagen chains having extension peptides of considerable size at both amino and carboxyl terminals. The procollagen chains must go through post-translational modifications by hydroxylation and glycosylation prior to helix formation. Prolyl and lysyl residues are hydroxylated by specific enzymes to hydroxylproline and hydroxylysine respectively. Ascorbic acid is an essential co-factor of the enzymes. The process is followed by the addition of galactose and galactose/glucose to some of hydroxylysine residues. A complex polysaccharide chain is also added within the carboxy-terminal domain of the procollagen. Disulphide bonds form both within one pro-α-chain and between three carboxy-terminal regions serving to catalyze triple helix formation.

The helical formation then proceeds and the intracellular post-translational modifications cease. Procollagen molecules are transported through the Golgi complex and exocytosed via the secretory granules (Linde, 1985; Gage *et al.*, 1989).

When procollagen is secreted into extracellular space, the extensions at both terminals of the procollagen molecule are excised by procollagen peptidases. The collagen molecules formed can self-aggregate into fibrils in a precise manner. The fibrils become stabilized by covalent intermolecular bonds (cross-links) primarily involving lysyl and hydroxylysyl residues. The elongated collagen molecules are arranged in a parallel fashion, with each molecule staggered, with respect to adjacent molecules, a distinct equal to ¼ of its length. This arrangement creates alternating spatial areas of the fibril with overlapping molecules (overlap zones) and with gaps (hole zones), thus accounting for the cross-striated pattern with a periodicity of about 68 nm as seen in the electron microscope (Shuttleworth *et al*., 1980; Linde, 1985; Gage *et al.*, 1989; Tjaderhane *et al*., 2001).

Type I collagen is not only a major collagenous protein in dentinal structure, but also plays a significant role in all phases of wound healing process and provides a fibrous matrix framework for mineralization process (Linde, 1985; Gage *et al.*, 1989). During mineralization, calcium phosphate inorganic crystals are deposited within and along with the long axes of the collagen fibrils (Hirschfeld *et al*., 1982; Schröder, 1985; Ohbayashi *et al*., 1999). Although collagen can be mineralized both *in vitro* and *in vivo*, the deposition of hydroxyapatite on the collagenous matrix requires the presence of non-collagenous matrix proteins such as phosphophoryn. The role of collagen fibrils thus seems to be a template that of giving attachment and stable support to other organic components and non-collagenous proteins, upon which mineral crystals can deposit (Gage *et al.*, 1989; Boskey, 1991).

One of the criteria used for evaluating osteoblast-like properties of cells *in vitro* is an osteogenic capacity which these cells can form mineralized nodules in laboratory condition

(Arceo *et al*., 1991). Mineral-like nodules can be formed both in periodontal cells (Arceo *et al*., 1991; Ramakrishnan *et al*., 1995; Nohutcu *et al*., 1997) and dental pulp cells (Tsukamoto *et al*., 1992). In human dental pulp cells, pulpal fibroblasts showed a typical, spindle-shaped, fibroblastic morphology, with prominent cytoplasmic processes. At 5-7 days after subcultured, the cells grew until confluent monolayer was formed. With continued culture, the cells formed nodules between 10-15 days (Tsukamoto *et al*., 1992). Generally, the calcified nodules were generally found in close proximity to collagen fibrils, suggestive of a relationship of collagen and mineralization process (Arceo *et al*., 1991; Tsukamoto *et al*., 1992; Ramakrishnan *et al*., 1995; Nohutcu *et al*., 1997). Therefore, type I collagen synthesis and calcification process are the important steps in laboratory condition which may represent the sign of pulpal wound healing *in vivo*.

2.2.4 Alkaline phosphatase activity

Robinson (1923) suggested that the enzyme alkaline phosphatase (ALPase) was important in the mineralization of bone and calcifying cartilage. This enzyme is thought to release phosphate ions from organic phosphate esters, leading to the precipitation of calcium phosphate salts. Alkaline phosphatase activity in pulp is as high as in bone and is thought to be essential for biomineralization (Goseki *et al*., 1990). Alkaline phosphatase activity in odontoblasts and subodontoblastic cells is higher than in undifferentiated mesenchymal cells (Yoshiki and Kurihashi, 1971).

Alkaline phosphatase activity is an index of early osteoblast differentiation, or a specific marker of odontoblasts as well as of dentine and its expression increases with differentiation development. The function of alkaline phosphatase activity is to hydrolyze organic phosphorus to form hydroxyapatite. It is the indispensable enzyme for bone formation, and its secretion indicates the occurrence of bone formation and the beginning of differentiation (Yang *et al*., 2003). Miller *et al*. (1976) suggested that alkaline phosphatase

activity is a prerequisite for cell differentiation because of its presence, during odontogenesis, in the cells from dental papilla differentiating into odontoblasts. This enzyme was functional in dentine mineralization (Goseki *et al*., 1990). Therefore, the experiment of fibronectin and alkaline phosphatase activity may use as indicators for determining the effect of any materials on the pulp tissue repair.

2.3 Pulp capping materials

Calcium hydroxide has been accepted as a gold standard of pulp capping materials. It is beneficial for induction of pulpal healing and calcified bridge formation. After capping procedure, calcium hydroxide dissociates into calcium and hydroxyl ions. The role of calcium ions is not well understood. It may be essential for cell proliferation, blood coagulation, mineralization, or other functions. The calcium ions released from the material may initiate the mineralization of collagen and induce hard tissue formation. The hydroxyl ions are responsible for maintaining a local state of high alkalinity that causes chemical trauma to soft connective tissue. A layer of coagulative necrosis or mummified zone is caused by the initial arrest of cellular activity due to early suppression of pulpal enzymes. The alkaline environment is also necessary for cell division and matrix formation (Franz *et al*., 1985; Schröder, 1985; Tziafas and Molyvdas, 1988; Mjör, 2002).

However, tissue irritation or necrosis may be more important than calcium hydroxide in initiating dentin bridge formation or calcification. In the absence of bacterial contamination, the successful bridge formation in germ-free rats occurred without any medicament covering the exposure sites (Kakehashi *et al*., 1965). Likewise, high alkalinity may not be an important factor in the bridge formation. The calcium hydroxide cements with lower pH have been proved to be effective in pulp capping procedures. In response to the materials, no or a thin layer of pulpal degeneration was found and vital pulp was seen in contact with the material. The soft connective tissue of the pulps showed only with minimal

inflammatory reactions followed by slow calcific bridge formation (Stanley and Lundy, 1972; Tronstad, 1974; Fitzgerald, 1979; Heys *et al*., 1981; Mjör *et al*., 1991; Kitasako *et al*., 2000).

However, the formation of incomplete dentinal bridges 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 in case of microleakage (Cox *et al*., 1996). Several researchers have reported failure associated with an incomplete dentinal bridge due to the presence of chronic inflammatory cell infiltration or necrotic pulp (Ulmansky *et al*., 1972; Cox *et al*., 1985; Heide and Kerekes, 1987; Schuurs *et al*., 2000; Mjör, 2002).

The softening and disintegration phenomenon of calcium hydroxide has also been demonstrated. Presence of cement particles in the connective tissue of the pulp indicates that the material or its filler components is in a progressive state of disintegration and may cause pulpal inflammation (McComb, 1983; Hwas and Sandrik, 1984). Most of calcium hydroxide medicaments disintegrate and wash out after six months, leaving a void under the restoration and leading to bacterial infection (Cox *et al*., 1996). Calcium hydroxide is also disintegrated by phosphoric acid-etching agents (Phillips *et al*., 1984) and allows long-term softening of the adjacent composite resin. Moreover, the inflammation or necrosis associated with the presence of dentinal bridge has been reported (Heys *et al*., 1981; Cox *et al*., 1985).

The recent experiments have been interested in a number of biologic molecules as an alternative way to stimulate pulpal regeneration. A number of biological dentinogenic molecules have been proposed as an alternative way to stimulate pulpal healing. Transforming growth factor-beta, multifunctional growth factor, implicated to function as an inducer of dentine matrix formation during dentinogenesis (Ruch, 1985). Bone morphogenic

proteins (BMPs), subgroup of transforming growth factor-beta family in tooth formation and dentinogenesis, were also shown to induce reparative dentine formation (Rutherford *et al.*, 1993; Rutherford and Fitzgerald, 1995). However, the processes of pulpal healing and repair from biologic molecules are not clarified.

More recently a mineral trioxide aggregate (MTA) was recommended for direct pulp capping materials (Pitt Ford *et al*., 1996). Mineral trioxide aggregate has been shown as an effective pulp-capping material and able to stimulate reparative dentine formation. It produced a similar tissue response to calcium hydroxide (Pitt Ford *et al*., 1996; Tziafas *et al.*, 2002) with less risk of dissolution over time. So far, clinical documentation on the efficacy of this material has to be studied.

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% (Haskell *et al*., 1978; Baume and Holz, 1981). However, further experiments are needed to discover more effective materials with additional options for the treatment of an exposed vital pulp with higher success rate. The medicament used in vital pulp therapy should ideally be non-toxic, possess anti-microbial and anti-inflammatory activities in order to control pre-existing inflammatory state of the exposed pulp and operative-induced inflammation (Ward, 2002). Considerably, the material should be able to stimulate pulpal healing process. An anti-inflammatory agent, especially topical corticosteroid, might be considered as a candidate for this purpose.

2.4 Topical corticosteroids

Topical corticosteroids are anti-inflammatory agents which have been successfully used in treatment of various dermatological diseases (Maibach and Stoughton, 1973; Sneddon, 1976). Some advantages of topical corticosteroids have been reported i.e. anti-inflammatory action (Rapoport and Abramson, 1958; Ulmansky *et al*., 1971; Fachin and Zaki, 1991), pain reduction or pain relief (Fry *et al*., 1960; Schröeder and Triadan, 1962), and do not inhibit dentin bridge formation (Schneider, 1968; Barker and Ehrmann, 1969; Barker *et al*., 1972). Although their disadvantages have been considered to be anti-proliferative and immunosuppressive effects which are believed to retard tissue healing, the stimulatory effects have been reported by some of the literatures. An *in vitro* study in mouse fibroblasts indicated that 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 was related to their relative glucocorticoid potency (Thrash *et al*., 1974).

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 (Ulmansky *et al*., 1971; Hume and Kenney, 1981). Severe inflammatory changes in the pulp are the same as that elsewhere in the body, but are different only by anatomic confines of rigid dentinal walls. Thus, inflammatory processes cause venous collapse, increase pressure on nerve ending and elicit pain. The corticosteroid pulp capping agent could control the inflammation 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 (Tziafas *et al*., 1995).

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 (Rapoport and Abramson, 1958). 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. Schroeder and Triadan were first proposed, and used successfully, a combination of triamcinolone, chloramphenicol, 4% xylocaine solution and ointment base in treatment of pulpitis (Schröeder and Triadan, 1962). In the same year, Schroeder developed the first proprietary products named "Ledermix®", which is a combined preparation of 1% triamcinolone and 3.21% demethylchlortetracycline.

Ledermix® has been used in vital pulp therapy for several years. When applied to normal pulp tissue, it did not impair pulpal vitality and evoked a moderate calcific response after prolonged period of contact. When applied to minimally inflamed pulps, resolution occurred in a proportion of cases but the outcome of treatment was unpredictable. Relief of symptom and continued positive response to vitality test were found in chronically inflamed degenerate pulps (Barker and Ehrmann, 1969). It was found to be successful in pulpotomy of normal human teeth (Ulmansky *et al*., 1971), but failed to induce healing in inflamed pulps. However, there were two of case reports which showed successful treatment of carious exposure with Ledermix® (Barker *et al*., 1972). In rat model, Ledermix® used in cariously exposed teeth resulted in pulpal necrosis limited to the coronal pulp, and bridge formation occurred in most of the cases (Paterson, 1981). However, failure to induce reparative dentin formation was reported when Ledermix® cement was also used as pulp capping agent in rat (Kirk and Meyer, 1992).

There have been some evidences regarding the effect of glucocorticoids on collagen synthesis. Cortisol and related glucocorticoids had two different effects on bone collagen synthesis *in vitro*. Collagen synthesis was stimulated in short term cultures and inhibited in long term cultures. The low physiologic concentrations of glucocorticoids might be essential for maintenance of the differentiated function of osteoblasts, the cells responsible for collagen synthesis (Dietrich *et al*., 1979). After short term treatment, low concentrations of cortisol, corticosterone and dexamethasone increased the incorporation of [^{3}H]proline into type I collagen in cultured rat calvariae. In vascular smooth muscle cells, 10^{-7} M dexamethasone showed an increase in the synthesis and secretion of collagen (Leitman *et al*., 1984). Therefore, specific concentrations of some corticosteroids might have stimulatory effect on collagen synthesis.

In contrast, the inhibitory effect of corticosteroids on collagen synthesis has been reported. In rabbit dental pulps, collagen synthesis was inhibited by some corticosteroids except prednisolone. Ledermix® also inhibited collagen synthesis in human teeth. The different glucocorticoids might exert different effects on collagen synthesis and this effect was dose-dependent (Uitto *et al*., 1975). In another study, high concentration of prednisolone was injected daily in rabbits. Collagen synthesis in the dental pulps was inhibited selectively by prednisolone treatment. It might disturb normal development and metabolism of teeth. The corticosteroid-induced inhibition of collagen biosynthesis seemed to be dose-dependent (Uitto and Manthorpe, 1983). In several laboratory experiments, dexamethasone strongly inhibited collagen synthesis, but enhanced alkaline phosphatase activity which is a marker for hard tissue biomineralization (Kasperk *et al*., 1995; Takada *et al*., 1996; Advani *et al*., 1997; Pei *et al*., 2003).

Interestingly, there was a case report that pulpal obliterations are observed in patients treated with long-term systemic corticosteroids. It was proposed that glucocorticoid therapy

may induce excessive dentin formation (Symons and Symons, 1994). The recent evidence reported that dexamethasone may be able to stimulate osteogenic differentiation in human dental pulp cultures. Although dexamethasone inhibited cell proliferation and markedly reduced the proportion of SMA-positive cells, but it strongly stimulated alkaline phosphatase (ALP) activity and induced the expression of the transcript encoding the major odontoblastic marker, dentine sialophosphoprotein (Alliot-Licht *et al*., 2005). Physiologic concentrations of dexamethasone and hydrocortisone induced the differentiation of osteoblastic cells and formation of bone nodules in rat calvaria cell culture (Bellows *et al*., 1987). The evidences showed that some topical corticosteroids may be able to induce mineralization process and hard tissue formation.

2.5 Corticosteriods and pulpal healing

Little data are available regarding to the specific effects of glucocorticoids on dental pulp cells. Dexamethasone is currently used to induce mineraization *in vitro* (Kasugai *et al*., 1993). Dexamethasone is a synthetic glucocorticoid that can induce odontoblastic differentiation and affects putative competent progenitor cells derived from dental pulp tissue. Indeed, dexamethasone has been widely used in culture to promote mineralization in bone marrow stromal cells (Bellows *et al*., 1990), pericytes (Brighton *et al*., 1992), and dental pulp cells (Kasugai *et al*., 1993). Dexamethasone has been used and found many advantages in pulpal regeneration. The acetonide derivatives of fluorinated steroids, triamcinolone and fluocinolone, have provided distinct advantages in topical therapy than Dexamethasone (David and Ann, 1967). An *in vitro* study in mouse fibroblasts indicated that steriods which posses 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 steriods was related to their glucocorticoid potency (Thrash *et al*., 1974). In the experiments of human osteoblastic cells or osteoprogenitors,

dexamethasone was found stimulate alkaline phosphatase activity, which is a marker for hard tissue biomineralization (Kasperk *et al*., 1995; Pei *et al*., 2003).

2.6 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 (Samman and Beer, 1962). It is a synthetic corticosteroid that is commonly used as topical application in treatment of various dermatologic disorders and also oral vesibuloerosive lesions (Hooley and Hohl, 1974; Lozada and Silverman, 1980; MacPhail *et al*., 1992; Buajeeb *et al*., 2000). The efficacy was superior to triamcinolone acetonide in treatment of oral lichen planus (Thongprasom *et al*., 1992). Topical 0.1% fluocinolone acetonide gel was safe, effective and easier to apply when compared with oral base form (Buajeeb *et al*., 2000).

When topically applied on the skin, 0.2% fluocinolone acetonide inhibited mitotic activity of the epidermis but 0.025% fluocinolone acetonide did not. Slightly increased mitotic activity was surprisingly found when low concentration of fluocinolone acetonide was used (Fisher and Maibach, 1971). In cultured human skin fibroblasts, a wide range of concentrations of fluocinolone acetonide had no inhibitory effect but additional produced a slightly increase in growth rate. It had a transient stimulatory effect on fibroblasts by promoting an earlier entry into period of DNA synthesis (S phase), which was also accompanied by a substantial increase in the length of S phase (Kirk and Mittwoch, 1977).

Fluocinolone acetonide and other glucocorticoids were tested in human skin collagen synthesis. The result showed that formation of radioactive hydroxyproline was inhibited by all corticosteroids tested, and the effect was dose-dependent. However, the lowest concentration of fluocinolone acetonide in this study (10 µg/ml) had no significant effect on

hydroxyproline formation. The authors concluded that higher, non-physiologic concentrations of fluocinolone acetonide inhibit the rate of collagen formation (Uitto *et al*., 1972).

As fluocinolone acetonide is a potent topical corticosteroid and available at the faculty of Dentistry, Chulalongkorn University, Thailand; it is interesting if fluocinolone acetonide would have potential to stimulate pulpal healing. An ideal pulp capping agent would have a capability to stimulate both extracellular matrix formation (i.e. type I collagen) and mineralization process. However, no evidence is available regarding the effects of fluocinolone acetonide on dental pulp cells. If fluocinolone acetonide in a suitable concentration is able to promote pulpal wound healing, the efficacy of this agent in vital pulp therapy should be further investigated. This study contributes to the knowledge of the inductive effects of fluocinolone acetonide on pulp cell proliferation, fibronectin and type I collagen synthesis, on alkaline phosphatase activity and *in vitro* calcification in cultured human dental pulp cells.

CHAPTER III

MATERIALS AND METHODS

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 the patients' informed consent. The teeth were extensively washed with sterile phosphate-buffered saline solution (PBS) and cracked open along the longitudinal axis. The pulps were 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 calf serum, 2mM 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 from Gibco BRL (Carlsbad, CA, USA). After the outgrowth of human cultured dental pulp cells reached confluence, they were subcultured into new culture dishes. The third and the fourth passages of three different donors were used in this study.

Fluocinolone aceonide preparation

Fluocinolone acetonide (FA) was purchased from FARMABIOS S.R.I. (Stabilimento e Direzione, Gropello Cairoli, Italy). To prepare stock solution, 4.52 mg fluocinone acetonide was dissolved in dimethyl sulfoxide (DMSO) solution to obtain 10 mM concentration. When the experiment was done, the stock solution was then serial diluted into 50 to 0.1 (50, 10, 1, 0.1) µM concentrations by mixing with culture medium. The

sterilization technique in all procedures was strictly controlled and new prepared solution was used in each experiment.

Colorimetric (MTT) assay for cytotoxicity and cell proliferation

Cultured human dental pulp cells were seeded at 20,000 cells/mL in 24 well plates (Nunc, Naperville, IL, USA) for 24 hours. After the overnight incubation, the medium was replaced by serum free medium at 3 hours intervals twice in order to wash out the serum. Cells were then treated with the dilutions of various concentrations (0.1, 1, 10, 50 μM) of fluocinolone acetonide for 24, 48 and 72 hours. Cells cultured in serum-free medium were used as control. At the end of the experiment, the media without phenol red containing 4.5 mg of MTT (Sigma Chemical Co., St. Louis, MO, USA) was added. After 30 minute incubation, 900 μL of dimethyl sulfoxide and 125 μL of glycine buffer 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 cell number, by comparing with control. The experiments were performed in triplicate using cells prepared from three different donors.

Fibronectin synthesis

Human cultured dental pulp cells were seeded at 50,000 cells/mL in 24 well plates (Nunc, Naperville, IL, USA) and treated with fluocinolone acetonide as described previously. After 48 hours, cells were extracted with RIPA Buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate). The amount of proteins from each extract was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of protein from each sample was mixed with reducing agent and subjected to electrophoresis on 12% polyacrylamide gel 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, Carlsbad, CA, USA) at 15 V for 90 minutes. The nitrocellulose membrane was incubated overnight in blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water) at room temperature.

Then, the nitrocellulose membrane was stained for 1 hour with primary antibody for fibronectin (Novocastra laboratories Ltd, Newcastle, United Kingdom) or β-actin (Chemicon international, Temecula, CA, USA) which was diluted to 1:1000 with blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water). Following 1 hour incubation, the nitrocellulose membrane was washed with PBS and incubated with biotinylated- secondary antibody (Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 30 minutes, respectively. The protein bands were detected using a commercial chemiluminescence system (Pierce, Rockford, IL, USA) and were exposed on CL-X Posture film (Pierce, Rockford, IL, USA). The band intensity was determined by scion image analysis software and the optical density was adjusted to percentage of expression, by comparing with the control condition. The experiments were performed from three different donors.

Type I collagen synthesis

The cells were cultured to 6-well plates at a density of 3×10^5 cells/well/mL in 2 mL of DMEM. After the overnight incubation, the cells were then treated with the selected concentrations of fluocinolone acetonide (0.1, 1, 10 µM), resulted from MTT experiment and fibronectin synthesis. The medium was changed every 48 hours and the cells are incubated for at least 5 days. Culture medium supplemented with ascorbic acid and DMSO solution was used as control. At the end of the experiment, the cells were washed three times with PBS. The 500 µL of 1 M acetic acid was then added to each well. The cells were

scraped and transferred to 1.5 mL tubes. The tubes were rotated at 4^{o}C overnight and centrifuged. The supernatant was collected and subjected to lyophilization.

The amount of total 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 and subjected to electrophoresis on 7.5% polyacrylamide gel 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, Carlsbad, CA, USA) at 15 V for 1 hour 45 minutes. The nitrocellulose membrane was incubated overnight in blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water) at 4^{o}C.

The nitrocellulose membrane was stained for 1 hour with primary antibody for type I collagen (L-19) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) which was diluted to 1:200 with blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water). The membrane was then washed with PBS and incubated with biotinylated-secondary antibody (Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 30 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 molecular weight of protein was obtained by determining the distance of bands driven onto the nitrocellulose membrane. The data was then compared with the protein marker to indicate the type of protein. The band intensity was determined by scion image analysis software 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.

Reverse-transcription polymerase chain reaction (RT-PCR)

The cells were cultured to 6-well plates at a density of 3 x 10^5 cells/well/mL in 2 mL of DMEM. After the overnight incubation, the cells were then treated with the selected concentrations of fluocinolone acetonide (1 μM) and culture medium. Culture medium mixed with DMSO solution was used as control. After 24 hours, total cellular RNA was extracted with Trizol (Gibco BRL) according to manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by reverse transcription using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) for 60 minutes at 42°C. Subsequent to the reverse transcription, polymerase-chain reaction (PCR) was performed for detection of type I collagen cDNA. The primers specific to type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared by GENSET (Singapore) using reported sequences from GenBank (type I collagen; GI: 34193787, GAPDH; GI: 83641890). The oligonucleotide sequences of type I collagen and GAPDH primers were:

Type I collagen	sense	5' CTGGCAAAGAAGGCGGCAAA 3'
	antisense	5' CTCACCACGATCACCACTCT 3'
GAPDH	sense	5' TGAAGGTCGGAGTCAACGGAT 3'
	antisense	5' TCACACCCATGACGAACATGG 3'

The PCR was performed using Tag polymerase (Qiagen, Hilden, Germany) with PCR volume 25 μL. The reaction mixtures contained 25 pM of primers and 1 μL of RT reaction. The PCR working condition was set at denaturation for 1 min at 94°C, primer annealing for 1 min at 60°C, and chain elongation for 1.45 min at 72°C on DNA thermal cycler (Omn-EThermal cycler, Hybaid, Middlesex, UK). The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide fluorostaining. The band intensity was determined by scion image analysis software and the optical density

was adjusted to percentage of expression, by comparing with the control condition. The intensity of bands indicated the relative amounts of the mRNA in the samples.

Alkaline phosphatase (ALPase) activity

Human cultured dental pulp cells were seeded at 20,000 cells/mL in 48 well plates (Nunc, Naperville, IL, USA). After overnight incubation, the medium was replaced by serum free medium at 3 hours intervals twice in order to wash out the serum. Cells were treated with the dilutions of various concentrations (0.1, 1, 10, 50 µM) of fluocinolone acetonide for 24 and 72 hours. The 50 µM and 72 hours experimemtal conditions were conducted in this experiment because these conditions also affected on stimulation of pulp cell proliferation. The control group was the cells cultured in 2% fetal bovine serum albumin. Cells was rinsed twice with PBS, scraped in 0.2 mL of alkaline lysis buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% triton-X100, pH 10) and kept in -20^{o}C condition until used. Half of each sample was incubated in the buffer containing 2mg/mL *p*-nitrophenyl phosphate in 0.1M 2-amino-2-methyl-1-propanal, 2 mM $MgCl_2$, pH 10.5 for the 30 minutes at 37^{o}C. The enzyme reaction was stopped by addition of 0.8 mL 50 mM NaOH to each well and the absorbance was measured at 410 nm wavelength. The other half of each sample was used for protein quantitation using BCA assay kit (Pierce, Rockford, IL, USA) with 2% fetal bovine serum albumin as standard. ALPase activities were calculated as nanomolar of *p*-nitrophenol /µg protien / minute and then adjust into percentage of expression, by comparing with the control condition. The experiments were performed in triplicate using cells prepared from three different donors. Three tripicate experiments were done to comfirm the reproducible results.

***In vitro* calcification**

The cells were cultured to 12-well plates at a density of 3 x 10^4 cells/well/ml in 1 ml of DMEM. At a 90% confluence, the cells were treated with the selected concentrations of fluocinolone acetonide (10, 1, 0.1 µM) and medium supplemented with 50 µg/ml ascorbic

acid and 10 mM β-glycerophosphate. The media for all groups was changed every 2 days for up to 28 days. Culture medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and DMSO solution was used as control. At the end of the experiment, the cells were washed with PBS and fixed with ice-cold methanol for 10 minutes. The cells were washed twice with deionized water and then stained with Alizarin red dye solution (0.1% NH_4OH, pH 6.5) for 5 minutes. The excess dye was washed several times with deionized water. Calcified nodules with red color were then observed on culture plates. Morphology of the cells and calcified nodules at various stages were recorded and photographed with a phase contrast microscope (Olympus CK2, Olympus America Inc., Mel ville, NY, USA).

Statistical analysis

All data from cytotoxicity, cell proliferation and alkaline phosphatase (ALPase) activity were analyzed using a one-way analysis of varience (ANOVA). Dunnett's T3 test was used for post-hoc analysis ($p < 0.05$). Fibronectin synthesis was examined by using descriptive statistical analysis.

The relative amounts of the collagen determined by scion image analysis software were evaluated by using SPSS version 11.5. At the 95% confidence interval, the data from Western blot analysis and RT-PCR was statistically analyzed by using One-way ANOVA followed by post hoc multiple comparison (Scheffe) test, and t test respectively.

CHAPTER IV

RESULTS

Cytotoxicity of fluocinolone acetonide and cell proliferation

The MTT assay was performed to examine both cytotoxicity and cell proliferation of fluocinolone acetonide (FA) on cultured human dental pulp cells. The data were presented as mean ± standard deviation (S.D.) of number of viable cells. The data were compared to each concentration groups to its corresponding control group and presented in Table 1. Three triplicate experiments were done to confirm the reproducibility of the data.

Concentration of fluocinolone acetonide (μM)	Number of viable cells (Mean ± S.D.) $x10^4$		
	24 hours	48 hours	72 hours
0.1	1.880 ± 0.023	3.430 ± 0.380	5.390 ± 0.130
1	1.870 ± 0.019	2.970 ± 0.160	4.660 ± 0.190
10	1.860 ± 0.028	2.640 ± 0.220	4.320 ± 0.120
50	1.850 ± 0.027	2.570 ± 0.100	4.100 ± 0.140
Control (Serum free medium)	1.850 ± 0.022	2.520 ± 0.026	3.740 ± 0.064

Table 1: Effects of fluocinolone acetonide (FA) on cytotoxicity and cell proliferation of cultured human dental pulp cells were examined by MTT assay at 24, 48 and 72 hours. Data were shown as mean ± S.D. of number of viable cells from three triplicate experiments.

As shown in figure 1, exposure of cultured human dental pulp cells to fluocinolone acetonide for the first 24 hours, did not affect both cytotoxicity and cell proliferation.

However, exposure of cultured human dental pulp cells to fluocinolone acetonide for 48 and 72 hours revealed that 0.1 to 50 µM of fluocinolone acetonide induced cell proliferation in reverse dose–dependent manner as shown in table 1 and figure 2. At 48 hours period, there was a statistically significant ($p < 0.05$) increase of cultured human dental pulp cells proliferation only when the cells were exposed to 0.1 and 1 µM of fluocinolone acetonide. In contrast, exposure of cultured human dental pulp cells to fluocinolone acetonide for 72 hours showed significant increase in cell numbers ($p < 0.05$) in all doses of fluocinolone acetonide (figure 2).

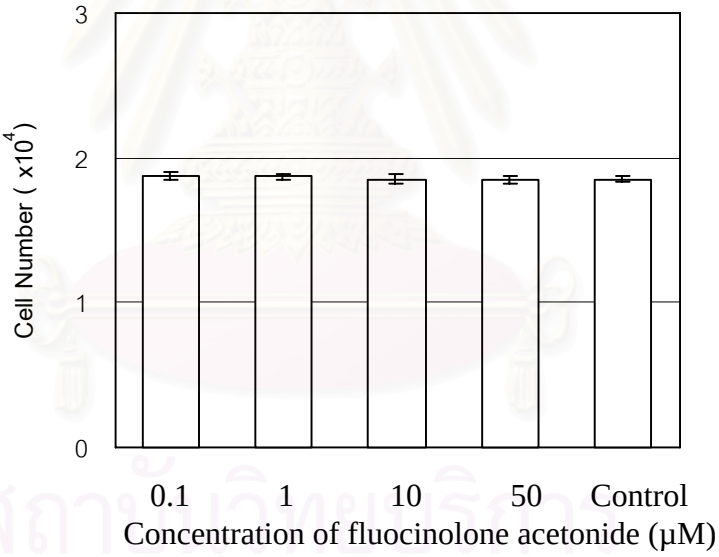


Figure 1: The cytotoxicity of fluocinolone acetonide on human cultured dental pulp cells was examined by MTT assay. Cells were treated with in a serum free (SF) medium with 0.1 to 50 µM of fluocinolone acetonide for 24 hours. The cytotoxicity of human cultured dental pulp cells was shown as cell number compared to control group. Data were shown as mean ± S.D. from three triplicate experiments. No significant difference was observed among group.

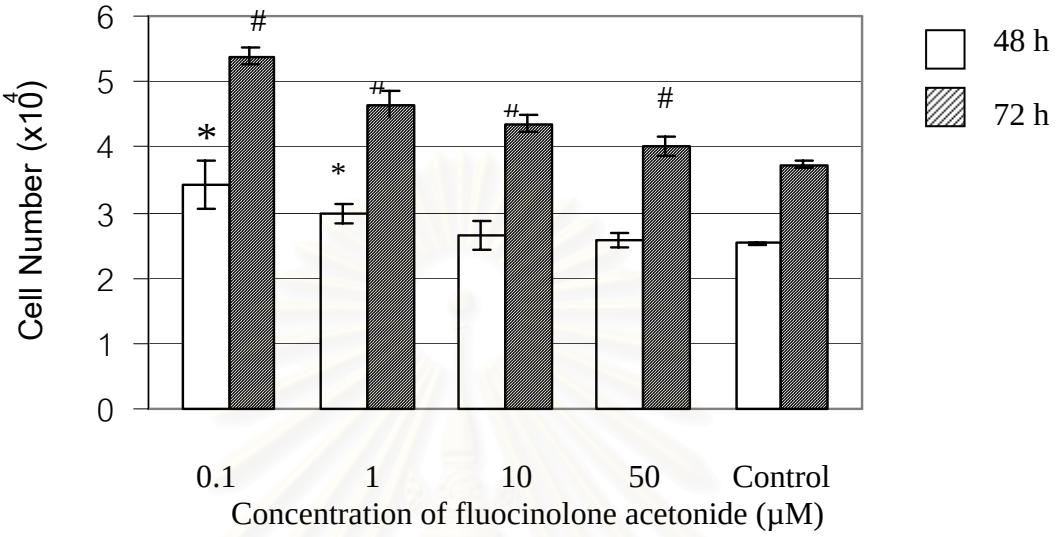


Figure 2: The proliferation of human cultured dental pulp cells after exposure to serum free medium with 0.1 to 50 µM fluocinolone acetonide for 48 and 72 hours. Data were shown as cell number comparing with control group as mean ± S.D. from three tripicate experiments.

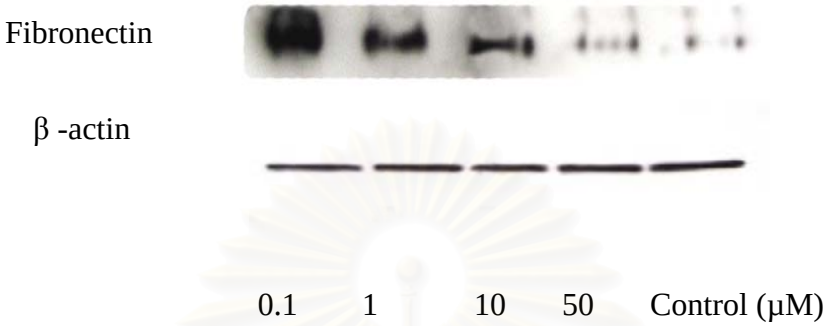
(*) Significant difference ($p<0.05$) when compared with control group at 48 hours.

(#) Significant difference ($p<0.05$) when compare with the control group at 72 hours.

Fibronectin synthesis

The effect of fluocinolone acetonide on the level of fibronectin synthesis was examined by Western blot analysis. The cell extracts were collected in RIPA buffer. The result demonstrated that fibronectin synthesis was increased in reverse dose–dependent manner as well as on cell proliferation (figure 3A). The lowest concentration at 0.1μM of fluocinolone acetonide increased the maximum amount of fibronectin as compared to the high concentration of fluocinolone acetonide as shown in figure 3B.

3A



3B

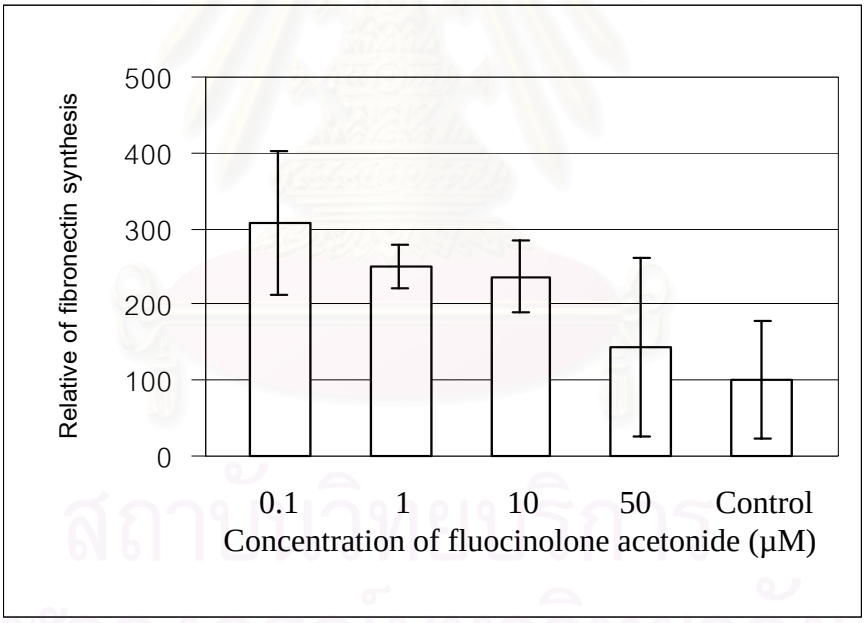


Figure 3: Fibronectin synthesis of cultured human dental pulp cells at 48 hours, cultured with different concentrations of fluocinolone acetonide.

(3A): Fibronectin synthesis in the cell extracted was examined by Western blot analysis.

(3B): Graph showed the quantitative of fibronectin synthesis.

Type I collagen synthesis

The ability of human dental pulp cells to synthesize and secrete type I collagen was examined by Western blot analysis. The synthesis of type I collagen in human dental pulp cells was influenced by fluocinolone acetonide (Fig. 4). The mean amount of type I collagen secreted by human dental pulp cells after being treated with selected concentrations of fluocinolone acetonide for 5 days was shown in figure 5. The results clearly demonstrated that 1 and 10 µM of fluocinolone acetonide could significantly increase the synthesis of type I collagen approximately 2-fold when compared with the control which was considered as 100% ($p < 0.05$)**.**

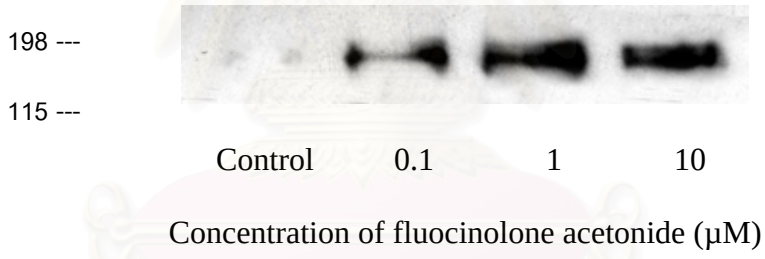


Figure 4: Type I collagen synthesis from human dental pulp cells cultured with 0.1, 1 and 10 µM fluocinolone acetonide at 5 days, examined by Western blot analysis

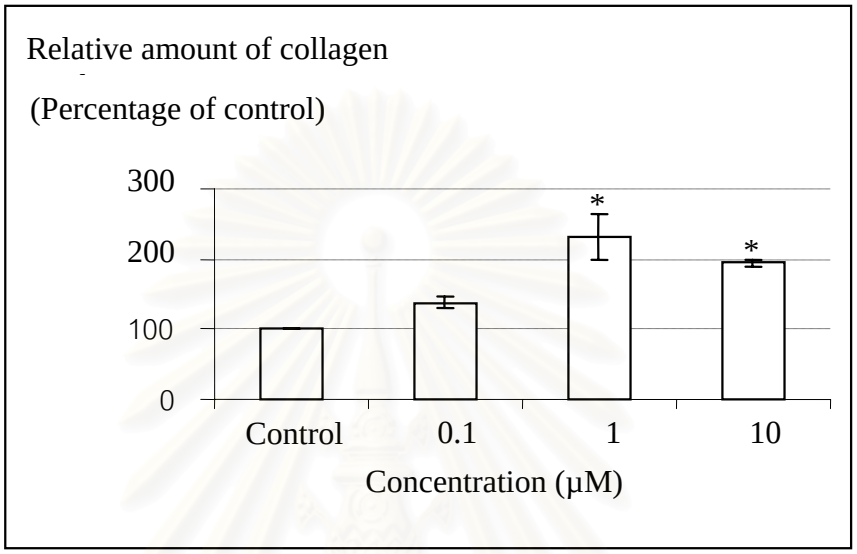


Figure 5: The effect of 0.1, 1 and 10 µM fluocinolone acetonide on type I collagen synthesis detected by Western analysis. Human dental pulp cells were treated with fluocinolone acetonide for 5 days. Fluocinolone acetonide at 1 and 10 µM increased the amount of type I collagen in while 0.1 µM had no significant effect. The relative amount of type I collagen from three separate experiments was shown in mean percentage ± standard deviation. (*) = statistically significant difference from the control group at $p < 0.05$ (Scheffe's test).

Reverse-transcription polymerase chain reaction (RT-PCR)

The effect of fluocinolone acetonide on the type I collagen synthesis was then confirmed by investigating the expression of type I collagen mRNAs in human dental pulp cells. At 48 hours, 1 µM fluocinolone acetonide could stimulate type I collagen mRNA expression. The expression of GAPDH was used as internal control (Fig. 6). The activity of type I collagen mRNA expression was significantly increased about 2.8-fold compared with the control ($p < 0.05$) as shown in figure 7.

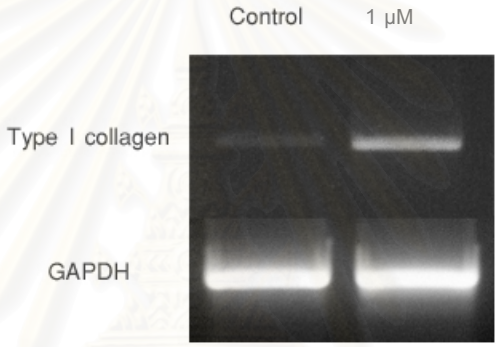


Figure 6: The expression of type I collagen of cultured human dental pulp cells at 48 hours cultured with 1 µM fluocinolone acetonide, *Upper row*: Type I collagen mRNA expression of cultured human dental pulp cells at 48 hours, cultured with 1 µM fluocinolone acetonide; *Lower row*: GAPDH mRNA expression was used as internal control.

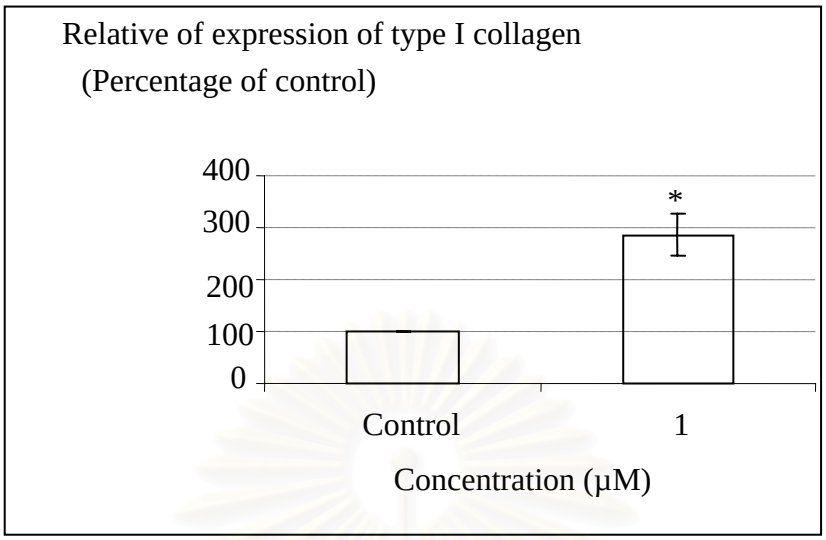


Figure 7: The effect of 1 µM fluocinolone acetonide on type I collagen mRNA expression detected by RT-PCR. Human dental pulp cells were treated with fluocinolone acetonide for 48 hours. Fluocinolone acetonide at 1 µM increased the expression of type I collagen mRNAs when compared to control. The relative amount of type I collagen mRNA expression from three separate experiments was shown in mean percentage ± standard deviation. (*) = statistically significant difference from the control group at $p < 0.05$ (t test).

Alkaline phosphatase (ALPase) activity

Human dental pulp cells were cultured in the presence of 0.1 to 50 µM of fluocinolone acetonide using 2% fetal bovine serum medium for 24 and 72 hours. The activity of ALPase was shown as percentage comparing with the control group in figure 4. The results showed that fluocinolone acetonide did not affect human cultured dental pulp cells in ALPase activity at every concentration used in this study at 24 hours.

However, fluocinolone acetonide at all doses in this study decreased alkaline phosphatase activity in a dose-dependent manner at 72 hours (figure 8). There was a statistically significant decreased this ALPase activity as shown in figure 8.

Concentration of fluocinolone acetonide (µM)	ALPase activity (Mean ± S.D.)	
	24 hours	72 hours
0.1	95.090 ± 4.160	60.59 ± 6.690
1	97.880 ± 4.650	71.46 ± 4.040
10	96.590 ± 3.980	76.160 ± 1.420
50	102.770 ± 2.970	87.750 ± 2.100
Control (Serum free medium)	100.000 ± 2.590	100.000 ± 3.580

Table 2: The effect of fluocinolone acetonide on alkaline phosphatase (ALPase) activity on cultured human dental pulp cells, cultured for 24 and 72 hours. The activity of (ALPase) was shown as percentage comparing with the control group (100%). Data were shown as mean ± S.D. of ALPase activity from three triplicate experiments.

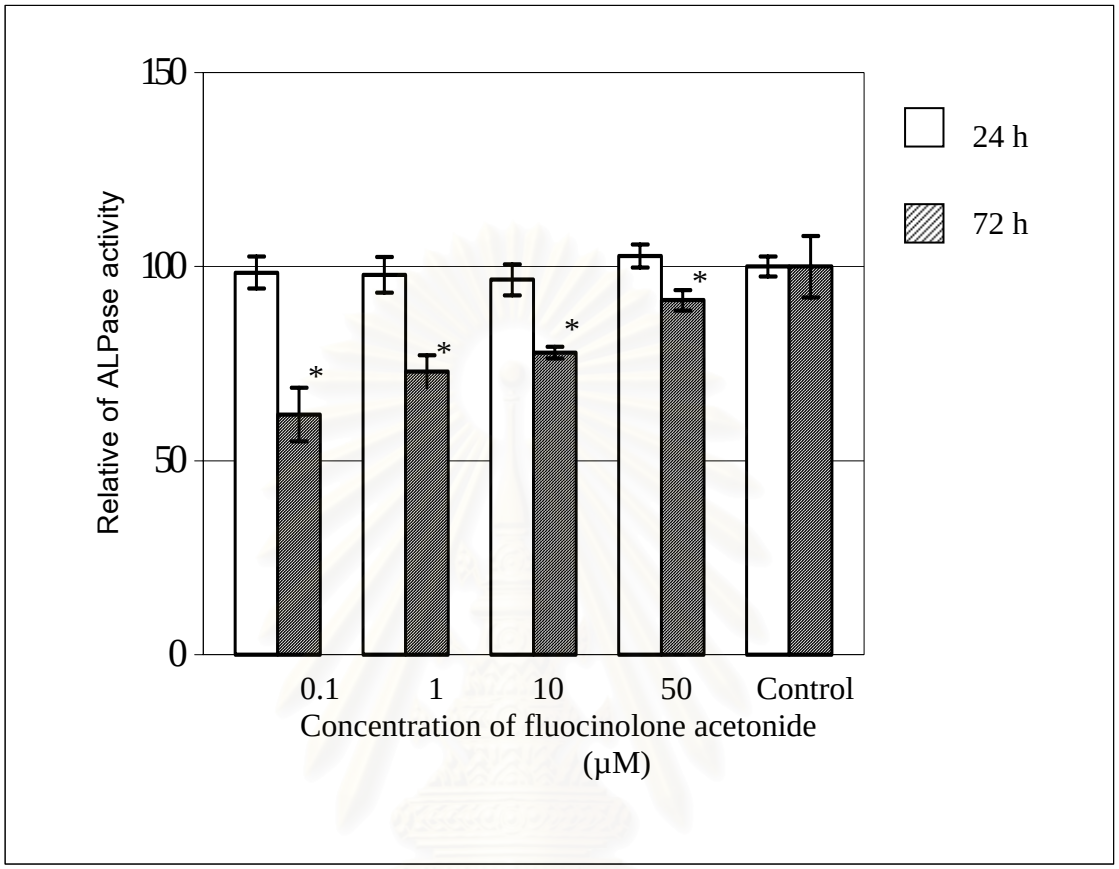


Figure 8: The effect of fluocinolone acetonide on alkaline phosphatase (ALPase) activity at 0.1 to 50 µM, cultured for 24 and 72 hours. The activity of ALPase was shown as percentage comparing with control group (100%). Data were shown as mean ± S.D. from three triplicate experiments. (*) Significant difference ($p<0.05$) when compared with the control group at 72 hours.

***In vitro* calcification**

Human dental pulp cells were cultured with selected concentrations (0.1, 1 and 10 μM) of fluocinolone acetonide for up to 28 days. Under phase contrast microscope, morphology of the cells and calcified nodules formation were observed. Initially, most of dental pulp cells in primary culture were fibroblast-like with a few short processes. Some of them gradually became broad and flattened. The cells became confluent at about one week of the cultures (Fig. 9A). When maintained in cultures, the pulp cells slowly became multilayered (Fig. 9B) and clustered forming many nodules (Fig. 9C). Calcified nodules were observed at about 2 weeks (Fig. 9D). The size and number of mineralized nodules were slowly increased with time. At the end of culture, alizarin red S positive staining was observed in all groups, and calcified nodules in the experimental groups seem not different from the controls (Fig. 10).

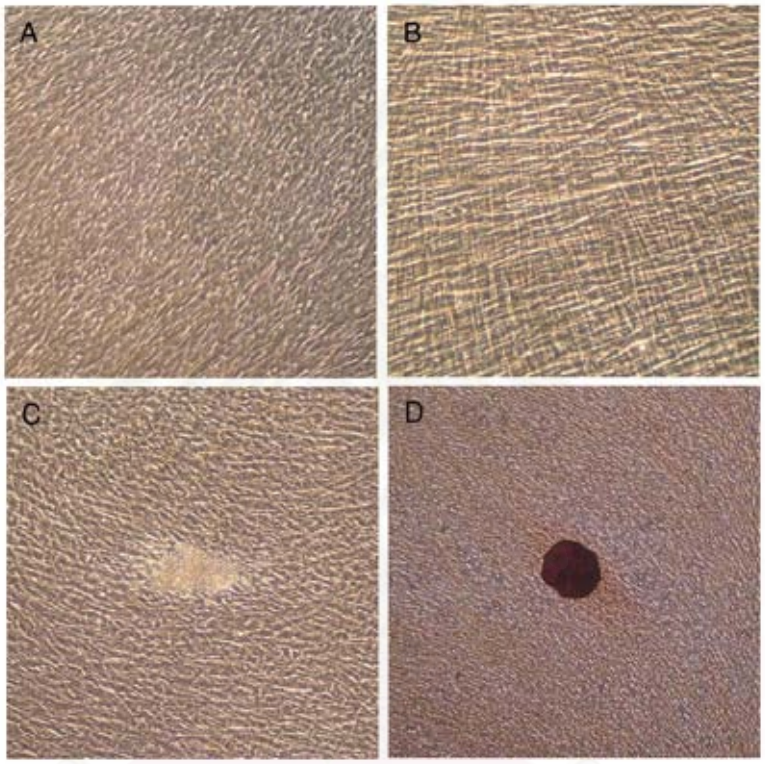


Figure 9: A photograph of 4 stages of cultured human dental pulp cells. Human dental pulp cell cultures, grown in the presence of ascorbic acid, β-glycerophosphate and fluocinolone acetonide, can be divided into four distinguished stages based on the appearance of cell alignment observed under phase contrast microscope. Four stages of cultures were identified as confluence (A), multilayer (B), nodule formation (C) and precipitation stage (D).

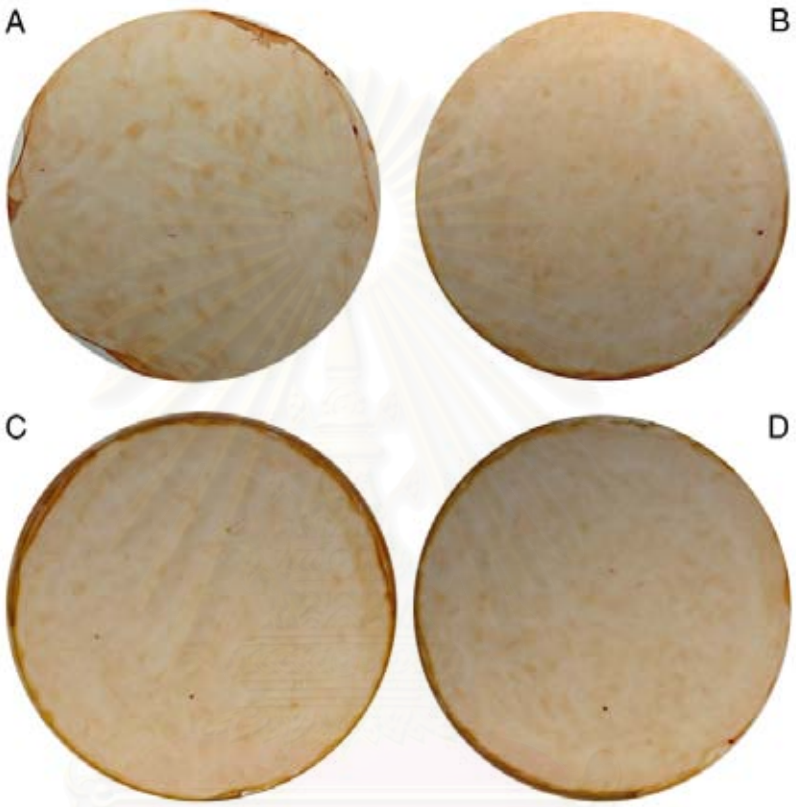


Figure 10: Photographs of calcified nodule formation demonstrated by alizarin red staining of human dental pulp cells in various concentrations of fluocinolone acetonide; Control (A), 0.1 µM (B), 1 µM (C) and 10 µM (D). The calcified nodules were macroscopically seen as red spots on the cells and seem not to different between the experimental and control groups.

CHAPTER V

DISCUSSION

Goal of direct pulp capping is to treat reversible pulp injury in order to maintain pulp vitality and function. In case of pulp exposure, the amputated pulp can repair itself or recover after application of suitable capping material (Nyborg, 1995). However, the success of self repair by dental pulp tissue depends on the extent of defensive reactions (Trowbridge, 1981). Treatment strategies to assist pulp tissue repair has been in much attention in the past four decades. However, controversy regarding mechanism of action of capping materials still exists. The use of topical corticosterioids such as Ledermix® treatment to treat vital pulp has been shown to be clinically effective regarding controlling of painful symtomps of pulpal inflammation (Barker *et al*., 1972). In the past two decades, dexamethasone becomes one of topical corticosterioids that has been of interest and studied *in vitro*. Dexamethasone has been shown to strongly stimulate alkaline phosphatase activity and induce the expression of transcript encoding the major odontoblastic marker, such as dentin sialophosphoprotein (Alliot-Licht *et al*., 2005). Dexamethasone has been shown to induce odontoblastic differentiation and effected the putative competent progenitor cells derived from dental pulp tissue in culture by promoting the compatability of dental pulp cells to induce mineralization (Kasugai *et al*., 1993). Therefore, it could play a role in both regulations of maintaining pulp tissue homeostasis and healing process of dental pulp repair. However, dexamethasone may not be the drug of choice for pulp capping due to its toxicity and the narrow gap of suitable concentration that makes it difficult to control practically.

Fluocinolone acetonide is commonly used as topical medicament in the treatment of various dermatologic disorders and oral vesibuloerosive lesions (Hooley and Hohl, 1974;

Lozada and Silverman, 1980; MacPhail *et al*., 1992; Buajeeb *et al*., 2000). It provides distinct advantages in the inhibition of inflammatory process and immunologic responses (Vogel *et al*., 1984). Interestingly, some evidence demonstrated that a range of concentrations of fluocinolone acetonide had stimulatory effect on cell proliferation (Fisher and Maibach, 1971; Kirk and Mittwoch, 1977). An *in vitro* study in mouse fibroblasts indicated that steroids, which possessed high glucocorticoid activity, were capable of stimulation both DNA synthesis and cell division. The relative potency of these active steroids was related to their relative glucocorticoid potency (Thrash *et al*., 1974). Fluocinolone acetonide is a synthetic glucocorticoid which has the most relative potency among various types of steroids (Berliner and Ruhmann, 1967). Thus, it may provide some advantages in the stimulation of cell proliferation and healing process.

In pulpal exposure, some odontoblasts are irreversibly damaged which may be due to dental caries, operative procedure, or traumatic injury. The main goal of regenerative pulp treatment is to induce a second generation of odontoblast-like cells that are capable of secreting a dentin-like matrix structure to seal off the pulp wound (Goldberg *et al*., 2003; Alliot-Licht *et al*., 2005). The formation of hard tissue barrier may be considered as a two-part phenomenon. The first stage is the formation of organic extracellular matrix, mainly composed of type I collagen, by the progenitor cells which are differentiated into matrix synthesizing odontoblast-like cells. Finally, the newly formed tissue is subsequently calcified to form a hard tissue barrier (Mjör *et al*., 1991; Linde and Goldberg, 1993).

The acetonide derivatives of fluorinated steroids have provided distinct advantages in topical anti-inflammatory condition over dexamethasone (David and Ann, 1967). The results of our experiments showed that the application of fluocinolone acetonide in a suitable concentration could stimulate cell proliferation as well as fibronectin synthesis. In addition, this study also demonstrated that the working dosage range of fluocinolone acetonide is wide

ranging from 0.1 to 50 µM, as compared to the critical dose of dexamethasone that was shown only 0.1 n M in previous report (Alliot-Licht *et al*., 2005; Yang *et al*., 2003). Since our results indicated that fluocinolone acetonide provided a stimulatory effect on proliferation and fibronectin synthesis, it supports the possibility of using fluocinolone acetonide in dental pulp tissue repair.

In this study, MTT assay was used to evaluate cell cytotoxicity and cellular proliferation. MTT assay is a colorimetric assay system, which measures reduction of tetrazolium products by mitochondria of viable cells. Cells with functional mitochondria convert tetrazolium dye into its reduced form. The MTT assay is an accurate and straightforward mean used to quantify changes in cell proliferation. In addition, the method is easy to handle. A large number of probes can be assayed in a relative short time and no radioactive materials are used.

The process of pulp tissue repair includes proliferation, migration, survival and differentiation of pulp cell lineages at the injured site forming a new generation of reparative dentin (Mitsiadis and Rahiotis, 2004). Up to now, no information is addressed about the biocompatibility of fluocinolone acetonide on human dental pulp cells. The results of this experiment were the first one to show that the range of concentration of fluocinolone acetonide, 0.1 to 50 µM, had no cytotoxicity effect on human dental pulp cells. In addition, these doses of fluocinolone acetonide also stimulated cell proliferation.

The MTT assay was also used to examine the effect of fluocinolone acetonide on human cultured dental pulp cells proliferation. The results revealed that cell proliferation significantly increased at low concentration (0.1 and 1 µM) of fluocinolone acetonide after 48 hours ($p < 0.05$). However, all doses of fluocinolone acetonide in this study show a statistically significant in cell proliferation at 72 hours ($p < 0.05$). Kirk and Mittwoch (1977)

also reported the same results that 50 µM to 50 mM of fluocinolone acetonide stimulated the growth of fibroblast, although this study used normal human diploid skin fibroblasts.

Moreover, Yang *et al*. (2003) revealed that corticosteroids had a significant effect on proliferation and differentiation of adult human osteoblasts *in vitro*. They treated adult human osteoblasts for 14 days with 10^{-8} M of dexamethasone and examined the proliferation, differentiation and apoptosis. The result showed after application of dexamethasone for 3 days, the number of osteoblast increased compared to control. In contrast, the conflicting result was reported from human dental pulp cultures using thymidine in–corporation. Alliot-Licht *et al*. (2005) reported that dexamethasone (0.1 nM) significantly inhibited dental pulp cell proliferation. This different result may be due to the difference in cell type as well as the differentiation stage of cells. The effect of corticosteroids on cellular growth had been suggested to depend on the stage of cell differentiation (Alliot-Licht *et al*., 2005). However, fluocinolone acetonide in this study showed the different result from dexamethasone, although the study was done on the same cell type, human dental pulp cells.

The ability of materials to promote protein synthesis may reflect better biocompatibility. The association between the certain type of protein in the extracellular matrix and the differentiation stage of odontoblast has been reported (Ruostlahti, 1981). Fibronectin is a high molecular weight extracellular glycoprotein found in connective tissues and basement membranes. This protein has been shown to play an important role in the regulation of cell behaviour during development and repair of several tissues including the dental pulp. Fibronectin is also implicated in the cytological modifications during odontoblast differentiation (Hynes, 1985).

The effect of fluocinolone acetonide on fibronectin synthesis was examined by Western blot analysis from cell extracts. The result showed that fluocinolone acetonide stimulated the synthesis of fibronectin in a reverse dose-dependent manner (0.1 to 50 µM,

but not significant at 50 µM). The concentration of 0.1 µM of fluocinolone acetonide has the strongest effect on fibronectin synthesis compared to the other concentrations used in the present study.

Fibronectin has been implicated in a variety of cell functions including an act as mediator of cell adhesion, both to other cells and to extracellular components, migration, growth and differentiation (Ruostlahti, 1981). Fibronectin can also influence behaviour of other cell types. It has been shown that fibronectin involved in the migration of endothelial cells, helping in the processes of angiogenesis, and probably increased the number of mesenchymal cells that usually associated with the newly formed blood vessels (Chon *et al*., 1997). Thus, increasing of fibronectin may provide an inductive effect not only on the pulp cells but also on the endothelial cells in the pulp tissue.

In contrast, the corticosterone was demonstrated to inhibit fibronectin production from cultured rat parietal bones (Gronowicz *et al*., 1991). The decrease in fibronectin synthesis may contribute to altered osteoblast organization and function during bone formation. This different effect may result from the different cell type or the different condition of the experiment, and different type of corticosterone used. Further analysis of fluocinolone acetonide *in vivo* model is needed to clarify this issue.

Because of numerous reports of a reciprocal and functionally coupled relationship between proliferation and differentiation, the effects of fluocinolone acetonide on alkaline phosphatase activity in human cultured dental pulp cells were the future investigation. Pulpal cells were exposed to fluocinolone acetonide in different concentrations (0.1 to 50 µM) for 24 and 72 hours in medium with 2% fetal bovine serum. Alkaline phosphatase activity was measured biochemically from the cell extracts. The results showed that fluocinolone acetonide did not affect statistically significant difference ($p>0.05$) in any groups at 24 hours. However, all doses of fluocinolone acetonide significantly decreased alkaline phosphatase

activity in a dose-dependent manner (0.1 to 50 μM) at 72 hours. This inhibitory effect was also reported in rat osteoblastic cell by dexamethasone (Iu *et al*., 2005). The application of cortisol decreased cell differentiation, and impaired maturation and mineralization (Weinstein *et al*., 1998).

Miller *et al*. (1976) suggested that alkaline phosphatase activity was a prerequisite for cell differentiation because of its presence during odontogenesis in the cells from dental papilla differentiating into odontoblasts. This enzyme was functional in dentine mineralization (Goseki *et al*., 1990) and was retained as a marker of fully differentiated odontoblast (Goseki *et al*., 1990). Since the function of alkaline phosphatase activity is to hydrolyze organic phosphorus to form hydroxyapatite, it is an indispensable enzyme for bone formation. Its secretion indicates the occurrence of bone formation and the beginning of differentiation (Yang *et al*., 2003). The effect of fluocinolone acetonide on alkaline phosphatase activity in this short term experiment may be in accordance with the hypothesis of Yamamura (1985) that pulpal cells will de–differentiated into mesenchymal cells before differentiating into an odontoblast–like cells.

The report from Alliot-Licht *et al*. (2005) indicated that corticosteroid (dexamethasone at 0.1 nM) strongly stimulated alkaline phosphatase activity. It was shown two folds increased after 7 days. This study also confirmed that dexamethasone had no effect on cell proliferation. This stimulation of alkaline phosphatase activity reached a maximum value after 14 days with a significant increased about 350%. The stimulatory effect of dexamethasone on alkaline phosphatase is also reported in the other cell types, such as bone marrow stromal cells (Kim *et al*., 1999), human dental papilla cells (Hao *et al*., 1997). In addition, stimulation of alkaline phosphatase is corresponding with the cease in cell proliferation. Thus, the differentiation stage of cells may be accounted in the correlation between alkaline phosphatase activity and proliferation. This concept is supported by the

work of Alliot-Licht *et al*. (2005). They showed that the increase in alkaline phosphatase activity in dental pulp cell was depended on the stage of differentiation of the culture.

This experiment demonstrated that fluocinolone acetonide enhanced proliferation and fibronectin synthesis. Conversely, fluocinolone acetonide reduced alkaline phosphatase activity on human cultured dental pulp cells. The findings might be attributed to the short-term culture (3 days) utilized with the aims to investigate early repair process, not to stage of cell differentiation. It appears that a sequence of *in vitro* cytotoxicity tests as well as biocompatibility assessment of dental materials need to be performed to demonstrate their biological properties and support any new proposed pulp therapy. The result from *in vitro* studies may support in the appropriate application of dental materials to the pulp-dentine complex.

The signaling pathway of fluocinolone acetonide is still unknown. The function of fluocinolone acetonide may involve glucocorticoid receptors. Glucocorticoid signal may act through the classical steroid hormone-receptor pathway. Glucocorticoids freely enter the cell and bind to glucocorticoid receptors in cytoplasm. This hormone–receptor complex translocates to the nucleus. Then, it binds to glucocorticoid response elements and regulates the transcription of target genes (Cato *et al*., 2002).

However, the exact mechanism of fluocinolone acetonide on human cultured dental pulp cell growth enhancement and differentiation remains unclear. Further experiment on long term effect of fluocinolone acetonide should be investigated.

In the present study, the effect of fluocinolone acetonide on type I collagen synthesis was examined by Western blot analysis from cell extracts. The result indicated that 1 to 10 μM fluocinolone acetonide was capable of stimulating type I collagen synthesis in human dental pulp cells. Type I collagen synthesis was maximized by the 1 μM fluocinolone acetonide when compared to the others (Fig. 5). This result was confirmed by RT-PCR. The

expression of mRNAs of type I collagen in 1 μM fluocinolone acetonide groups was stronger than controls. In addition, we investigated the expression of integrin receptors which were corresponding to type I collagen. The expression of mRNAs of α1β1 integrin receptors were also stimulated (data not shown). This result strongly supported the effect of fluocinolone acetonide in stimulation of type I collagen synthesis in human dental pulp cells in laboratory condition.

It was previously reported that fluocinolone acetonide topically applied for 7 days in mice might reduce fibroblast proliferation and collagen formation. In the wound biopsies, the extracellular space contained non-banded filamentous material and decreased mature collagen when compared with control (Berliner *et al*., 1967). The *in vitro* effect of fluocinolone acetonide on human‘s skin collagen was also studied and demonstrated that formation of radioactive hydroxyproline was inhibited by all corticosteroids tested. The corticosteroid-induced inhibition of collagen biosynthesis was found to be dose-dependent. Large non-physiologic concentrations of fluocinolone acetonide inhibited the rate of collagen formation, but the lowest concentration (10 mM) of fluocinolone acetonide had no significant effect on hydroxyproline formation (Uitto *et al*., 1972).

The result of these studies was different from our experiment which might be due to different methodology used. The earlier study was conducted in mice which applied topical glucocorticoid on the skin, and microscopic observation was used to determine the appearance of collagen fibrils intra- and extracellularly. The result was descriptive and unable to compare the quantity of collagen formation. In addition, the concentration of steroid used was higher than in our study. The later experiment used skin fibroblast and analyzed the radioactive hydroxyproline formation. The result indicated that the 10 mM fluocinolone acetonide had no significant effect on collagen synthesis. This concentration was also much higher than in our study, but within a range of physiological concentrations

(100 nM to 10 mM) in human (Berliner and Ruhmann, 1967). The inhibitory effect was in dose-dependent manner, thus lower concentration might be able to stimulate collagen synthesis. In our study, human dental pulp cells were used and different cells might exhibit different response. Western blotting and RT-PCR were used in our study because they have been widely accepted as highly sensitive and reliable techniques in the detection of protein synthesis and gene expression respectively.

Several literatures, however, reported that some glucocorticoids had ability to stimulate collagen synthesis in different tissue. In fetal rat calvaria, short term treatment of some concentrations of cortisol, corticosterone and dexamethasone increased bone collagen synthesis. On the other hand, long term treatment of cortisol (96 hours) inhibited collagen synthesis (Dietrich *et al*., 1979). After that, Canalis used the similar method and confirmed the stimulatory effect of short term treatment of these steroids in collagen synthesis and inhibitory effect of high concentrations (1-10 μM) of cortisol on type I collagen labeling after 24-hour treatment. The inhibitory effect was dose related (Canalis, 1983). In cultured bovine aortic smooth muscle cells, 0.1 μM dexamethasone produced an approximate two folds increasing in the incorporation of [^{3}H]proline into collagenase-digestible protein (Leitman *et al*., 1984). Recently, RT-PCR was used to measure the up-regulation of type I collagen mRNA expression in tooth organ cultures. The treatment of 10 nM dexamethasone exhibited four folds greater than in the control group (Ritchie *et al*., 2004). Hence, these studies have supported the possibility in the use of some glucocorticoids in order to stimulate type I collagen synthesis.

The *in vitro* calcification of human dental pulp cells was studied by long term cultures for up to 28 days. The results in all groups demonstrated small amount of calcified nodules formed in a variety of size and time (Fig. 10). Hence, the quantification of nodules and statistical analysis was not possible. However, the formation of mineralized nodules

observed in the experimental groups was not different from the control group. It might be explained that fluocinolone acetonide might lack of the ability to enhance the differentiation of dental pulp cells. In another way, the cells treated by fluocinolone acetonide might need more time to exhibit the calcification process. Therefore, further investigations are needed with regard to the markers of cellular differentiation into hard tissue forming cells.

However, the formation of calcified nodules in this study was consistent to the previous study (Tsukamoto *et al*., 1992). Tsukamoto and colleagues (1992) reported the alterations of cell morphology and mineralized nodule formation in human dental pulp fibroblasts in cultures. The nodules were formed after 10-15 days and their characteristics were similar to our study.

In the development of mineralized nodules by rat periodontal cells, four distinct stages based on the appearance of cultures under the phase contrast microscope were identified. The first stage was the formation of confluent monolayer of cells which generally exhibit spindle or polygonal morphology. When treated with steroids, ascorbic acid and β-glycerophosphate, cell proliferation began to occur in certain areas with the formation of several cell layers appearing like clusters of cells referred to as the multilayer formation stage. The third stage was associated with the formation of nodules formed by deposition of matrix in the clusters of cells. Finally, mineralization of the matrix occurred and was referred to as mineralized nodule stage. The mineralized nodules showed densely stained mineral deposits with globular shape that appeared to increase in size and fuse towards the lower cell layers of the nodules. As mineralization progressed, the deposits increased in size and fused together, forming a contiguous mineral deposit in the intercellular space (Ramakrishnan *et al*., 1995). In contrast, the observation of calcified nodules in our study demonstrated that the nodules were rarely fused together. The isolated nodules were frequently found which was

different from the periodontal cell cultures. These results may be because of the different cell type in experiment.

Glucocorticoids demonstrated some adverse effects from using in human. The major adverse effects of corticosteroids particularly applicable to dental usage are inhibition of fibroblastic proliferation which favors dissemination of microorganisms, and inhibition of cellular response (both vascular and local tissue) to an irritant (Sinkford and Harris, 1964). Thus, living noxious irritants are able to multiply and disseminate into systemic circulation at an increased rate. The increased susceptibility to infection resulting from corticosteroid administration appears to be secondary to the suppressed protective inflammatory response (Sinkford and Harris, 1964). Klotz and co-workers suggested that bacteremia might occur by topical prednisolone treatment in infected monkey pulps, especially in young permanent teeth with wide apexes (Klotz *et al*., 1965). In rat models, prednisolone and triamcinolone acetonide produced poor responses when applied to the mechanically exposed pulps. Bacteremia was observed to be able to distribute widely throughout the necrotic pulp tissues with the presence of inflammation in the periapical tissues (Watts and Paterson, 1988).

Abbott discussed about the release of triamcinolone acetonide from Ledermix® which applied onto the dental pulp. It was initially rapid in the first day, then drastically diluted, and reduced once it entered the circulation. The released concentration appeared to be an extremely low possibility of producing systemic side-effects from intra-dental use of corticosteroids as pulp capping and cavity lining materials (Abbott, 1992). In the present study, the low physiological concentrations of fluocinolone acetonide which were probably too low to induce any serious adverse effect were used. No evidence has been reported that fluocinolone acetonide enhances the spread of infection when used topically. However, *in vivo* study is needed to examine the side-effect of this material. In addition, the results from this study showed that fluocinolone acetonide stimulated dental pulp cell proliferation,

fibronectin and type I collagen synthesis, which were in healing process. In vital pulp therapy, case selection is a crucial step for predictable outcome. An ideal case should be a mechanical exposure from operative procedure or, otherwise, an infected pulp has to be eradicated before application of pulp capping agent. In addition, sterilization has to be strictly controlled by using rubber dam and aseptic technique (Cox *et al*., 1985; Stanley, 1989). With proper clinical conditions, the problem of using topical fluocinolone acetonide would be minimized.

There were also some limitations in the present study. It was an *in vitro* experiment using human cultured pulp cells. The results obtained from this trial can only assess the efficacy of fluocinolone acetonide in the enhancement of pulpal healing process. The whole mechanisms of pulpal healing *in vivo* are more complex, involved both cellular and extracellular events. Due to the nature of experiment, the number of samples was limited. Thus, the results cannot be completely judged to total populations.

The data supported that application of fluocinolone acetonide to a dental pulp might promote pulp cell proliferation, increase fibronectin and type I collagen synthesis, the major component of extracellular matrix in dentin-like structure. It may be efficient to promote early stage of pulpal healing and may be beneficial in short term pulpal treatment. Thus, it is probable that this substance may be developed as a new commercial effective pulp capping agent in the future. However, the induction of pulpal cell differentiation in this study is unclear. Other markers in hard tissue formation (such as osteopontin, osteocalcin) are needed to investigate for this purpose (Arceo *et al*., 1991). Moreover, further *in vivo* investigations are needed before this material can be recommended for routine use in clinical practice.

In conclusion, fluocinolone acetonide could promote extracellular matrix formation especially fibronectin and type I collagen synthesis of human dental pulp cells. In addition, the ability to form *in vitro* calcification in the presence of fluocinolone acetonide is

comparable to the control. Regulating the collagen synthesis of pulp cells by fluocinolone acetonide could be crucial in the repair and regeneration of human dental pulp.

CHAPTER VI

CONCLUSIONS

The results of this study can be concluded as follows:

1. Fluocinolone acetonide did not affect cell cytotoxicity of cultured human dental pulp cells at 24 hours.

2. Fluocinolone acetonide affects cultured human dental pulp cells proliferation.

2.1 Fluocinolone acetonide at 0.1 and 1 µM stimulates human cultured dental pulp cells proliferation at 48 hours.

2.2 Fluocinolone acetonide at 0.1, 1, 10 and 50 µM also stimulate cultured human dental pulp cells proliferation at 72 hours.

3. 0.1, 1, 10 µM of fluocinolone acetonide stimulate fibronectin synthesis of cultured human dental pulp cells at 48 hours.

4. Fluocinolone acetonide at 1 and 10 µM concentrations enhanced type I collagen synthesis in human cultured dental pulp cells at 5 days.

5. 1 µM fluocinolone acetonide stimulated mRNA expression of type I collagen of human cultured dental pulp cells at 48 hours.

6. Fluocinolone acetonide decreased alkaline phosphatase activity of cultured human dental pulp cells at 72 hours in all concentrations, but no effect on alkaline phosphatase activity of cultured human dental pulp cells at 24 hours.

7. Fluocinolone acetonide had no effect in the *in vitro* calcification in human cultured pulp cells.

SUGGESTION FOR FURTHER WORK

The results from this study demonstrated that fluocinolone acetonide stimulated pulp cell proliferation, fibronectin and type I collagen synthesis which related to the early stage of repair and healing processes. However, some results did not show the increase of alkaline phosphatase activity and calcification process. These may be because this cell culture experiments were done in short term or the duration of experiment did not long enough to induce calcification process.

The results suggest that fluocinolone at low concentration (0.1-10 μM) is suitable to be applied as a pulp capping material to induce early stage of pulpal healing. The further researches should conduct in producing pulp capping material, containing fluocinolone acetonide, and compare physical properties with standard materials such as Dycal. The animal experiment should conduct to investigate the whole process of repair and healing before clinical trial is done.

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APPENDICES

Table 1: The optical density (O.D.) and number of viable human dental pulp cells were examined by MTT assay at 24 hours.

Number of viable cells(x10^4)	O.D. 1	O.D. 2	O.D. 3	Mean	S.D.
0.5	0.065	0.078	0.091	0.078	0.013
1	0.156	0.162	0.159	0.159	0.003
2	0.328	0.297	0.352	0.326	0.028
4	0.607	0.614	0.653	0.625	0.025
8	1.102	1.234	0.929	1.088	0.153

Figure1. Standard curve of number of viable human dental pulp cell.

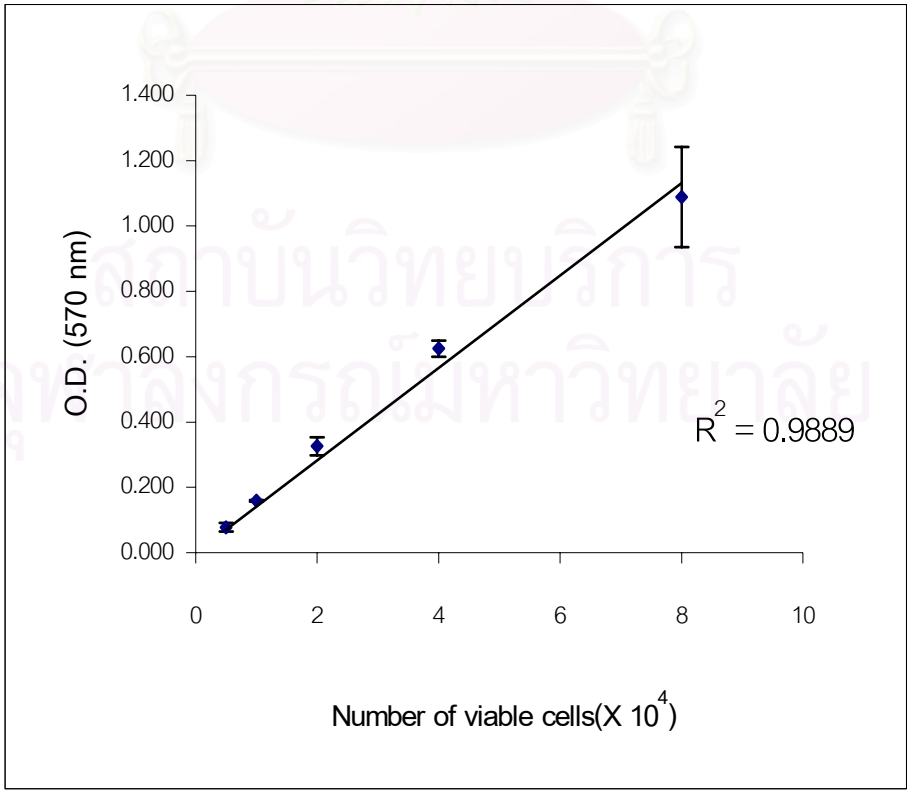

Table 2: The cytotoxicity and cell proliferation effect of fluocinolone acetonide (FA) on cultured human dental pulp cells were examined by MTT assay at 24 hours. Data were shown as number of viable cells from three triplicate experiments.

Concentration of fluocinolone acetonide (μM)	Number of viable cells (Mean) $x10^4$ Line1		
	1	2	3
Control (Serum free medium)	1.870	1.830	1.860
50	1.850	1.810	1.880
10	1.870	1.870	1.830
1	1.910	1.850	1.850
0.1	1.870	1.890	1.880
Line 2			
Control (Serum free medium)	1.880	1.850	1.820
50	1.830	1.880	1.860
10	1.870	1.810	1.860
1	1.870	1.850	1.870
0.1	1.830	1.870	1.890
Line 3			
Control (Serum free medium)	1.870	1.850	1.820
50	1.850	1.870	1.810
10	1.870	1.890	1.820
1	1.890	1.850	1.870
0.1	1.870	1.870	1.910

Number of viable cells $= \frac{(O.D. \times 2 \times 10^4)}{0.326}$

Table 3: The cell proliferation effect of fluocinolone acetonide (FA) on cultured human dental pulp cells were examined by MTT assay at 48 hours. Data were shown as number of viable cells from three triplicate experiments.

Concentration of fluocinolone acetonide (μM)	Number of viable cells (Mean) $x10^4$ Line1		
	1	2	3
Control (Serum free medium)	2.530	2.500	2.550
50	2.440	2.530	2.790
10	2.440	2.610	2.680
1	2.800	3.080	2.990
0.1	3.340	3.010	3.560
Line 2			
Control (Serum free medium)	2.510	2.520	2.560
50	2.580	2.560	2.470
10	2.530	2.470	2.730
1	3.080	2.980	2.810
0.1	2.810	3.140	3.710
Line 3			
Control (Serum free medium)	2.480	2.560	2.500
50	2.550	2.610	2.620
10	2.770	2.460	3.120
1	2.710	3.170	3.090
0.1	3.960	3.580	3.690

Number of viable cells = $\frac{(O.D. \times 2 \times 10^4)}{0.326}$

Table 4: The cell proliferation effect of fluocinolone acetonide (FA) on cultured human dental pulp cells were examined by MTT assay at 72 hours. Data were shown as number of viable cells from three triplicate experiments.

Concentration of fluocinolone acetonide (μM)	Number of viable cells (Mean) $x10^4$ Line1		
	1	2	3
Control (Serum free medium)	3.690	3.770	3.670
50	4.050	3.960	4.210
10	4.310	4.280	4.200
1	4.570	4.820	4.390
0.1	5.480	5.450	5.360
Line 2			
Control (Serum free medium)	3.670	3.730	3.800
50	3.820	3.880	3.960
10	4.330	4.280	4.370
1	4.630	4.570	4.790
0.1	5.190	5.390	5.530
Line 3			
Control (Serum free medium)	3.850	3.790	3.690
50	3.960	4.210	4.040
10	4.130	4.370	4.570
1	4.390	4.920	4.820
0.1	5.190	5.360	5.530

Number of viable cells $= \frac{(O.D. \times 2 \times 10^4)}{0.32}$

Table 5: The effect of fluocinolone acetonide on the level of fibronectin synthesis was detected by Western blot analysis. Data were shown as the optical density, adjusted to percentage of expression, by comparing with the control condition. The experiments were performed from three different donors.

Concentration of fluocinolone acetonide (μM)	The optical density ($x10^4$ pixel)		
	Line1		
	Fibronectin	Actin	Percentage of expression
Control (Serum free medium)	0.150	1.070	44.950
50	0.260	0.940	92.380
10	1.310	2.100	205.270
1	1.490	1.990	246.120
0.1	1.760	1.380	417.890
Line 2			
Control (Serum free medium)	0.270	1.330	65.990
50	0.280	1.560	59.440
10	1.020	1.570	214.290
1	1.160	1.700	224.880
0.1	1.540	1.980	256.090
Line 3			
Control (Serum free medium)	0.470	0.820	189.060
50	0.750	0.890	277.080
10	1.710	1.930	291.480
1	1.500	1.760	281.100
0.1	1.260	1.650	250.290

Percentage of expression = $\frac{(\text{Fibronectin} / \text{Actin}) \times 10000}{30.3904}$

by comparing with the control condition

Table 6: The effect of fluocinolone acetonide on the level of fibronectin synthesis was detected by Western blot analysis. Data were shown as mean ± S.D. of percentage of expression, by comparing with the control condition. The experiments were performed from three different donors.

Concentration of fluocinolone acetonide (μM)	Percentage of expression (Mean ± S.D.)
Control (Serum free medium)	100.000 ± 77.840
50	142.970 ± 117.300
10	237.010 ± 47.380
1	250.700 ± 28.380
0.1	308.090 ± 95.130

Table 7: The effect of fluocinolone acetonide on alkaline phosphatase (ALPase) activity on cultured human dental pulp cells, cultured for 24 hours. The activity of (ALPase) was shown as percentage comparing with the control group (100%).

Concentration of fluocinolone acetonide (μM)	ALPase activity Line1		
	1	2	3
Control (Serum free medium)	98.780	98.780	98.780
50	99.820	98.630	100.420
10	93.890	96.110	93.340
1	93.360	95.610	94.480
0.1	94.150	90.290	91.950
	Line 2		
Control (Serum free medium)	101.070	94.960	99.950
50	100.700	104.650	102.940
10	97.200	89.880	95.660
1	95.730	94.670	96.800
0.1	91.780	92.300	95.950
	Line 3		
Control (Serum free medium)	103.750	102.570	101.380
50	106.940	105.720	105.110
10	101.280	100.120	101.870
1	106.600	104.260	99.580
0.1	98.310	103.330	97.750

ALPase activity = $\frac{\text{(ALPase activity x100)}}{\text{by comparing with the control group}}$ 164.305

Table 8: The effect of fluocinolone acetonide on alkaline phosphatase (ALPase) activity on cultured human dental pulp cells, cultured for 72 hours. The activity of (ALPase) was shown as percentage comparing with the control group (100%).

Concentration of fluocinolone acetonide (μM)	ALPase activity Line1		
	1	2	3
Control (Serum free medium)	102.430	103.430	102.480
50	88.400	84.870	88.330
10	74.300	77.430	77.430
1	77.750	72.430	72.430
0.1	52.010	52.010	54.310
Line 2			
Control (Serum free medium)	102.830	101.840	100.950
50	86.460	86.460	85.130
10	75.390	77.410	77.810
1	63.180	70.780	74.190
0.1	62.900	57.660	67.780
Line 3			
Control (Serum free medium)	96.150	95.550	94.340
50	89.990	89.520	90.600
10	76.260	74.310	75.100
1	73.170	68.500	70.650
0.1	64.670	65.000	68.950

ALPase activity by comparing with the control group = $\frac{\text{(ALPase activity x100)}}{162.649}$

Statistical analysis

All data from cytotoxicity and cell proliferation were analyzed using a one-way analysis of varience (ANOVA). Dunnett's T3 test was used for post-hoc analysis ($p < 0.05$).

Tests of Normality

	Concentration of fluocinolone acetonide (µM)	Kolmogorov-Smirnov Statistic	df	Sig.
Number of viable cells at 24 hours	Control	.191	9	.200*
	50	.217	9	.200*
	10	.226	9	.200*
	1	.235	9	.165*
	0.1	.230	9	.184*
Number of viable cells at 48 hours	Control	.168	9	.200*
	50	.208	9	.200*
	10	.173	9	.200*
	1	.203	9	.200*
	0.1	.188	9	.200*
Number of viable cells at 72 hours	Control	.201	9	.200*
	50	.206	9	.200*
	10	.222	9	.200*
	1	.205	9	.200*
	0.1	.197	9	.200*

* This is a lower bound of the true significance.

Descriptives

	Concentration of fluocinolone acetonide (µM)	N	Number of viable cells (Mean)	Std. Deviation	95% Confidence Interval	
					Lower Bound	Upper Bound
Number of viable cells at 24 hours	Control	9	18513.974	220.726	18344.308	18683.639
	50	9	18493.524	271.105	18285.133	18701.9145
	10	9	18624.389	210.544	18522.551	18846.228
	1	9	18684.389	194.274	18535.057	18833.722
	0.1	9	18755.739	233.836	18565.997	18925.482
Number of viable cells at 48 hours	Control	9	25228.357	263.4785	25025.829	25430.884
	50	9	25725.971	1001.053	24956.493	26495.449
	10	9	26441.717	2147.896	24790.698	28092.737
	1	9	29672.801	1602.155	28441.276	30904.327
	0.1	9	34321.745	3725.840	31457.810	37185.679
Number of viable cells at 72 hours	Control	9	25228.357	263.4785	25025.829	25430.884
	50	9	25725.971	1001.053	24956.493	26495.449
	10	9	26441.717	2147.896	24790.698	28092.737
	1	9	29672.801	1602.155	28441.276	30904.327
	0.1	9	34321.745	3725.840	31457.810	37185.679
Number of viable cells at 72 hours	Control	9	37355.146	636.415	36865.954	37844.339
	50	9	40995.432	1357.378	39052.059	41138.806
	10	9	43156.100	1216.253	42221.205	44090.995
	1	9	46550.783	1925.1436	45070.987	48030.580
	0.1	9	53885.480	1297.440	52888.179	54882.781

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Number of viable cells at 24 hours	Between Groups	461189.105	4	115297.276	2.226	.083
	Within Groups	2071754.467	40	51793.862		
	Total	2532943.573	44			
Number of viable cells at 48 hours	Between Groups	518916866.356	4	129729216.589	29.306	.000
	Within Groups	177070186.224	40	4426754.656		
	Total	695987052.580	44			
Number of viable cells at 72 hours	Between Groups	1477112089.695	4	369278022.424	202.537	.000
	Within Groups	72930441.074	40	1823261.027		
	Total	1550042530.769	44			

Multiple Comparisons

Dunnett T3

Dependent Variable	Concentration of fluocinolone acetonide (µM) (I) TX	Concentration of fluocinolone acetonide (µM) (J) TX	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Number of viable cells at 24 hours	Control	50	20.449	116.532	1.000	-354.345	395.244
		10	-170.415	101.679	.636	-495.740	154.908
		1	-170.415	98.0150	.594	-484.599	143.767
		0.1	-231.765	107.185	.333	-574.757	111.226
Number of viable cells at 48 hours	Control	50	-497.614	345.0488	.781	-1719.781	724.553
		10	-1213.360	721.332	.640	-3832.699	1405.978
		1	-4444.444*	541.225	.000	-6398.060	-2490.827
		0.1	-9093.387*	1245.048	.001	-13638.178	-4548.597
Number of viable cells at 72 hours	Control	50	-2740.286*	499.722	.002	-4429.002	-1051.570
		10	-5800.954*	457.565	.000	-7329.816	-4272.092
		1	-9195.637*	675.8700	.000	-11553.353	-6837.921
		0.1	-16530.334*	481.707	.000	-18150.654	-14910.01

* The mean difference is significant at the .05 level

Alkaline phosphatase (ALPase) activities were analyzed using a one-way analysis of varience (ANOVA). Dunnett's T3 test was used for post-hoc analysis ($p < 0.05$).

Tests of Normality

	Concentration of fluocinolone acetonide (µM)	Kolmogorov-Smirnov Statistic	df	Sig.
ALPase activity at 24 hours	Control	.207	9	.200*
	50	.202	9	.200*
	10	.146	9	.200*
	1	.260	9	.080*
	0.1	.193	9	.200*
ALPase activity at 72 hours	Control	.271	9	.056*
	50	.174	9	.200*
	10	.255	9	.094*
	1	.199	9	.200*
	0.1	.191	9	.200*

* This is a lower bound of the true significance.

Descriptives

	Concentration of fluocinolone acetonide (µM)	N	Number of viable cells (Mean)	Std. Deviation	95% Confidence Interval	
					Lower Bound	Upper Bound
ALPase activity at 24 hours	Control	9	100.000	2.590	98.009	101.990
	50	9	102.769	2.975	100.482	105.057
	10	9	96.594	3.979	93.535	99.652
	1	9	97.898	4.649	94.324	101.472
	0.1	9	95.090	4.163	91.890	98.290
ALPase activity at 72 hours	Control	9	100.000	2.590	98.009	101.990
	50	9	102.769	2.975	100.482	105.057
	10	9	96.594	3.979	93.535	99.652
	1	9	97.898	4.649	94.324	101.472
	0.1	9	95.090	4.163	91.890	98.290

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ALPase activity at 24 hours	Between Groups	324.870	4	81.218	5.773	.001
	Within Groups	562.758	40	14.069		
	Total	887.628	44			
ALPase activity at 72 hours	Between Groups	8292.690	4	2073.173	128.964	.000
	Within Groups	643.023	40	16.076		
	Total	8935.713	44			

Multiple comparision

Dunnett T3

Dependent Variable	Concentration of fluocinolone acetonide (µM) (I) TX	Concentration of fluocinolone acetonide (µM) (J) TX	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
ALPase activity at 24 hours	Control	50	-2.769	1.314	.364	-6.986	1.446
		10	3.406	1.58260	.347	-1.768	8.580
		1	2.101	1.774	.911	-3.787	7.991
		0.1	4.909	1.634	.084	-.457	10.275
ALPase activity at 72 hours	Control	50	12.248*	1.385	.000	7.674	16.823
		10	23.839*	1.284	.000	19.425	28.253
		1	28.544*	1.799	.000	22.778	34.310
		0.1	39.412*	2.530	.000	30.976	47.849

* The mean difference is significant at the .05 level.