CHAPTER II

Literature review

2.1 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 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 hydroxyproline 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 specific 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 proteinases. 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 67 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; Cho *et al.*, 1992; 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; Cho *et al.*, 1992; 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*.

2.3 Pulp capping material

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 (Cotton, 1974). 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.*, 1980; 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.*, 1982; 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 (Cox and Suzuki, 1994). Moreover, the inflammation or necrosis associated with the presence of dentinal bridge has been reported (Heys *et al.*, 1980; Cox *et al.*, 1985).

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; Schroeder 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 (Schroeder 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 (Clarke, 1971). 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 (Canalis, 1983). 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, dentin 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 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 type I collagen synthesis and *in vitro* calcification in cultured human dental pulp cells.