CHAPTER 2

Review of the Literature

Regenerative endodontics

Regenerative Endodontic Procedures (REPs) have been defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex (27). The goal of REPs is to regenerate the pulp-dentin complex, resulting in the continuation of root development or restoration of functional pulp tissue (27). In 1961, Nygaard-Östby reported a newly formed tissue inside the root canal after intracanal bleeding was stimulated (28). He proposed that evoked-bleeding played an important role as a scaffold to support growth of new tissue in the root canal. Later, investigation of pulpal changes in replanted and autotransplanted immature teeth revealed an ingrowth of a vascularized and cell-rich connective tissue occupying the entire root canal space in the first six months (29). After a 180 day observation period, a newly-formed, atubular, hard tissue was present. Regrettably, discussion of this useful information was limited to the treatments of traumatized teeth and not to treatment of causes other than trauma.

A successful case report by Banchs and Trope (10) inspired and stimulated the possibility of regenerative treatment in dentistry. That study showed that an immature tooth with necrotic pulp regains its vitality together with continued root formation after the treatment. After the publication of that study, regeneration became a topic of interest, especially in endodontics, in which several case reports and scientific studies have been published. Therefore, the term “Regenerative Endodontics” has been proposed and generally used since then.

Treatment of immature permanent teeth with necrotic pulp

Treatment of immature teeth with necrotic pulp presents some difficulties. A standard protocol for root canal treatment is impractical because of the thin and weak

dentinal walls. Root canal obturation in a tooth with a large apical opening is also a challenge, since without an apical termination, the root canal filling material might be extruded into the periapical tissue (Figure 1) (1).

Traditionally, $Ca(OH)_2$ apexification has been suggested for the induction of an apical barrier. After the formation of a calcific barrier at a root terminus, root canal filling materials can be introduced without extrusion. Even though a high success rate has been reported (74-100%) (30), this technique still has some disadvantages (15). For example, multiple visits are required, since calcific barrier formation is unpredictable. Therefore, good patient co-operation is necessary, because several recall sessions are compulsory in order to evaluate the amount of remaining $Ca(OH)_2$ inside the canal and also to assess the formation of a calcific barrier. Moreover, the risk of tooth fracture may be increased, since long-term $Ca(OH)_2$ medication has negative effects on the strength of the tooth dentin (6, 27).

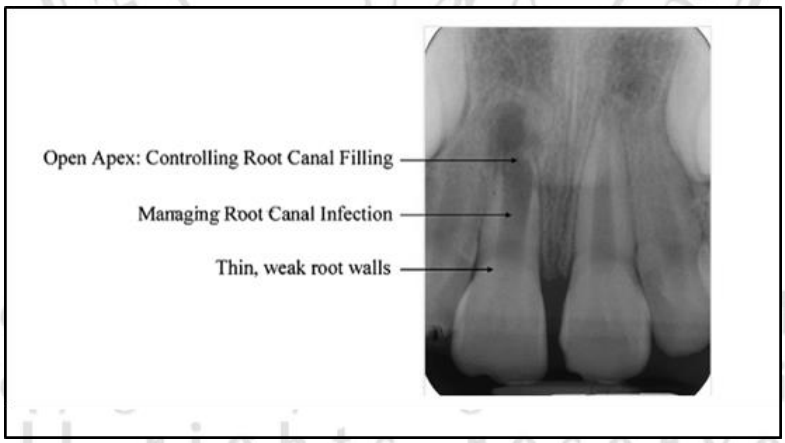

Figure 1 The immature tooth with necrotic pulp presents many problems to root canal treatment (Modified from Trope 2006) (31)

Consequently, one visit apexification using MTA has been introduced. This technique provides an immediate hard tissue barrier by MTA apical filling. Thus, root canal obturation can be performed and a bonded restoration can be immediately applied to prevent these teeth from fracture (1, 2). Many studies have reported that MTA apical barriers revealed more predictable and favorable outcomes than those of conventional $Ca(OH)_2$ methods (5, 32)

Although both $Ca(OH)_2$ and MTA apexification result in apical closure, these procedures are unable to allow root continuation. The tooth is still short and weak (1, 6). Therefore, many clinicians have struggled to develop a new approach to stimulate a continuation of root development.

In 2001, Iwaya and colleagues (8) described the first case report of treatment of the necrotic immature permanent tooth, using a new procedure called revascularization treatment. In that technique, a mixture of metronidazole and ciprofloxacin (double antibiotic paste, DAP) was used as the root canal medication without mechanical cleaning. After a 30-month recall period, root closure with thickening of the root canal wall was observed. Moreover, the treated tooth exhibited a positive response to electric pulp testing. Later, another case report from Banchs and Trope (10) was published. A necrotic immature mandibular second premolar with a large apical lesion was chemically irrigated with 5.25% NaOCl without instrumentation. A triple antibiotic paste (TAP) composed of metronidazole, ciprofloxacin and minocycline was applied into the canal for 26 days. At the second appointment, the medication was removed and an intracanal blood clot was created. MTA was carefully placed over the blood clot and then a permanent restoration was placed two weeks later. After a two-year follow-up, a periapical radiograph showed thickening of the dentinal wall and the closure of the root apex. The tooth also responded positively to cold test. Thenceforward, the protocol described in that case report has become widely used in treating an infected immature tooth with necrotic pulp.

American Association of Endodontists (AAE) clinical considerations for regenerative procedures (33)

To date, many case reports and scientific studies in regenerative endodontics have been published. Therefore, the revascularization protocol has been modified, based on current evidence, in order to improve the treatment outcome. The American Association of Endodontists (AAE) has recently introduced the latest protocol for the revascularization procedure as follows:

First appointment

- An informed consent is signed after an explanation of alternative treatment, risks and benefits.
- After local anesthesia, the tooth is isolated and the access is performed.
- Each root canal is gently irrigated with 20 mL of 1.5% NaOCl for five minutes, followed by 20 mL of saline for five minutes, with the irrigating needle positioned about 1 mm from the root end.
- The canals are dried with paper points, then $Ca(OH)_2$ or a low concentration of triple antibiotic paste is delivered into the canal system.
- The access is sealed with temporary restorative material.

Second appointment

- The second treatment visit is appointed one to four weeks after the first visit.
- A clinical examination is conducted to evaluate the response of the initial treatment.
- The tooth is anesthetized with 3% mepivacaine without vasoconstrictor, rubber dam isolation is placed and the access is opened.
- The antibiotic paste is washed out with 20 mL of 17% EDTA and the canals are dried with paper points.
- Bleeding is created inside the canals by rotating a pre-curved sterile K-file, 2 mm beyond the root apex, in order to fill the entire canal with a blood clot to the level of the cemento-enamel junction.

- After a blood clot has formed, a piece of CollaPlug^{TM} is inserted over the blood clot to support the placement of MTA.

- About 3 mm of white MTA is placed, followed by a permanent restoration.

- The tooth should be followed-up for 12 to 24 months to evaluate increasing root canal wall thickness and root length.

As a result of minimal to no mechanical debridement, chemical debridement and intracanal medication are mentioned to be the crucial steps in REPs to accomplish the root canal disinfection. However, chemical agents used in regenerative procedures should be considered not only in terms of antibacterial properties but also for their ability to promote the survival and proliferation of the patient's stem cells (7).

Intracanal medications used in REPs.

3Mix, a combination of Metronidazole, Ciprofloxacin, and Minocycline, is the most widely used medicament in revascularization procedures (7). Topical application of this antibiotic combination sterilizes the carious and endodontic lesion of deciduous teeth within one day (34). Moreover, it has been reported that a concentration of 25 µg/mL of this combination drug was effective in eliminating bacteria from the infected root dentin, whereas these drugs, used alone, were not sufficient to kill all bacteria (35). Another study revealed that placing the 3Mix in the root canal was efficient in eradicating bacteria in the deep layer of root canal dentin (36). Metronidazole is a nitroimidazole antibiotic medication used particularly against anaerobic bacteria and protozoa (37, 38). The mechanism of metronidazole is to inhibit nucleic acid synthesis by permeating the cell membrane of bacteria, binding to the DNA and then disorganizing its helical structure. The *in vitro* study of Roche and Yoshimori (39) found that metronidazole is very effective in killing anaerobic bacteria isolated from odontogenic abscesses but has no activity against aerobic bacteria. Thus a combination of medicaments should be considered for mixed infections.

Ciprofloxacin, a synthetic fluoroquinolone, is a broad-spectrum antibiotic which has bactericidal effects. It functions by inhibiting the DNA gyrase result in degradation of DNA (37, 40). Ciprofloxacin is very active against gram-negative but less effective for gram-positive bacteria. Most anaerobic bacteria are resistant to ciprofloxacin.

Minocycline, a derivative of tetracycline, is a broad-spectrum bacteriostatic agent (37, 41). It exhibits activity against a wide range of gram-positive and gram-negative microorganisms, and also is effective against mycoplasmas, protozoans, spirochaetes, and many anaerobic and facultative bacteria. Minocycline works by inhibiting bacterial protein synthesis on ribosomal surfaces.

Approximately 51% of published regenerative endodontic cases have used this antibiotic mixture as an intracanal medicament (Figure 2) (7). Even though favorable outcomes have been consistently reported when 3Mix was used (9, 10, 21), some studies have mentioned that the results were unpredictable (11, 12).Recent studies have reported negative effects of 3Mix on cells involved in dental regeneration, for example dental pulp cells (DPC) and SCAPs. Ruparel *et al* (20) found that 3Mix had a detrimental effect on the survival of SCAPs in a concentration-dependent manner. A clinical manipulation of 3Mix (1,000 mg/mL) resulted in total cell death whereas lower concentrations (0.1 and 0.01 mg/mL) had no effect on cell viability. As a result of that study, 3Mix at 0.1 mg/mL has been recommended by the AAE to be used in the clinical revascularization procedure (33). Another similar study by Chuensombat *et al* (18) has reported that 3Mix at high concentrations had undesirable effects on human DPC and APC viability. Also, that study proposed that a concentration of 0.39 µg/mL was the best candidate for use because it caused less cytotoxicity than did higher concentrations, while its antibacterial efficacy was still maintained. This concentration has further been investigated regarding its effect on the regenerative capacity of APCs. The study revealed that APCs after exposure to 3Mix at 0.39 µg/mL for seven days showed no effects on their dentinogenic differentiation potential (19).

Currently, even though the use of 3Mix in REPs is generally accepted, $Ca(OH)_2$ has also been mentioned as an effective medicament for revascularization as published in 37% of regenerative endodontic cases (Figure 2) (7). Cehreli *et al* (42) presented six successful cases after treatment using $Ca(OH)_2$ in the coronal third of the root canal of immature teeth with necrotic pulp. On the basis of clinical and radiographic evaluation, all cases demonstrated favorable outcomes since complete periapical healing and continued root development was observed after a follow-up period of 10 months. Moreover, a positive response to cold test was shown in two cases. Later, favorable results were also reported in a 20-tooth case series by Chen *et al* (43). Therefore, $Ca(OH)_2$ has

now been a medication of interest for regenerative endodontics since 3Mix showed some deleterious effects. However, the effects of $Ca(OH)_2$ on regenerative endodontics have not been extensively studied. One recent study by Ruparel *et al* reported that $Ca(OH)_2$, at concentrations of 0.01-100 mg/mL, was non-toxic to SCAPs, and appeared to promote cell proliferation (Figure 3) (20). Based on their findings, they have proposed that $Ca(OH)_2$ might be the first drug of choice for regenerative endodontic procedures.

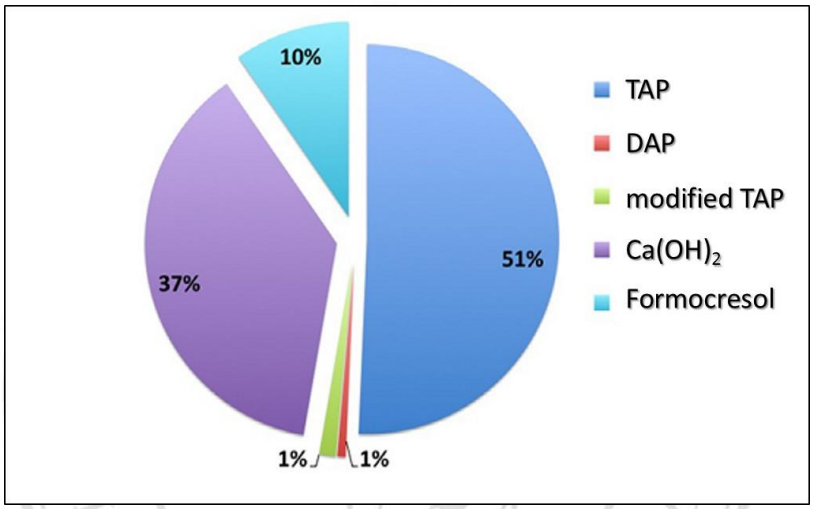

Figure 2 Frequency of use of different intracanal medicaments in regenerative endodontic published cases (modified from Diogenes *et al*., 2013) (7).

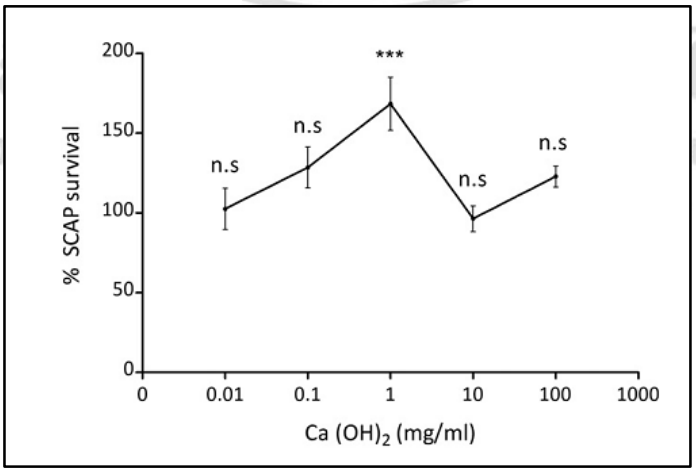

Figure 3 The effect of $Ca(OH)_2$ in a 10-fold serial dilution on survival of SCAPs (modified from Ruparel *et al*., 2012) (20).

Recently, the effect of medicaments used in regenerative endodontic procedures on the chemical structure of human immature radicular dentin was investigated by Yassen *et al* (44). The relative loss of organic and inorganic components (phosphate/ amide I ratios) and SEM examination of the radicular dentin treated with intracanal medicaments ($Ca(OH)_2$, TAP, and DAP) were measured. They reported that the highly alkaline property of $Ca(OH)_2$ caused degradation of dentinal collagen, whereas the acidity of antibiotic paste, especially TAP, induced dentin demineralization and formation of a collagen-rich matrix on the dentin surfaces (Figure 4). They assumed that the exposed collagen matrix on the dentin surface treated with antibiotic paste might play a significant role in pulp regeneration by enhancing stem cell attachment and growth. However, no additional investigations have been reported regarding cell growth and attachment on the differently treated dentin surfaces.

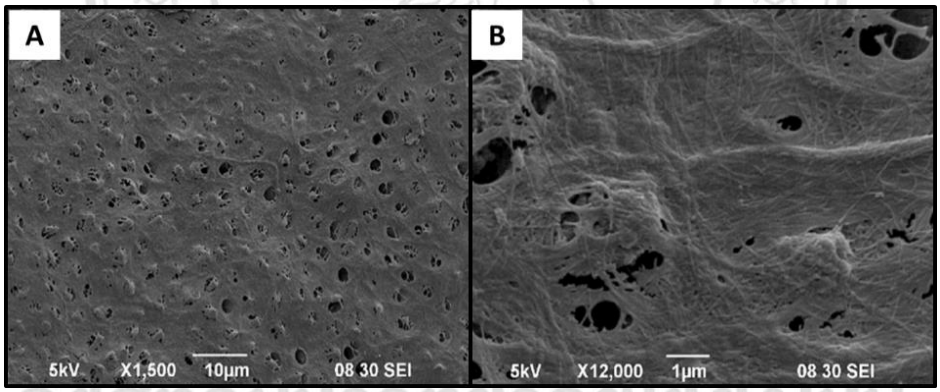

Figure 4 SEM examination of a TAP-treated radicular dentin surface at x 1,500 magnification (A), and x 12,000 magnification (B). Strong demineralization with intensive collagen exposure was observed (modified from Yassen *et al*., 2013) (44).

Cells in regenerative endodontics

One of the expected outcomes after revascularization in an immature tooth with necrotic pulp is the continuation of root development. To date, evidence supporting the mechanism of root development after the revascularization procedure is still limited. It is possible that surviving cells in the apical papilla play some important roles in root continuation (16). The apical papilla is a vascularized connective tissue attached to the developing root apex (Figure 5). Histological observation has revealed that this tissue is located apically to the epithelial diaphragm containing an apical-cell rich zone between the pulp and the apical papilla (Figure 6) (45). A recent study has shown that mesenchymal stem cells, called stem cells from the apical papilla (SCAPs), have been found inside this tissue (46).

Since the discovery of stem cells in the apical papilla, the role of these cells in apexogenesis and regeneration of immature teeth with necrotic pulp and apical periodontitis has been discussed (16, 45). SCAPs have some characteristics which are distinct from those of DPSCs. SCAPs present significantly greater amounts of population doubling, significantly greater numbers of positive cells to a mesechymal stem cell marker (STRO-1), and significantly greater dentin regeneration capacity than with DPSCs (46). Moreover, it has been reported that developing apical complex cells display higher proliferation and mineralization potential than dental pulp cells in culture (45).

In the developing tooth, it has been hypothesized that pulpal infection might pass through the root apex while allowing the survival of dental pulp cells and apical papilla tissue (Figure 7) (16). It is possible that the evoked-bleeding step in the revascularization procedure provides an abundant influx of survived SCAPs into the root canal allowing regeneration of the tooth root (7).

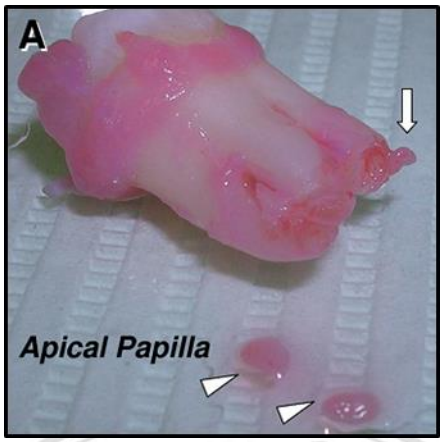

Figure 5 An extracted human immature third molar with two pieces of separated apical papilla (arrow heads) and one piece that is incompletely detached from the root apex (arrow) (modified from Huang *et al*., 2008) (16).

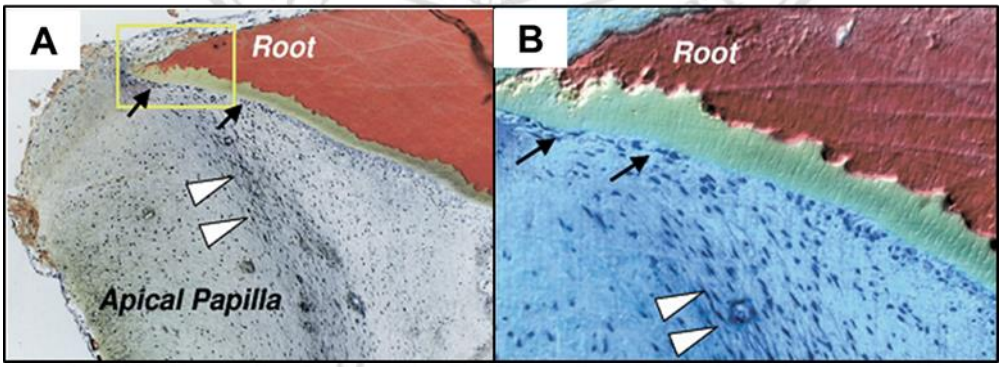

Figure 6 A hematoxylin and eosin staining of human developing root apex with attached apical papilla. (A) Odontoblasts (black arrows), an apical cell-rich zone (arrow heads), and apical papilla tissue are presented. (B) Magnified view of the area presented in the yellow rectangle (modified from Huang et al., 2008) (16).

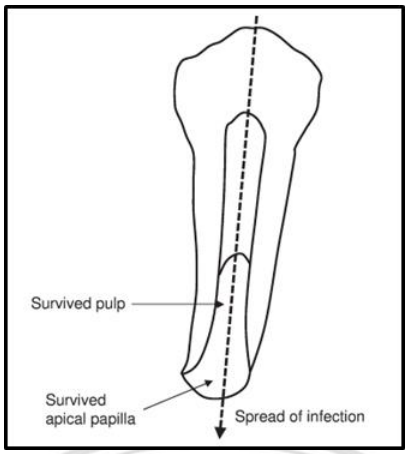

Figure 7 The possible pathway of infection of immature permanent teeth (modified from Huang et al., 2008) (16).

Studies on cell attachment related to REPs

Root canal wall thickening is an essential event to complete the root development. The cells and pathways related to this process are unclear. Ideally, undifferentiated mesenchymal cells would attach and differentiate into dentin-forming cells and form dentin on the root surfaces, similar to the general reparative process of dental pulp. Cell attachment is a crucial step in cell growth, cell division, and cell survival, since many types of normal animal cells, such as fibroblasts and epithelial cells, are anchorage-dependence (47). In order to achieve pulp-dentin complex regeneration, the cells that migrate into the root canal should attach on the root dentin surface. Therefore, any chemical reagent used in regenerative endodontic procedures must not cause undesirable effects on the root dentin surface or on attaching cells. Many techniques, such as LIVE/DEAD fluorescence staining, SEM, and also the expression level of adhesion protein, have been performed to investigate the cellular attachment to root dentin surfaces (23-25). Galler *et al* (48) reported that dentin conditioning influenced cellular behavior at the cell-dentin interface. In their study DPSCs were seeded on two groups of pretreated dentin cylinders (NaOCl or NaOCl followed by EDTA) and transplanted subcutaneously into immunocompromised mice. The histological observations presented an intimate association of DPSCs with the dentin surface in the EDTA-conditioned group. The cells that were in contact with the dentin had differentiated into odontoblast-like cells, expressing dentin sialoprotein, and extended cellular processes into the dentinal tubules. In contrast, resorptive lacunae with multinucleated cells were found at the cell-dentin

interface in the NaOCl-treated group. So, the study concluded that preconditioning the dentin with NaOCl and EDTA promotes DPSC attachment and differentiation. Another interesting study has also investigated the effect of surface wettability of root canal dentin, treated either with MTAD or 17% EDTA, on cell attachment. The study revealed that both irrigation steps did improve dentin surface wettability and, then, promoted cell attachment (Figures 8 and 9) (23).

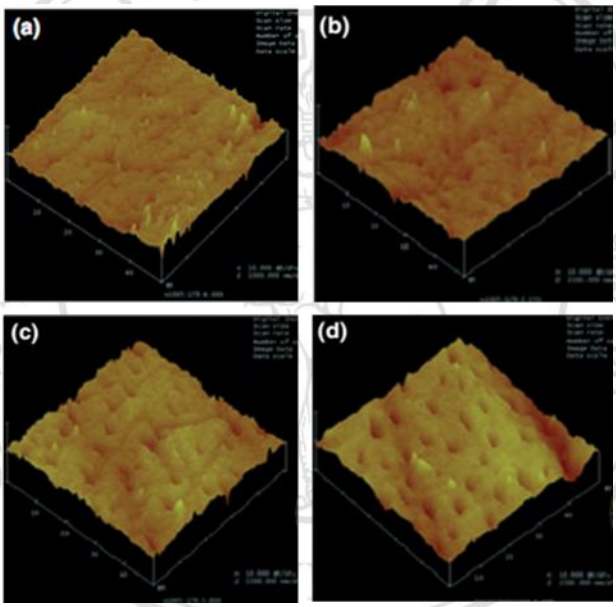

Figure 8 Topography images of dentin surface (50 µm x 50 µm). (a) H_2O irrigated dentin, all dentinal tubules are blocked. (b) 5.25% NaOCl irrigated dentin, dentinal tubules are partially open. (c) 17% EDTA irrigated dentin, exposed dentinal tubules with relatively clean surface are shown. (d) MTAD irrigated dentin, wide open dentinal tubules with clean surface are seen (modified from Huang *et al*., 2012) (23).

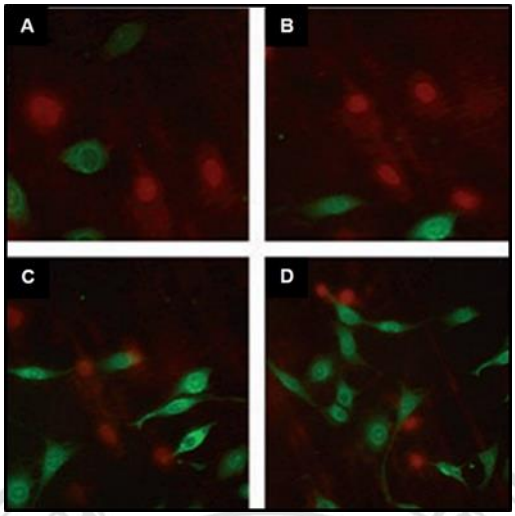

Figure 9 LIVE/ DEAD fluorescence staining images of cells attached to irrigated dentin surfaces. The live cells exhibit green fluorescence, and the nuclei of dead cells present red fluorescence. (A) H_2O irrigated dentin, (B) 5.25% NaOCl irrigated dentin, (C) 17% EDTA irrigated dentin, and (D) MTAD irrigated dentin (modified from Huang *et al*., 2012) (23).

From those and other current studies, it can be implied that EDTA should be used in regenerative endodontic treatment, including the revascularization procedure, because EDTA can positively influence initial cell attachment to the root dentin (23, 26, 48). Recently the effects of EDTA on the revascularization procedure were investigated. Pang and colleagues have studied DPSC attachment and differentiation (24). In their study, DPSCs were seeded on EDTA-treated and untreated dentin slices for three days, then cell attachment was evaluated by cell density, fibronectin 1 gene expression level using quantitative real-time polymerase chain reaction (qRT-PCR), and SEM. Moreover, the expression of dentinogenic and osteogenic differentiation genes were investigated after 21-day culture in differentiation medium. After three days in culture, both cell density and fibronectin 1 expression level were significantly higher in the EDTA-treated group (Figure 10). For the SEM examination, DPSCs in the EDTA-treated group were attached and stretched over the dentinal tubules with the longer cytoplasmic process containing many granules (Figure 11).

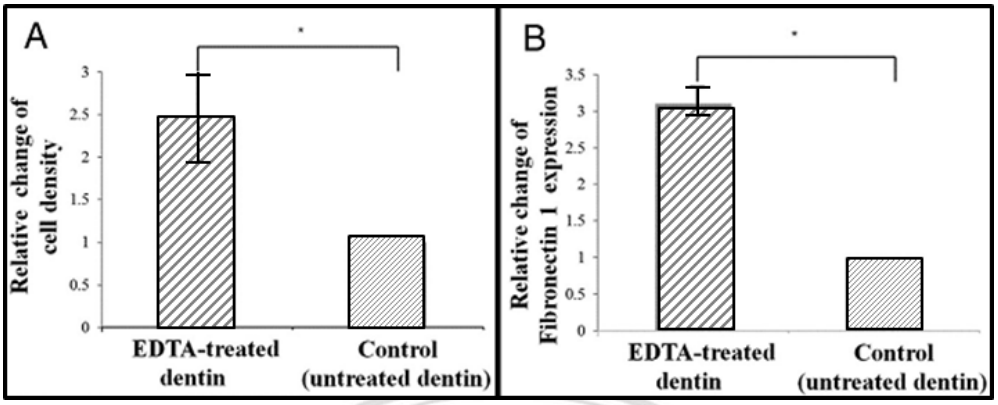

Figure 10 Cell density (A) and fibronectin 1 expression level (B) in the EDTA-treated and untreated dentin slices (modified from Pang *et al*., 2014) (24).

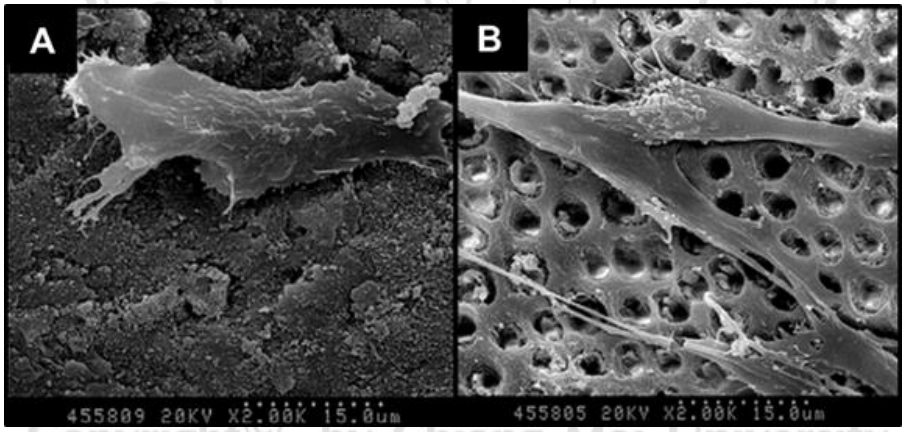

Figure 11 SEM views of untreated (A) and EDTA treated (B) dentin surfaces. All dentinal tubules in the untreated dentin group were blocked, whereas a clean surface with wide-open dentinal tubules were observed in the EDTA-treated group (modified from Pang *et al*., 2014) (24).

Fibronectin is the high molecular weight glycoprotein that has been associated with a variety of cell functions, including adhesion, growth, morphology, migration, differentiation, and hemostasis . This adhesion molecule is synthesized by a wide variety of cells, especially fibroblasts and endothelial cells (49). Fibronectin can be found abundantly in the extracellular matrix (ECM) of embryonic and regenerating or injured tissues, and also in most ECMs. Fibronectin interacts with cell via integrins, the heteromeric transmembrane receptors linking the ECM to the intracellular cytoskeleton (50, 51) (Figure 12). Fibronectin is considered to be a mediator of cell and extracellular matrix adhesion. Moreover, this adhesion determines the polarization and migration of odontoblasts (52). One study has reported that the up-regulation of fibronectin was related to increased cell attachment (24).

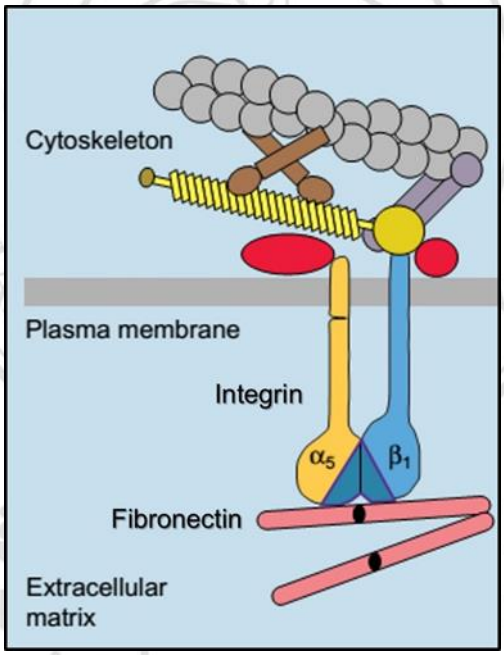

Figure 12 Transmembrane connection between ECM and intracellular cytoskeleton. The extracellular domains of integrin bind to the specific site of fibronectin in the ECM and the cytoplasmic domains bind to submembranous cytoskeletal proteins (modified from Hynes, 1999) (50)

The goal of regenerative endodontic treatment is to regenerate the pulp- dentin complex; the survived cells that migrate into the root canal space should attach to the root canal dentin surface and differentiate to odontoblast-like cells. Therefore, the attachment ability of cells to root dentin surfaces is necessary for this procedure. However, some chemical agents used in regenerative endodontic procedures seem to have an effect on root dentin surfaces that might impact on the attachment ability of cells to the dentin surfaces. Various studies have focused on the effects of irrigants on cell attachment. However, in a clinical situation, disinfection steps of revascularization procedures include both medications and irrigations. Moreover, the study of $Ca(OH)_2$, which has recently been recommended in revascularization, is still limited. Therefore, in this study, the effects of either 3Mix or $Ca(OH)_2$ (at various concentrations) on cell attachment to root canal dentin was investigated.