CHAPTER 2

Literature Review

2.1 Regenerative endodontic procedures

Regenerative endodontic procedures are biologically based procedures, designed to repair/replace damaged dental structures, including the pulp-dentin complex (21). Several case studies have reported successful clinical outcomes in which teeth that have undergone revitalization show continuation of root development (22). Therefore, REPs have been accepted as alternative approaches for necrotic permanent teeth with immature root development. However, some recent histological data have revealed that the regenerated tissues of the treated teeth are not dentin and pulp, but rather cementum and bone (3). In general, REPs comprise two treatment steps. Initially, infection is controlled by means of irrigations and medications, using either triple antibiotic mixture or CH (1, 23). After a period of time, the regenerative step is accomplished by induction of a blood clot to the root canal from the apical region of the tooth. SCAPs, within the induced blood clot, are suspected of acting as modulators of healing processes (6). However, scientific evidence is still lacking in order to clearly explain the consequences of REPs. Therefore, various aspects, for example the effects of recruited cells and of the chemical agents used, need to be further examined.

Due to the principle of tissue engineering, stem cells and signaling molecules directly affect tissue generation. Stem cells have been identified in various dental structures, including dental pulp, apical papilla tissue, periodontal ligament, etc. (5, 24, 25). SCAPs have been suggested as playing roles in regenerative endodontics, since they are situated around the root and could survive after infection (26). A previous study has also confirmed the presence of stem cells in blood after apical tissue was intentionally

stimulated with instruments (6). Consequently, it is valuable to understand these stem cells in regenerative endodontic purposes. Furthermore, stem cell behavior is determined by appropriate growth factors and other mediators (27). The molecules within the environment are important to determine stem cells differentiation. In dentin, a variety of growth factors are sequestrated. These active molecules play roles in the self- protecting process of the dental pulp and also exert some effects on odontoblastic differentiation (28-31). Therefore, the application of these bioactive molecules in regenerative treatment is of interest.

A significant challenge in clinical regenerative procedures is the development of protocols to promote sufficient disinfection while establishing a suitable root canal environment for stem cell proliferation and differentiation. Knowing the bioactive properties of dentin enables the development of methods that can expose the sequestrated growth factors. Local release of the growth factors as a result of dentin conditioning procedures, such as instrumentation or irrigation, or after using certain agents and materials, improves the predictability of pulp-dentin generation (32).

2.2 Stem cells in apical papilla

Apical papilla tissue is located at the root ends of developing teeth. Histologically, it is a connective tissue underlying the dental pulp tissue. Between the two tissues, there is a band of dense cellular component termed the “apical cell-rich zone”. The vascular and cellular density of the apical papilla appears lower than those of dental pulp (33). Many types of cells can be found in papilla tissue, for example fibroblasts, endothelial cells and undifferentiated mesenchymal cells (34). Current evidence has established the presence of stem cells in this tissue. These are categorized as SCAPs (5). Cell marker analysis has confirmed that SCAPs exhibit expression profiles, such as STRO-1, CD73, and CD90, exactly similar to those of typical mesenchymal cells (34, 35). SCAPs also have a high proliferation rate and a self-renewal property, confirmed by high positive markers for cell proliferation, high rate of population doubling time, and high bromodeoxyuridine (BrdU) uptake (33). More importantly, SCAPs are of interest as they can differentiate into odontoblast-like cells being able to secrete dentin when implanted into animal models (35).

In relation to tooth development, the role of the apical papilla has been confirmed in a minipig model (33). Removal of the apical papilla results in cessation of root development, even though the pulp tissue is intact. On the other hand, when apical papilla is intact, the normal processes of root growth and development continue. Huang et al (26) suggested that apexogenesis can occur in infected immature teeth with apical periodontitis or abscess because stem cells in apical papilla can survive after infection. Thus, evidence suggests that SCAPs would be outstanding candidates for tissue regeneration (5, 26, 35).

In a routine revascularization procedure, the apical papilla tissue is intentionally lacerated in the regeneration step after disinfection. The influx of stem cells from apical tissue has been elucidated by Lovelace et al (6), who examined the content of blood provoked into root canals from the periapical tissues. The mesenchymal stem cell population within provoked intracanal blood, confirmed by positive CD73 and CD105 stem cell markers, contains a 400- to 600-fold greater concentration of cells than does systemic blood. Cherpa et al further showed that human apical papilla tissues have the ability to maintain stem cell viability and stemness, in spite of the presence of inflammation (7). In that study, the inflamed apical papilla tissue collected from immature teeth with apical periodontitis exhibited mesenchymal stem cell markers, CD73, CD90, and CD105, which are comparable to those of normal SCAPs. Therefore, it is appropriate to investigate the roles of cells together with the proper environment in order to promote ideal regeneration in regenerative endodontics.

2.3 Dentin as a bioactive extracellular matrix

Dentin has been considered as an inert structure of mineralized collagenous matrix. In fact, dentin contains a variety of bioactive molecules playing roles in pulpal protection and repairing processes (36, 37). These bioactive components include non-collagenous proteins, growth factors and cytokines, neuropeptides, and plasma proteins (37). Among those molecules, growth factors play important roles in dentin-pulp regeneration. These bioactive growth factors include the TGF-β superfamily, insulin-like growth factors-1 and -2 (IGF-1 and -2), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (38-40). TGF-β is one of the

essential molecules found in dentin responsible for immune responses, tissue repairing processes, and odontoblast-like cell differentiation (30, 41-43). TGF-β can be locally solubilized, mostly by dental caries, activating the reactionary dentin formation (44). Several studies have investigated the effect of TGF-β1 on cellular responses and reported that: it is a potent chemo-attractant for DPSC migration (45-47); it induces type I collagen (46, 48); and, interestingly, it promotes dentinogenesis (48, 49). In conclusion, such evidence clearly indicates that TGF-β1 can upregulate dentinogenic activity. In 2015, Galler and colleagues determined the influence of root canal disinfectants on growth factor release (13). They evaluated the TGF-β1 level after conditioning dentin with demineralizing solutions and reported that 10% EDTA at pH 7 is the most effective reagent. FGF-2 and VEGF were also released but in much lower concentrations than those of TGF-β1. In addition, they also evaluated the effects of different disinfectants and medicaments on the TGF-β1 release have been also evaluated. They measured the TGF-β1 level after dentin treatment with different irrigants and showed that immersion in 0.12% CHX for five minutes followed by 10% EDTA pH 7 for 20 minutes results in the highest release, whereas a longer CHX treatment time (10 minutes) diminishes the release. In contrast, a prior rinse with 5.25% sodium hypochlorite (NaOCl) causes a lower release of TGF-β1. Therefore, they suggest that the acidic pH of CHX has a beneficial chelating effect, but that prolonged use may interfere with the growth factor release because of its acidity and hard tissue binding ability. On the other hand, NaOCl supresses growth factor release. Among medicament groups, water-based CH does not suppress growth factor release, whereas other medicaments, including corticoid-antibiotic paste, oil-based CH, triple antibiotic paste (TAP), and CHX gel, demonstrate significant reduction of TGF-β1 level compared with the effect of water-based CH. The suppression of growth factor release may be the result of difficulty in medicament removal. TAP, oil-based medicament, and CHX gel cannot be efficiently removed because of their effective dentin affinity. A more recent study has also proved that several growth factors, one of which is TGF-β1, are moderately released after irrigation with either EDTA or NaOCl followed by EDTA (50). These irrigation methods have been tested in root segment models; besides, it has been found that the growth factors released from the root segments are functional as they induce dental pulp stem cell migration (50). In fact, it has been

proposed that the local expression of these molecules may play an important role in clinical regenerative procedures (51).

2.4 Effects of dentin conditioning procedures on stem cells

In regenerative endodontics, it is emphasized that infection control must be achieved before engaging the regenerative processes. Furthermore, to promote root canal dentin favorable for stem cells, suitable chemical agents should be selected. Thus, several studies have focused on dentin conditioning regimens influencing cellular activities, including irrigation and medication.

In 2011, Galler et al demonstrated stem cell behavior after dentin cyllinders containing progenitor cells were subcutaneously implanted into animal models (9). In that study, dentin cylinders were pre-treated either with 5.25% NaOCl for 10 minutes or with 5.25% NaOCl followed by 17% EDTA for two minutes as a final rinse. After six weeks of implantation, in the dentin treated with NaOCl, multinucleated cells were observed on the cell-dentin interface with resorption lacunae reflecting a dentinoclastic phenotype. On the other hand, in the dentin treated with EDTA, the cells contacting the dentin surface showed a pulp-like structure expressing dentin sialoprotein. The authors supported the idea that EDTA is a suitable dentin conditioner in REPs. More recently, the benefits of EDTA on stem cell attachment and differentiation have been investigated by Pang et al in 2013 (52). In their study EDTA-treated dentin demonstrated significantly greater cell attachment potential, including cell density and fibronectin gene expression, than did untreated dentin. Moreover, the EDTA-treated dentin also showed a higher expression of odontoblastic markers, DSPP and dentin matrix protein 1 (DMP-1). However, the authors noticed that the differentiation effect occurred only when the cells directly contacted the EDTA-treated dentin surface, implying that the local expression of bioactive molecules in dentin extracellular matrix is important in inducing stem cell differentiation. Huang et al have reported that the surface wettability of dentin is increased when the dentin is treated with 17% EDTA, or with a mixture of Doxycycline, citric acid and a detergent (MTAD) (10). Human dental pulp cells grown on MTAD-treated dentin surface exhibit the greatest viability followed by those treated with 17% EDTA. In contrast, NaOCl- and water-treated dentin exhibit the lowest number of viable cells. The benefits of the use of

EDTA as a dentin conditioner are conclusive, since it improves the physical and biochemical properties of dentin. Considering the desirable effects of dentin conditioning with EDTA on dental pulp cell adhesion and differentiation, the guidelines for clinical regenerative endodontic procedures of the American Association of Endodontists (AAE) suggest that EDTA should be used as a final irrigant before blood clot provocation in the regenerative step of the revascularization procedure (53).

Practically, common intracanal medicaments in regenerative endodontic procedures are TAP, double antibiotic paste (DAP), and CH (22). In regenerative endodontics, pragmatic concentrations of various medicaments should not only be able to eradicate the infection but also to be compatible with stem cells. A recent study investigated an appropriate antibiotic concentration that does not negatively interfere with stem cells viability (54). The authors used various concentrations of TAP and DAP (0.125, 0.25, 0.5, 1, and 10 mg/mL) to test their antibacterial efficacy against *E. faecalis* biofilm, and to test their cytotoxicity and their effect on the viability of stem cells. They reported that all dilutions provided the optimum antimicrobial effect, but in the viability and toxicity tests, 0.125 mg/mL of both TAP and DAP were the only concentrations that showed no deleterious effects on stem cells. Moreover, it is important that dentin after medication may affect stem cell attachment or even differentiation, so several studies have investigated these effects. Althumairy et al in 2014 conducted an experiment by conditioning dentin specimens with antibiotic combinations (TAP and DAP), or CH and determined the effects on cell viability (8). The results demonstrated that no viable cells were observed in specimens treated with either antibiotic at 1,000 mg/mL (a clinical-mixed concentration), whereas 1 mg/mL concentration of TAP and DAP exhibited no significant adverse effects. On the other hand, CH-treated dentin promoted stem cell survival and proliferation. It is important that, in REPs, treated dentin surface must enhance both stem cell survival and attachment. Kitikuson and Srisuwan in 2016 evaluated attachment ability of apical papilla cells on dentin samples treated with various concentrations of TAP or CH. They found that clinical-mixed TAP depressed cell attachment, whereas concentrations of 0.39 µg/mL and 0.1 mg/mL did not significantly show negative effects on cell attachment. Interestingly, both CH concentrations (1 and 1,000 mg/mL) demonstrated increased attachment ability (12).

To determine cell attachment ability, previous studies have evaluated either FN-positive cell count using immunofluorescence (Figure 2.1) (12) or FN-related mRNA (11). In fact, fibronectin is key adhesion gycloprotein molecule and is found in dental pulp and predentin at different stages of dentinogenesis (55).

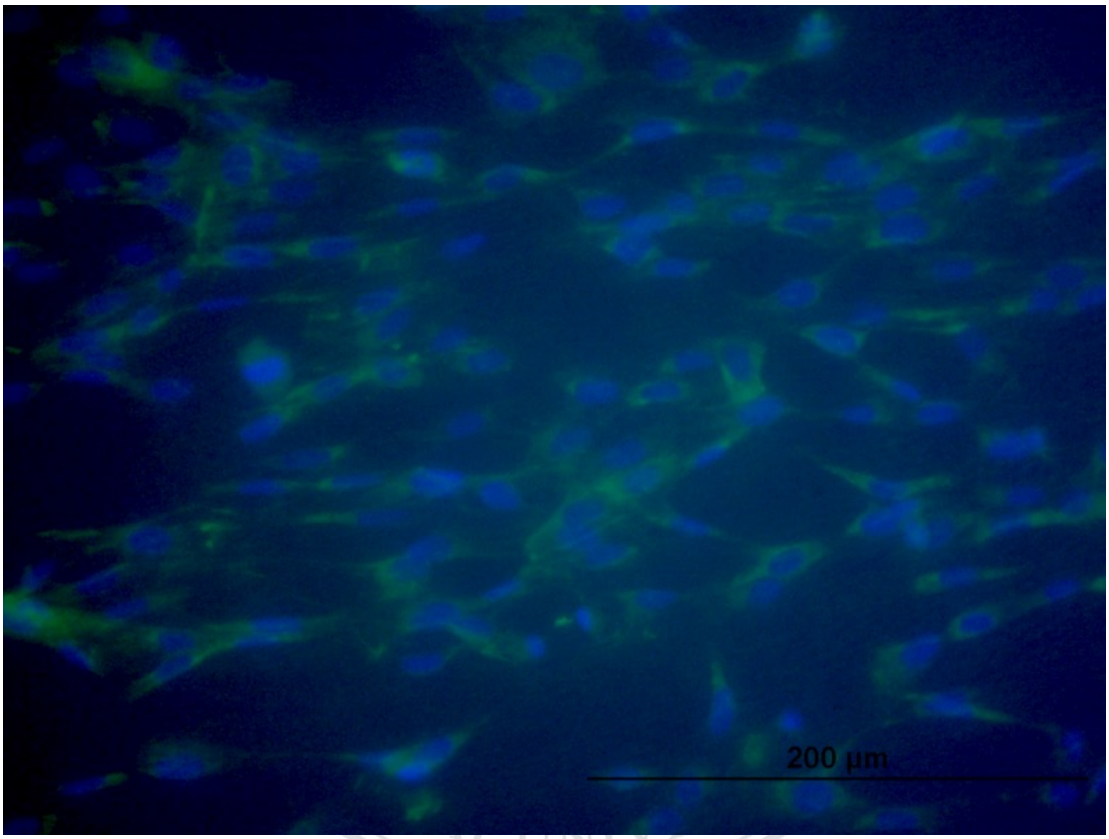

Figure 2.1 An example of immunofluorescent image illustrating fibronectin positive cells. Nuclear marker (DAPI) is visualized in *blue*, whereas expression of fibronectin is in *green*

From those pieces of evidence, it would be beneficial if effective regenerative treatment protocols could be developed in respect to the stimulation of self-bioactive resources in dentin. To date, several factors, including applications of various chemical agents, have been initially investigated. Therefore, it is of interest to investigate other possible methods to improve the regenerative technique.

2.5 Ultrasonically and sonically activated irrigation

During conventional irrigation (needle irrigation), an irrigant is simply delivered and flows within the canal space. Sonic and ultrasonic irrigation provide an additional dynamic mechanism when the irrigant is being activated. In fact, a variety of dynamic irrigation techniques has been developed, but the techniques which are generally mentioned are sonic and ultrasonic irrigations (14). The use of sonic or ultrasonic irrigation techniques provides effectiveness and efficiency of root canal debridement (19). Moreover, between these two techniques, a major difference is the distinct frequency used in each method to agitate an irrigating solution. Ultrasonic irrigation has higher frequencies and lower amplitudes than does sonic (56). Ultrasonic energy was introduced to endodontics by Richman (57). The ultrasonic frequency used to prepare and debride root canals is 25 to 30 kHz. After a period of time, the term "passive ultrasonic irrigation" (PUI) was introduced by Waller in 1980 (58). This term refers to a non-cutting action during treatment, since the activated file must be placed without contacting the canal wall. The effectiveness of this technique in terms of root canal cleanliness has been supported by several studies (59-62). Malki et al, in 2012, examined the influence of file insertion level on root canal wall cleaning, and suggested that ultrasonically activated instrument insertion within 3 mm in front of the file tip is essential to clean the root canal (63). The visualization of irrigant flow also has confirmed the association between the flow and cleaning outcome (3 mm beyond the file's tip).

Another dynamic irrigation technique is sonic irrigation. The EndoActivator System is a sonic activated irrigation device whose working frequency is at 160 to 190 Hz. Movement of a flexible polymer tip at 10,000 cycles per minute has been shown to promote effective debridement and smear layer removal (64). However, a difference in tip size does not impact cleaning efficacy of the root canal wall (16).

2.5.1 Results of sonically and ultrasonically activated irrigation. Activated irrigation results in two hydrodynamic phenomena: (i) acoustic microstreaming and (ii) cavitation. Acoustic microstreaming occurs after a file is oscillated in the presence of fluid, resulting in enhancement of irrigant distribution, creating a shear stress on root canal walls (14). The patterns of nodes and antinodes can be observed, in a translucent

cast, along the activated files (Figure 2.2). A previous study has shown that acoustic microstreaming is more intense in the presence of the following conditions: a greater displacement amplitude of the file, a thinner file, and a higher frequency. Besides, a higher streaming velocity has been observed around the apical region of files, due to the smaller file diameter in the tapered region of the file, and the maximum file displacement in that region (65). Nevertheless, the flow is more rapid in ultrasonic than in sonic irrigation, because the amplitude of the sonically activated tips is greater than the root canal diameter (a root canal is usually as small as 0.5 mm); thus, the greater chance of file-to-canal wall contact inhibits the effective oscillation and then reduces streaming velocity (16).

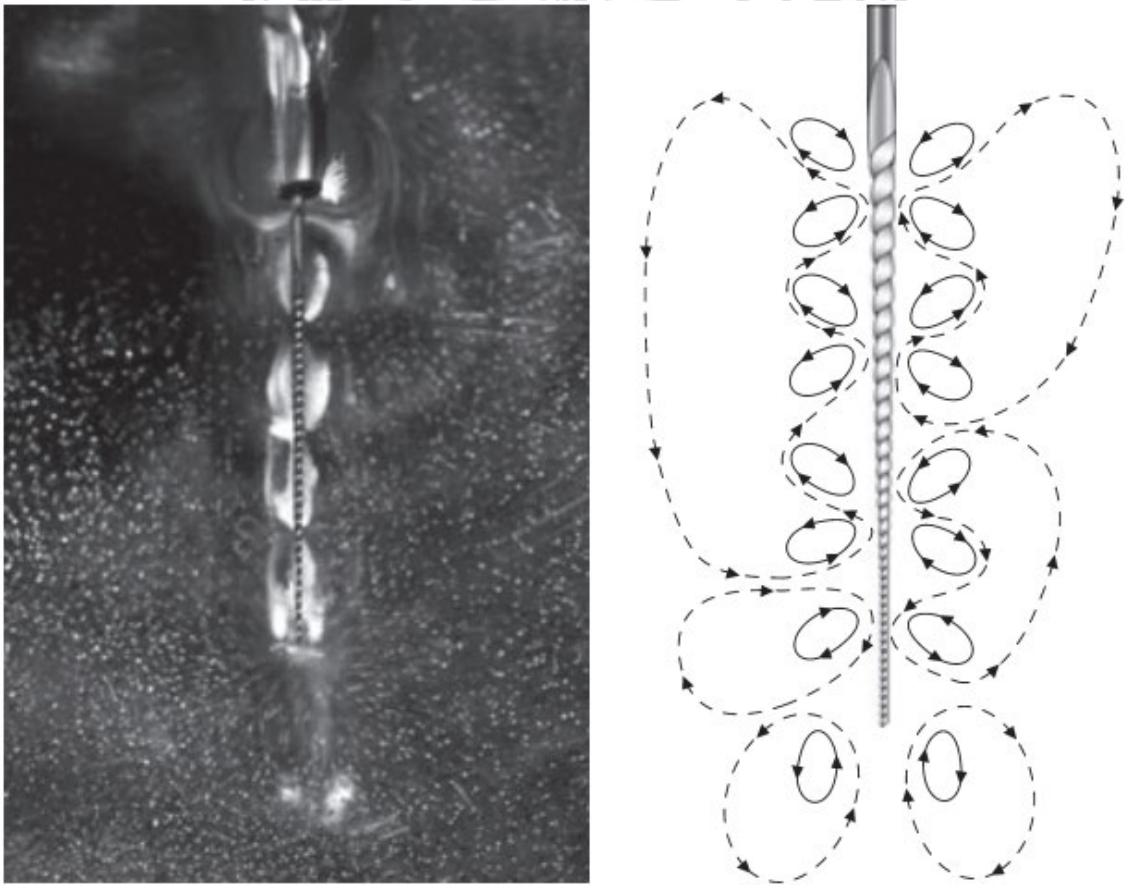

Figure 2.2 Photographic appearance and schematic diagram of PUI

Photographic appearance (left) and schematic diagram (right) of acoustic streaming around the file (14).

Cavitation is the other effect after dynamic irrigation. It has been described by van der Sluis et al as “the impulsive formation of cavities in a liquid through tensile forces induced by high-speed flows or flow gradients” (14). Forceful energy form the cavitation within a solution creates sound and damage (14). There are two types of cavitation classified by Roy et al: stable and transient cavitation (66). Stable cavitation is a linear pulsation of gas bubbles in a low amplitude ultrasonic field, whereas transient cavitation is produced when acoustic streaming is high enough to collapse the bubbles, resulting in radiating shock waves, high gas pressures, and temperatures. Transient cavitation is found appearing all along the ultrasonic file, including at the apical end. When the vibrating file touches the canal wall, the effect is reduced (66). However, several studies have reported that cavitation is not present in sonic activation, even when the highest frequency is applied with various sizes of the sonic tips (67).

2.5.2 Clinical applications of sonically and ultrasonically activated irrigation. Dynamic irrigations have long been utilized in clinical endodontic practice for various purposes, e.g., improvement of antibacterial effect, removal of medicaments, and elimination of the smear layer. Thus, various studies have confirmed the effectiveness of solution penetration into inaccessible areas when activated irrigation systems, either sonic or ultrasonic, were applied (68, 69). Medicament removal is another beneficial feature of both dynamic irrigation systems. The removal of modified triple antibiotic paste (mTAP) using activated irrigation techniques has been studied and it has been reported that Self-Adjusting File (SAF; ReDent-Nova, Ra’anana, Israel), EndoVac (EV; Discus Dental, Culver City, CA, USA), EA, and PUI significantly improve the removal of mTAP from the root canal wall compared with the conventional syringe technique (70). Another similar study has also reported that PUI with 1% NaOCI showed effective removal of TAP (71). The use of irrigant activation methods for removing antibiotic paste from the root canal have been, therefore, advocated. However, none of the techniques is able to completely remove antibiotic medication (18). In addition, calcium hydroxide is more effectively removed after irrigation using the PUI technique (96.8%) compared with using the irrigation needle alone (87.4%) (18). It has also been reported that sonic and ultrasonic irrigation are more effective than rotary file agitation and the conventional irrigation technique in medicament removal (72).

Dynamic irrigation methods are able to remove smear layer and debris effectively. Caron et al in 2010 examined the effects of different final irrigation regimens and activation techniques on smear layer removal from the root canal (17). They found that activated irrigation (EA and gutta-percha agitation) with 17% EDTA and 3% NaOCl show significantly better smear layer removal than non-activated irrigation at all levels along the working length. Many studies also have reported similar findings confirming that activation techniques are important for effective smear layer removal (73, 74).

Another benefit of ultrasonic irrigation recently reported is that it improves growth factor release from dentin (20). The authors that report found that TGF-β1 can be detected after the dentin is irrigated with EDTA; moreover, this finding is improved by ultrasonic irrigation. Interestingly, PBS can also be used following EDTA, as it is a physiologic irrigant. TGF-β1 can be detected in moderate amounts in PBS after 10-min EDTA needle irrigation followed by 5-min PBS ultrasonic irrigation. Encouraging endogenous growth factor release by means of irrigation techniques seems worth mentioning, since, in clinical regenerative procedures, the expression of those molecules on the dentin surface and the release of such molecules into the scaffold are believed to support the induced progenitor cells from periapical tissues in terms of cell attachment, proliferation, and differentiation.

From the available evidence, it can be summarized that dynamic irrigation techniques exert effects on the root canal surface, resulting in many positive consequences. However, the investigation of these techniques on REPs is still limited. Whether root canal dentin conditioning, as a result of dynamic irrigation, promotes subsequent cellular attachment needs to be elucidated.