CHAPTER 5

DISCUSSION

Regenerative endodontics aims to regenerate functional pulp-like tissue supporting tooth function and development (27), especially in diseased immature teeth. Many types of cells, for example apical papilla cells, survived dental pulp stem cells, etc., have been hypothesized to play roles in regenerative endodontics (16). Therefore, attempts should be made in order to recruit those cells into the empty root canals of diseased immature teeth. Ideally, attached cells would differentiate into dental pulp-like cells, producing mineralized matrix. These steps may be of benefit not only in dentin-pulp generation, but also in the continuation of root development (7, 16, 53, 54). Thus, one of the most interesting initial aspects is to search for the appropriate dentin surfaces to which specific cells could attach before they differentiate. To date, a variety of regenerative procedures (revascularization techniques) have been advocated (1, 7). The Lesion Sterilization and Tissue Repair (LSTR) concept is promising, whereas a standard protocol supported by scientific evidences is still lacking. In order to keep mechanical instrumentation in regenerative procedures to a minimum, root canal irrigation and medication are deemed to be very important. Therefore, properties of these chemical reagents should be considered, especially in their abilities to enhance cell attachment on the dentin surfaces, while still offering little cytotoxicity.

In this study, the effects of medicated root dentin, with either 3Mix or $Ca(OH)_2$, at previously suggested concentrations or in routine preparations, on APC attachment and viability on dentin were investigated using fibronectin immunofluorescence, alamarBlue® assay and SEM. Dentin treated with 3Mix paste showed the lowest amount of viable cell attachment, whereas that treated with $Ca(OH)_2$ had significantly higher cell attachment. Only APCs grown on $Ca(OH)_2$-treated dentin surfaces presented with cellular projections attaching to the dentin.

In this study, three concentrations of 3mix (0.39, 100 µg/mL and paste) and two of $Ca(OH)_2$ (1 mg/mL and 1,000 mg/mL paste) were chosen to treat the dentin slices. 3Mix, a mixture of metronidazole, minocycline and ciprofloxacin, has become widely used, since it has strong anti-bacterial properties, which can eliminate bacteria isolated from endodontic infections (34-36). It has been recognized in regenerative endodontics since Banch and Trope reported success after 3Mix was placed in necrotic immature teeth (10). However, the high cytotoxicity of 3Mix is negatively regarded, (18, 19) and is unfavorable for regenerative endodontics. Recent studies have suggested the use of 3Mix at 0.39 µg/mL (18) and 100 µg/mL (34) for revascularization procedures because these concentrations caused less cytotoxicity. Even though 3Mix paste is still generally used in the routine clinical setting, its high concentration would definitely cause negative effects to the cells in regeneration processes. Interestingly, the benefits of $Ca(OH)_2$ in regenerative endodontics have been recently reported (20). Ruparel and colleagues revealed that $Ca(OH)_2$ had no toxicity to SCAPs and appeared to promote cell proliferation, particularly when used at 1 mg/mL. Thus, the use of $Ca(OH)_2$ has become of interest in regenerative endodontics. Therefore, scientific information encouraging the use of this medicament should be established.

Cell attachment is a crucial step in cell growth, cell division, and cell survival, since many types of normal animal cells are anchorage-dependent (47). Various techniques, for example cell counting and fibronectin staining, can be used to investigate the attachment of cells to the surfaces. Fibronectin, a high molecular weight glycoprotein associated with a variety of cell functions (49), is one of the most common mediators for cell and ECM (52). A recent study reported higher fibronectin expression in dental pulp stem cells after preparing dentin surfaces with EDTA (24). Moreover, fibronectin is also considered to function in the terminal differentiation of odontoblast (55, 56). Dental pulp cells cultured on fibronectin-coated surfaces had phenotypes of mineralized tissue-forming cells (57).

Various types of cells have been reported as having a role in regenerative endodontics (45, 58). APCs extracted from apical tissues have been advocated because of their high proliferation rate and mineralization potential. An evoked-bleeding step in regenerative procedures could recruit stem cells, including APCs into the root canal (54). These cells may attach to the dentin surfaces and differentiate into mineralized-tissue-

forming cells, which are important in the continuation of root development (7, 16, 54). Therefore, it is significant to evaluate the effects of regenerative treatment procedures in order to promote specific cell attachment and differentiation.

Currently, revascularization procedures use a variety of medicaments and irrigants, including 3Mix, $Ca(OH)_2$, NaOCl, and EDTA. However, more information is required regarding whether these products have negative consequences on cell attachment. Some recent studies related to regenerative endodontics have now focused on the improvement of cell attachment on dentin and have reported that EDTA could promote DPSC attachment when cells are grown in contact with EDTA-treated dentin (24). Furthermore, $Ca(OH)_2$, when used to precondition the dentin, increased SCAP survival and proliferation in the dentin lumen containing a Matrigel scaffold (59).

In the experiment, all samples were final rinsed with 17% EDTA before cell seeding in order to promote cell attachment, using a protocol as previously described (24). The viability of all attached cells was confirmed after each dentin disc was repositioned into a new well. These steps were taken so as to eliminate the bias of counting non-attached or dead cells. Therefore, the attachment ability of viable APCs on dentin was exactly analyzed. In this study, the viability of APCs was investigated using the alamarBlue® assay. This assay was used because it is a rapid, sensitive and reliable fluorometric/colorimetric growth indicator. Moreover, it is non-toxic to cells, making it safe for evaluating the viability of cells in kinetic fashion (60-62).

Collectively, the results showed that treating dentin with 3Mix had undesirable effects on APC attachment. Treating dentin with 100 µg/mL of 3Mix still had a detrimental effect on cell viability compared with other. Moreover, the 3Mix paste group had the lowest number of fibronectin-positive cells. The results were similar to those of a study by Althumairy *et al* (59), who reported that dentin conditioning with 3Mix paste, a clinical preparation, resulted in no SCAP survival on conditioned dentin. In contrast, Yassen *et al* (44, 63) suggested that 3Mix paste had a demineralization effect on dentin surfaces, possibly enhancing the attachment and growth of stem cells on root dentin. This converse result might be explained by the toxicity of the residual 3Mix on the dentin. Berkhoff *et al* (64) reported that 88% of the 3Mix remained bound to the dentin and was retained in the root canal system even after extensive irrigation. The substantivity of minocycline, a derivative of tetracycline, has been reported to form a strong, reversible

bond with enamel and dentin, exhibiting a slow release and diffusion through dentin for several weeks (65, 66). Another recent study also revealed that the mixed antibiotic paste, even without minocycline (named as DAP), still had a deleterious effect on cell attachment (59). Therefore, it is possible that residual antibiotics on dentin may result in a long-lasting negative effect on cell attachment and viability. Moreover, from the results of this study, it can be speculated that the effect of APC attachment might be concentration-dependent, since higher cell numbers were noticed when a lower concentration of 3Mix was used. Similar results have been reported, suggesting that high concentrations of 3Mix create more destructive effects than do low concentrations (18-20). In the SEM segment of this study, 3mix-treated dentin had fewer cells on the surface than did the dentin treated with $Ca(OH)_2$. Cell projections were not frequently detected. Obviously, thin irregular patches were generally found on the dentin when 3Mix paste was used (Figure 22 D). Such patches might act as barriers for cell attachment. From these results, it can be speculated that, even after thoroughly flushing dentin with EDTA after medication with 3Mix, residual effects or remnants of 3Mix could still be discerned. These might hamper cell attachment. However, the lower concentration of 3Mix showed higher cell attachment with less excessive products. Thus, these results confirm the benefit of using of 3Mix at lower concentrations in regenerative endodontics.

Interestingly, in the $Ca(OH)_2$-treated dentin, a high number of fibronectin-positive staining cells was observed and tended to increase when $Ca(OH)_2$ at the higher concentration was used. This finding corresponds with that of Mizuno and Banzai (57), who reported that calcium ions from $Ca(OH)_2$ promoted fibronectin synthesis in dental pulp cells, especially at high concentrations. In similar circumstances, Althumairy *et al* (59) also reported that $Ca(OH)_2$ promoted SCAP survival and proliferation. A previous study has shown that $Ca(OH)_2$ remained in the dentin even after root canal irrigation (64). The solubilizing effect of $Ca(OH)_2$ on dentin, when used as a root canal medicament, has also been reported (67, 68). It stimulates the release of various growth factors, including Transforming growth factor-beta 1 (TGF-β1), that might promote cell proliferation and mineralization (69, 70). Those effects after $Ca(OH)_2$ medication might help cells to attach, proliferate and differentiate. The results of the SEM segment of this study showed that APCs with cellular projections were found in abundance on the $Ca(OH)_2$-treated dentin. Clear dentinal surfaces with exposures of dentinal tubules were generally observed. These

surface conditions, together with the residual effects of $Ca(OH)_2$ itself, might offer appropriate areas for cell attachment and differentiation.

These findings suggest that medicament used in current regenerative procedures have some effects on APC attachment. Dentin treated with 3Mix at 0.39 or 100 µg/mL had lower negative effects on viable APC attachment than that treated with 3Mix with paste-like consistency. Even after final rinsing with EDTA, the 3Mix-medicated dentin still had some deleterious effects on APC attachment. In contrast, $Ca(OH)_2$-medicated dentin provided a better condition for viable APC attachment. Ultimately, the medicaments used in regenerative endodontics should be carefully considered, since efficient, viable cell attachment should offer cell differentiation, leading to successful regenerative consequences. However, the differentiation potential of these cells needs to be further investigated.

Conclusions

3Mix at low concentration, as well as $Ca(OH)_2$, had a lower effect on cell viability than it did at higher concentrations. However, $Ca(OH)_2$ tended to promote fibronectin synthesis that might enhance the attachment ability of APCs to dentin surfaces. In order to determine valuable procedures in regenerative endodontics, one interesting aspect that must be verified in the future is the differentiation potential of APCs attached to treated dentin surfaces.