CHAPTER 5

DISCUSSION

A three-antibiotic mixture (3Mix) has been generally used in regenerative endodontic procedures due to the LSTR concept. It has been reported as an effective medication against endodontic bacteria in a concentration-dependent manner (26, 27). Recently, an interesting issue has been raised regarding the strong toxicity of 3Mix on human dental cells (29, 30). Therefore, the new regimen of 3Mix, especially for regenerative endodontics, has been changed to use low concentrations. However, the regenerative capacity of cells after exposure to 3Mix has never been evaluated. Therefore, this study determined the regenerative capacities of DPCs/APCs after treatment with a very low concentration of 3Mix (0.39 µg/mL) for seven days. The results revealed that cells treated with 0.39 µg/mL of 3Mix had a significantly lower proliferation rate than untreated cells. Mineralized matrix formation was observed in both groups, but it was lower in the 3Mix-treated groups than in the control groups. However, the expressions of DMP-1 and BSP genes were not significantly different when cells were exposed to 3Mix for 21 days.

There are several ways for treating immature teeth, diagnosed with necrotic pulp. Conventional calcium hydroxide apexification has been widely practiced for many decades. The osteo-inductive properties (74) and high pH of calcium hydroxide may be the contributing factor for the induction of a hard tissue barrier, which prevents the extrusion of root canal filling materials from the root apex. However, there are several drawbacks to the technique, including the requirement of patient

compliance and a risk of root fracture as a result of long-term calcium hydroxide medication. Since Torabinejad's publication, alternative methods for apexification, for example, the formation of an MTA apical barrier, have been suggested (75). Even though successful results have been reported, these procedures provide only small increases in root length and width (5, 6). Principally, the ideal outcome of treatment of immature teeth with necrotic pulps is stimulating continued root growth. So, the wide apex of the tooth should be closed, and the thin dentin wall should also be thickened. Therefore, a new treatment protocol known as revascularization has been proposed as an alternative for treating immature teeth with necrotic pulps (9).

The revascularization procedure aims to stimulate the regeneration of a functional pulp-dentin complex. It has been a topic of interest since successful continuation of the tooth root has been radiographically reported after this technique was carried out in an immature tooth with necrotic pulp (9-11). However, it has not been clarified that a complete pulp-dentin complex had occupied the root canal system. Only clinical and radiographic presentations revealed asymptomatic functional teeth with continued root development (10-14, 76-79). To date, some clinical and histological studies have constantly reported unpromising results after revascularization (17, 18, 21, 22). Moreover, scientific evidence supporting the pathway of this revascularization procedure is lacking. Several factors affecting the outcome have been studied, for example the role of blood clot formation (38), the induction of cells into the root canals (31, 53, 80, 81), and the action of materials and drugs used during the treatment (17).

A three-antibiotic mixture has been generally used in endodontic procedures due to the LSTR concept (48). It has been suggested for use in the revascularization technique since 2004 (9). However, an interesting issue regarding the strong toxicity of 3Mix on human dental cells has recently been raised (29, 30). Those studies reported that 3Mix had negative effects on the survival of SCAPs, dental pulp, and apical papilla cells. One of those studies attempted to determine the cytotoxicity and antibacterial efficacy of 3Mix and suggested that 3Mix at a concentration of 0.39 µg/mL had sufficient antibacterial efficacy and caused less damage to DPCs and APCs than currently used concentrations (30). Therefore, 3Mix, especially in regenerative procedures, has been recommended for use at a very low concentration. In this study, a low concentration (0.39 µg/mL) of 3Mix was tested on DPCs/APCs focusing on the "recovery" of the regenerative capacity of these cells. The experiment was designed based on real procedures normally performed in the clinic, in which 3Mix was left in the root canal of the treated tooth for a period of time, and then removed. Cells were cultured in a medium containing 3Mix for seven days, and then the medium was changed to a normal one without 3Mix. Later, cells were tested for their regenerative capacities. The results showed that cells after exposure to 3Mix for seven days had a reduced proliferation rate and diminished mineralization potential compared to the control group, implying that a low concentration of 3Mix, even with a short contact time, still exerted some effects on cells.

Regenerative treatment aims to regenerate/repair tissue or organs lost, using a tissue engineering concept. Cells are one of the most interesting tissue components, since different tissues/organs need particular cell types. In regenerative endodontics, a dentin-pulp complex regeneration is ideal, since keeping or retrieving tooth vitality is

the ultimate goal. There are several cell populations involved in the natural formation of dentin and pulp, including perivascular cells, cells from the layer of Höhl, undifferentiated mesenchymal cells, and fibroblasts (82). However, during the regeneration and repair processes, progenitor cells in the dental pulp play a more significant role than do other cells by differentiating into cells that are able to form new dentin (54). To date, several types of stem cells have been recovered in the dental pulp and surrounding tissues, for example DPSCs, SCAPs, PDSCs, etc. (55, 61). These stem cells have the potential to be used in dental tissue engineering (83). In this study, different populations of cells, from dental pulp and apical papilla, were used, since regeneration involves a complex pathway. Additionally, the selection of these cells was based on the idea that surviving cells, including dental pulp and apical papilla cells, could be observed in necrotic teeth (9, 53, 61). These surviving cells may play a part in continued root formation. Therefore, in order to promote the growth of immature teeth using the regenerative procedure, care should be taken not to damage the surviving cells since fewer cells at impaired sites had some effects on wound healing (84). Moreover, various capabilities of cells, especially regenerative function, should still be maintained.

The ideal medicament used in regeneration should maintain cell proliferation, or should allow the contacted cells to re-proliferate after medicament removal. In this study, the consequences of treatment of DPCs and APCs with 3Mix were evaluated; 1) the proliferative capacity and 2) the mineralization potential. Interestingly, a pilot study using 1 mg/mL, an LC50 dose of 3Mix (as recommended by another study) (29), was attempted to culture both cell types for seven days. Unfortunately, cells after exposure to 3Mix at that concentration died afterward (Figure 39). Various factors

might be responsible for cells not surviving in the presence of an LC50 dose. One factor may be the great variety of drugs, on the market. Further studies are required in order to confirm the hypothesis that different drugs may have different effects on cell survival. Nevertheless, the materials used in this study complied with the Thai FDA standard, and the stock 3Mix solution was carefully kept at a concentration within its solubility. The composition of each antibiotic is shown in Table 3.

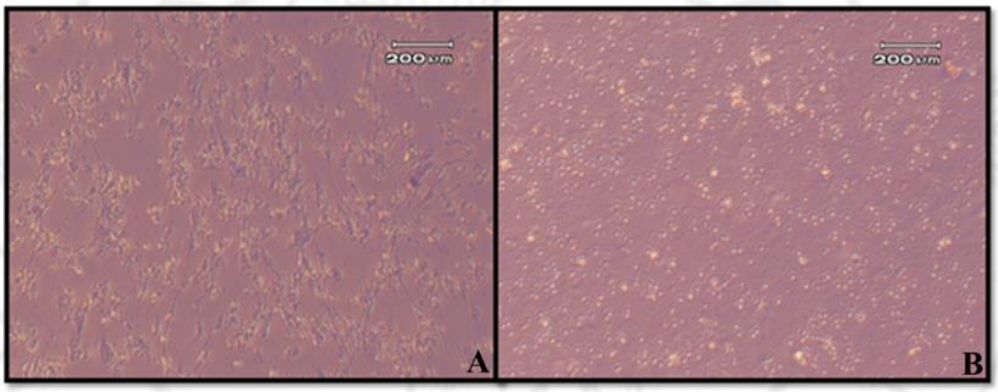

Figure 39: *Cell morphology of DPCs (A) and APCs (B) after treatment with an LC50 dose of 3Mix. On the 4th day, both cell types had irregular cell surfaces with body shrinkage, and dissolved afterward.*

Table 3: *3Mix composition*

Antibiotic	Active ingredient	Powder
Ciprofloxacin hydrochloride tablet	250 mg	a white hygroscopic crystalline powder
Metronidazole Benzoate tablet	200 mg	a white odourless crystalline powder
Minocyline hydrochloride capsule	50 mg	a yellow crystalline powder

The proliferative capacity of cells is important, since the regeneration and repair processes of damaged tissues require cells. Therefore, in regenerative treatment, keeping or promoting cell proliferative function should be one of the key factors. In this study, the experiment revealed that 0.39 µg/mL of 3Mix-treated DPCs/APCs had a significantly lower proliferation rate than untreated DPCs/APCs at all time points of the experiment. Moreover, the proliferative capacity of these cells could not be completely recovered, even with continued culture under regular conditions for seven days. Thus, it can be implied that the exposure of cells to 3Mix, even at very low concentrations for seven days, negatively affected the cell number. Mineralized matrix formation was observed in both 3Mix-treated and untreated cells, but it was lower in the 3Mix-treated groups than in the control groups. Interestingly, it was visualized that APCs had higher osteo/dentinogenic potency than that of DPCs. The mineralized nodules in the APCs were formed faster than in the DPCs. In addition,

alizarin red-S staining revealed that APCs stained clearer than DPCs. This result can be explained by previous findings reporting that SCAPs have higher mineralization potential than DPSCs (60).

The expressions of genes involved in mineralization were also investigated in this study. Dentin matrix proteins-1 (DMP-1) is an odontoblast-specific gene involved in odontoblast differentiation and mineralization (54). Cells that express one of these genes might play some roles in dentin production (57, 58). Bone sialoprotein (BSP) is an acidic, non-collagenous glycoprotein in the SIBLING family. BSP is expressed in several cell types associated with mineralized tissue, such as bone and dentin, but expressed especially in osteoblasts, hypertrophic chondrocytes and osteoclasts (71). In this study, under dentino-/osteogenic-stimulating conditions, there were no statistically significant differences in DMP-1 and BSP gene expressions between the 3Mix-treated groups and the control groups at all time periods. However, there were some tendencies showing that these genes were altered. DMP-1 gene expression was down-regulated in 3Mix-treated DPCs /APCs, whereas the BSP gene was up-regulated in the 3Mix-treated group. This result may imply that the production of mineralized matrix might relate to the bone pathway, assuming that the 3 Mix-treated DPCs/APCs had a tendency to differentiate into bone-forming cells. These findings may support the histological outcome in which bone formation was regularly observed after the revascularization procedure (17, 18, 21). Further investigation is strongly recommended, since excessive bone formation would impede dental pulp regeneration. Finally, from this study, it can be concluded that 3Mix not only affected the cell numbers but also affected their dental mineralization potential. These results

suggest that using 3Mix in the revascularization procedure may compromise the "dental" tissue regeneration.

Conclusions

After treatment with 3Mix, even at a very low concentration, there were negative effects on the proliferative capacity and mineralized matrix formation of DPCs and APCs *in vitro*. Alternative regimens for regenerative endodontic procedures, including other influencing factors, still need to be clarified in order to promote appropriate pathways for dental tissue regeneration and to achieve predictable treatment outcomes.