Regenerative Capacity of Human Dental Pulp and Apical Papilla Cells after Treatment with a 3-Antibiotic Mixture

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Abstract

Introduction: A 3-antibiotic combination (3Mix) has been widely used in regenerative endodontics. Recent studies recommend that a safe concentration of 3Mix is in the range of 0.39 μg/mL and 1 mg/mL because higher concentrations may limit tissue regeneration. The aim of this study was to determine the regenerative capacity of isolated human dental pulp cells (DPCs) and apical papilla cells (APCs) after a 7-day treatment with selected doses of 3Mix. **Methods:** Primary human DPCs/APCs from the third passage were divided into control and experimental groups. In the control group, cells were cultured in regular complete media. In the experimental group, cells were cultured in complete media containing 0.39 μg/mL or 1 mg/mL of 3Mix for 7 days. After the treatment period, the media were changed, and the cells were further tested for proliferation and differentiation potential. For cell proliferation, a colorimetric qualification of 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide was used on days 1, 3, 5, and 7. For differentiation potential, a dentinogenic differentiation medium was added into treated cells and cultured for 7, 14, and 21 days. Results were analyzed using quantitative alizarin red S staining and real-time reverse-transcription polymerase chain reaction. **Results:** After 7 days of treatment, 100% cell death was discovered in the 1-mg/mL 3Mix group. The proliferative capacity of 0.39 μg/mL 3Mix-treated DPCs and APCs was significantly lower than that of untreated cells at all time points ($P < .05$). Mineralized nodule formation was found both in the 3Mix-treated and control groups, but it was significantly less in the 3Mix-treated groups at 7, 14, and 21 days ($P < .01$). Quantitative reverse-transcription polymerase chain reaction showed no statistically significant difference (95% confidence interval) in bone sialoprotein, alkaline phosphatase, and dentin matrix protein 1 gene expression in either 3Mix-treated DPCs or APCs compared with control groups. **Conclusions:** One milligram per milliliter of 3Mix had strong toxicity to DPCs/APCs when applied for 7 days, whereas 0.39 μg/mL 3Mix showed no toxicity but still affected cell proliferation and mineralization potential. However, no differences in dentinogenic gene expressions were observed between the 3Mix-treated and untreated groups. *(J Endod 2014;40:399–405)*

Key Words

3MIX, apical papilla cells, dental pulp cells, regenerative capacity

The treatment of immature teeth with necrotic pulps is a challenge in dentistry. For many decades, clinicians have relied on conventional calcium hydroxide apexification or the use of artificial apical barriers to treat these teeth. Even though successful results have been reported (1, 2), these procedures provided a small increase in root length and width after a period of time (3, 4). Recently, a revascularization method has been proposed as a new treatment protocol for immature teeth with necrotic pulp (5, 6) because successful continuation of the tooth root has been radiographically reported (7–10).

Regenerative endodontics can be defined as biologically based procedures to replace damaged structures, including dentin and root structures and cells of the pulp-dentin complex (11). The ideal objective of regenerative endodontics is to generate/regenerate the dentin-pulp complex (11). Therefore, vital pulp tissue would be preserved or regenerated, keeping the tooth in homeostasis. Regenerative endodontic procedures depend on the disinfection of the root canal without mechanical disruption. Various chemical disinfectants, such as sodium hypochlorite and chlorhexidine, are used for chemical debridement. To date, antibiotic combinations and calcium hydroxide have been selectively used as interappointment medications for regenerative endodontic procedures. A 3-antibiotic combination (ciprofloxacin, metronidazole, and minocycline) (3Mix) has been reported as an effective medication against endodontic bacteria in a concentration-dependent manner (12, 13). However, the question of the cytotoxicity of 3Mix has been raised recently. Therefore, calcium hydroxide, one of the most common intracanal medicaments used, has been alternatively mentioned because it could stimulate the proliferation of stem cells from the apical papilla (SCAPs) (14).

The concept of regenerative endodontics has become of interest because conventional calcium hydroxide and mineral trioxide aggregate apexification could neither strengthen nor ensure further root development. In an immature tooth, with regenerative endodontics, root development can continue, ensuring a thicker dentinal wall that is resistant to fracture and closure of the apex, which makes subsequent endodontic therapy, if required, more predictable (15). In addition, the importance of noninfected, vital pulp is that it maintains apical periodontal health (16).

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Recently, several studies describing the histologic characteristics of teeth treated with the revascularization technique have been published (17–22). One interesting study in infected immature dog teeth treated with the revascularization procedure revealed that tissue that grew into the root canal system after revascularization mainly resembled cementum, periodontal ligament, and bone. Only 1 case from 60 teeth in that study revealed partially survived pulp tissue (17).

Various reasons have been discussed regarding the ambiguous results in the current literature. One of those reasons is that the high concentration of the 3-antibiotic paste used might be toxic to living tissue (17). The clinical dosage of 3Mix generally used to date may be excessive and might affect the host tissue, causing cell death, which limits tissue regeneration. A recent study has shown that the 3-antibiotic paste affected the viability of SCAPs in a concentration-dependent manner; 1 mg/mL 3Mix caused 50% cell death on SCAP viability (14).

Furthermore, another study by Cheunsombat et al (23) reported that the cytotoxicity of 3Mix was induced when concentration and time increased. The 0.39-μg/mL concentration of 3Mix was the best candidate for use because it produced less cytotoxicity on human dental pulp cells (DPCs) and apical papilla cells (APCs) than higher concentrations, whereas it was able to significantly reduce bacteria isolated from necrotic teeth.

However, there is no evidence showing that DPCs or APCs, after treatment with this noncytotoxic dose of 3Mix, would promote mineralization of dentin, a property that is important for the regeneration process. Therefore, the aims of this study were as follows: (1) to determine the proliferative capacity of DPCs/APCs after treatment with 1 mg/m and 0.39 μg/mL 3Mix for 7 days; (2) to compare the mineralization potential of 3Mix-treated DPCs/APCs and untreated DPCs/APCs, and (3) to identify the messenger RNA expression of mineralization markers of 3Mix-treated and untreated DPCs/APCs.

Materials and Methods

Patient Recruitment

This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. After verbal and written informed consent, nonpathologic impacted third molars with/without immature roots from healthy patients (aged 18–25 years) were extracted. The teeth were rinsed using sterile normal saline solution and stored on ice in separate containers with serum-free media.

Culture of Primary Human DPCs and APCs

To obtain pulp tissue, teeth were soaked and inverted in 5.25% sodium hypochlorite except for 3 mm from the root apex. The teeth were longitudinally sectioned into buccal and lingual parts, and then pulp tissue was removed using a sterile endodontic spoon and immediately transferred into serum-free alpha minimum essential medium (α-MEM; Sigma-Aldrich, St Louis, MO). For the apical papilla cells, after tooth extraction, the root apical papilla was gently separated from the root surface by tweezers, copiously irrigated with sterile normal saline, and then transferred into serum-free α-MEM transport medium (Sigma-Aldrich). Pulp tissue and apical papilla were minced and digested separately in 3.00 mg/mL collagenase type I (Gibco Invitrogen, Gaithersburg, MD) and 4.00 mg/mL dispase (Sigma-Aldrich) for 45 minutes at 37°C. After that, cells were centrifuged at 1500 rpm at 25°C for 5 minutes and then cultured in complete α-MEM (Sigma-Aldrich) containing 10% fetal bovine serum (Gibco Invitrogen), 1% penicillin-streptomycin (Sigma-Aldrich), and 100 μmol/L L-ascorbic in a humidified atmosphere of 5% CO_2 and 95% air. Cells from the third passage were used in the experiment.

3Mix Preparation and Treatments

To prepare a 150-μg/mL stock solution, ciprofloxacin (Khandelwal, Mumbai, India), metronidazole (Piramel Healthcare, Gujarat, India), and minocycline (Qualimed, Samut Prakarn, Thailand) were weighed and dissolved separately in deionized distilled water and then sterilized by passing through 2-μm filtering papers (Whatman; Maidstone, Kent, UK) and 0.2-μm microfilters (Corning, Oneonta, NY). Then, a known concentration of 3Mix solution was prepared by mixing each drug at an equal volume and diluting it to 1 mg/mL and 0.39 μg/mL using complete culture media. Generally, a clinical protocol recommended for the revascularization technique includes the placement of intracanal medication for some period. Therefore, to mimic this situation in patients, DPCs/APCs were treated either with 3Mix or without 3Mix for 7 days before further investigation. The cells were divided into 3 groups as follows: (1) control group: DPCs/APCs were cultured in regular complete media for 7 days, (2) experimental group A: DPCs/APCs were cultured in complete media containing 1 mg/mL 3Mix for 7 days, and (3) experimental group B: DPCs/APCs were cultured in complete media containing 0.39 μg/mL 3Mix for 7 days.

In all groups, after 7 days, culture media were replaced with regular α-MEM. Then, samples of 30,000 cells were plated into 6-well plates and samples of 5000 cells into 24-well plates for further investigation.

MTT Cell Proliferation Assay

3Mix-treated and untreated DPCs/APCs, which were plated and cultured in regular α-MEM, were used to determine their proliferative capacity. Briefly, DPCs and APCs were separately seeded into 24-well plates at 5000 cells/well in regular complete media (n = 6, triplicates). Cell proliferation was measured at the specific time intervals of 1, 3, 5, and 7 days using colorimetric qualification of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich). A freshly mixed MTT solution (5 mg/mL MTT in phosphate-buffered saline [PBS] solution) was added to growing cells at 80 μL/well and incubated at 37°C and 5% CO_2 for 3 hours. After that, the medium was removed, each well was flushed with sterile PBS, and 800 μL dimethyl sulfoxide (Sigma-Aldrich) was added into each well. One hundred microliters of the mixed solution was transferred into a 96-well plate, and absorbance at 550 nm was measured using a spectrophotometer (Sunrise; Tecan, Mannerdorf, Switzerland).

Dentinogenic/Osteogenic Differentiation Capacity

3Mix-treated DPCs/APCs and control groups (without 3Mix treatment) were seeded at a density of 5000 cells/well on 24-well plates. After 70% confluence in culture, the culture medium was replaced by differentiation medium containing complete α-MEM, 50 μg/mL ascorbic acid (Sigma-Aldrich), 10 nmol/mL dexamethasone (Sigma-Aldrich), and 10 mmol/mL ß-glycerophosphate (Sigma-Aldrich). Then, cells were cultivated for 7, 14, and 21 days in a humidified atmosphere of 5% CO_2 and 95% air with the medium changed regularly every 3 days. The cells were monitored every day under an inverted-light microscope (DP12; OLYMPUS, Melville, NY).

Alizarin Red S Staining and Quantification Assay

Alizarin red S staining was used to detect calcium deposits. 3Mix-treated cells and control groups in dentinogenic differentiation medium were evaluated at 7, 14, and 21 days. All specimens were fixed with 4% paraformaldehyde in PBS solution (4% paraformaldehyde in PBS). Then, they were rinsed with 1 mL PBS/well, and 0.5 mL alizarin red S

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