Effects of a Triple Antibiotic Solution on Pulpal Dynamics after Intentionally Delayed Tooth Replantation in Mice

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Abstract

Introduction: This study analyzed the detailed biological events underlying pulpal dynamics evoked by 3Mix (the mixture of ciprofloxacin, metronidazole, and minocycline) solution after intentionally delayed tooth replantation because 3Mix improves pulpal healing after tooth injuries. **Methods:** The maxillary first molars of 3-week-old mice were extracted and immersed in 3Mix solution for 30 minutes in comparison with phosphate buffered saline (PBS) alone. Cell proliferation, apoptosis, and differentiation were assessed in extracted/replanted teeth during days 0–14 using immunohistochemistry, apoptosis assay, and reverse-transcriptase polymerase chain reaction. **Results:** 3Mix solution accelerated odontoblast differentiation in the coronal pulp on day 7 and tertiary dentin formation on day 14, whereas the regenerative process was delayed in the PBS group. Cell proliferation and apoptosis occurred in the pulp of the 3Mix group during days 5–7 and subsequently decreased from days 7–14. On day 5, dentin sialophosphoprotein and nestin were first recovered in the 3Mix group, whereas expression levels for alkaline phosphatase, osteopontin, and osteocalcin increased in the PBS group. The expression levels for *octamer-binding factor 3/4A* and *3/4B* reached the maximum level on day 1 and were sharply decreased on day 3 in both groups. High expression levels of *Cd11c* were first observed in the 3Mix group on day 1 and later at days 5 and 7. **Conclusions:** The results suggest that the application of 3Mix may suppress osteoblast differentiation by the migration of dendritic cells to the injury site and via the activation of stem/progenitor cells, resulting in the acceleration of odontoblastlike cell differentiation. *(J Endod 2014;40:1566–1572)*

Key Words

Antimicrobial, apoptosis, cell differentiation, cell proliferation, dental pulp, odontoblasts, regeneration, tooth replantation, Crlj:CD1 (ICR) mice

Dental pulp is a connective tissue uniquely situated within the rigid encasement of mineralized dentin (1). Dental pulp not only provides nutritional and sensory properties to dentin but also has its own reparative capacity. Dental caries, attrition, abrasion, or restorative treatments, such as cavity preparation, lead to local dentin formation in the pulp chamber (2). Tooth injuries such as cavity preparation and tooth replantation induce destructive changes in the odontoblasts at the affected site and an acute inflammatory reaction (3–5). After odontoblast cell death, stem or progenitor cells residing in the adult dental pulp replace the degenerated cells to differentiate into odontoblastlike cells. Numerous clinical studies have shown that pulp may heal after tooth injuries even if complete severance of the neurovascular supply takes place. Hence, the mode of pulpal responses may vary according to the origin of the progenitor cells involved and the extent of tissue injury (6).

Tooth replantation, defined as a therapeutic method in which the avulsed or extracted tooth is replaced in its original socket, has become widely used in clinical dentistry. However, this procedure causes interruption of the nerve and vascular supply to the dental pulp. Pulpal responses to tooth replantation can be divided into at least 2 types of healing patterns: dentin and/or bonelike tissue formation in the pulp tissue (7–12). Although the mechanisms for determining the divergent healing processes after tooth replantation remain to be fully clarified, they may be directly linked to the death or survival of odontoblast-lineage cells. An intentionally prolonged operating time for tooth replantation induces the total death of odontoblast-lineage cells because of the lack of a properly oxygenated medium (12). The presence of bacteria in the root surface as well as bacteria associated with the blood clot in the socket appears to worsen the chances of a successful outcome (6). Therefore, the establishment of an adequate environment that regulates these external factors seems to be critical for the regeneration of the afflicted dental pulp after tooth replantation.

The combination of antibacterial drugs such as ciprofloxacin, metronidazole, and minocycline (referred to as 3Mix) is currently widely used as an intracanal medicament in the regenerative endodontic/revascularization procedures for the treatment of immature teeth with pulpal necrosis (13). The high antibacterial effect and biological compatibility of 3Mix as well as the clinical outcomes have been reported elsewhere (14–31). However, there are some drawbacks related to the clinical application of 3Mix (32). These include the risk of developing antibiotic resistance in certain strains of root canal bacteria (33, 34), the fear of triggering an allergic reaction in sensitive patients (35–37), and the discoloration of the tooth crown caused by tetracycline (38–41). Recently, the use of calcium hydroxide has been reported in regenerative endodontic procedures with successful outcomes (42, 43). Despite several concerns raised about the use of calcium hydroxide in revascularization (13, 44, 45), this could become a safe alternative in patients with sensitivity to 1 of the 3Mix components. Our recent article showed the usefulness of 3Mix antibiotic solution in

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improving the healing process of intentionally delayed replanted teeth in mice. 3Mix solution effectively accelerated the pulpal regeneration process of replanted teeth in concentration- and immersion time–dependent manners (46). Although the pulpal healing patterns have already been investigated in terms of cell proliferation and apoptosis in different types of 3Mix solutions, more detailed analysis of the biological events underlying the pulpal dynamics evoked by 3Mix solution is necessary for the proper understanding of its implications for dental therapy at the molecular level. This study analyzed the detailed biological events underlying the pulpal dynamics evoked by 3Mix solution after intentionally delayed tooth replantation. The progression of the pulpal healing was assessed by immunohistochemistry for nestin and Ki-67 and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Furthermore, we analyzed the gene expression profile by reverse-transcription polymerase chain reaction (RT-PCR) using *dentin sialophosphoprotein (Dspp), nestin, alkaline phosphatase (Alp), cyclinD1, caspase3, osteopontin (Opn), osteocalcin (Ocn), octamer-binding factor (Oct) 3/4A, Oct3/4B,* and *Cd11c* primers on each observation point.

Materials and Methods

Tooth Replantation

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations or under the conditions proposed by the Committee. One hundred five Crlj:CD1 mice (3 weeks old) were divided into 2 groups: (1) 1 group using phosphate buffered saline (PBS) solution and (2) the other using 3Mix solution (ciprofloxacin 0.01 mg/mL, metronidazole 0.02 mg/mL, and minocycline 0.01 mg/mL diluted in distilled water). Under anesthesia with an intraperitoneal injection of chloral hydrate (maximum dose of 350 mg/kg), the maxillary right first molars of each animal were extracted and then repositioned in their original socket after immersion for 30 minutes in PBS or 3Mix solution. The current concentration of 3Mix solution and the immersion time were established based on the results provided in our previous publication (46).

Tissue Preparation

Materials were collected from groups of 8–9 animals immediately after immersion (n = PBS: 8, 3Mix: 8) or at 1 (n = PBS: 9, 3Mix: 9), 3 (n = PBS: 8, 3Mix: 9), 5 (n = PBS: 9, 3Mix: 8), 7 (n = PBS: 9, 3Mix: 10) and 14 (n = PBS: 9, 3Mix: 9) days after tooth replantation. At each stage, the animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH = 7.4) under deep anesthesia induced by an intraperitoneal injection of chloral hydrate. The maxillae were removed en bloc and immersed in the same fixative for an additional 12 hours at 4°C. After decalcification in Morse solution (10% sodium citrate and 22.5% formic acid) for 4–6 days at 4°C, the specimens were processed for embedding in paraffin and cut sagittally at a thickness of 4 μm. Sections were processed for hematoxylin-eosin staining and immunohistochemistry.

Immunohistochemistry and TUNEL Assay

Immunohistochemistry was conducted using a mouse antirat antinestin monoclonal antibody diluted 1:100 (Millipore, Temecula, CA; catalog number: MAB353) and a rat antimouse Ki-67 monoclonal antibody diluted 1:100 (Dako Japan, Tokyo, Japan; catalog number: M7249). The Envision + Horseradish Peroxidase System (Dako Japan, catalog number: K5027) and the avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) method using biotinylated antirat immunoglobulin G (Vector Laboratories; catalog number: BA-4000) were used for nestin and Ki-67 immunohistochemistry, respectively. For the final visualization of the sections, 0.05 mol/L Tris-HCl buffer (pH = 7.6) containing 0.04% 3-3′-diaminobenzidine tetrahydrochloride and 0.0002% H_2O_2 was used. The immunostained sections were counterstained with hematoxylin. Apoptosis was quantified by TUNEL with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore; catalog number: S7100). Negative controls were performed by replacing the primary antibodies or terminal deoxynucleotidyl transferase enzyme with PBS.

RT-PCR

Total RNA was isolated from the dental pulp tissue of replanted teeth at each observation stage (days 0–14) using the Trisol system (Invitrogen, Life Technologies, Carlsbad, CA). Complementary DNA was synthesized from the RNA with the SuperScript First-Strand Synthesis System (Invitrogen). The sequences of the PCR primer pairs for *β-actin, Dspp, nestin, cyclinD1, caspase3, Alp, Opn, Ocn, Oct3/4A, Oct3/4B,* and *Cd11c* are listed in Supplemental Table S1 (Supplemental Table S1 is available online at www.jendodon.com). The thermocycling protocol during 30 or 35 amplification cycles was as follows: denaturation at 94°C for 1 minute, annealing at 58°C or 60°C for 1 minute, and extension at 72°C for 1 minute. The amplified DNA fragments were separated by electrophoresis on 2% agarose gels. The relative densities of each band against those of β-actin on monochrome photographs were determined with Image J software (Image J 1.45; National Institutes of Health, Bethesda, MD).

Cell Counting

The numbers of Ki-67- and TUNEL-positive cells in the coronal and root pulp of each specimen (3.4×10^4 grid was selected) were calculated separately. All data were presented as the mean and standard deviation of each group. Furthermore, the number of cells in the coronal and root pulp at different times after tooth extraction or replantation (days 0–14) and between groups were compared by the Bonferroni test (1-way analysis of variance) using statistical software (SPSS 16.0 J for Windows; SPSS Japan, Tokyo, Japan).

Statistical Analysis of Nestin-positive Perimeters and Newly Formed Hard Tissue Areas in the Dental Pulp after Tooth Replantation

The percentage of nestin-positive perimeters in the total perimeter of the pulp-dentin border was calculated on days 7 and 14 using Image J software (Image J 1.45s, National Institutes of Health). Similarly, the percentage of newly formed hard tissue areas in the total area of the pulp chamber was quantified on day 14 using WinRoof image processing software (WinRoof Version 7.4; Mitani Corporation, Tokyo, Japan). All data were presented as the mean and standard deviation of each group and analyzed by the Student's t test using statistical software (SPSS 16.0 J for Windows).

Results

Nestin Immunoreaction in the Dental Pulp of Controls

Coronal odontoblasts showed that pseudostratified features and blood capillaries were located in the odontoblast layer (Fig. 1*A*). Intense immunoreactivity for nestin was recognized in coronal (Supplemental Fig. S1*A*) and root odontoblasts (data not shown) within their cell bodies and processes. (Supplemental Figure S1 is available online at www.jendodon.com.)