Effect of Dentin Conditioning with Intracanal Medicaments on Survival of Stem Cells of Apical Papilla

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Abstract

Introduction: Regenerative endodontics is a valuable treatment modality for immature teeth with pulpal necrosis. A common feature in regenerative cases is the use of intracanal medicaments. Although these medicaments are chosen because of their antibacterial properties, their enduring effect on dentin (conditioning) and the subsequent impact on stem cell survival has never been evaluated. In this study, we hypothesized that triple antibiotic paste (TAP), double antibiotic paste (DAP), or $Ca(OH)_2$ has an indirect adverse effect on the survival of stem cells of apical papilla (SCAP) by dentin conditioning. **Methods:** Human dentin disks were created with a standardized root canal diameter of 3.2 mm. The disks were then exposed to either TAP or DAP (at concentrations of 1 mg/mL or 1000 mg/mL), $Ca(OH)_2$ (Ultracal), or Hank's balanced salt solution for 7 or 28 days. Next, the medicaments were removed with copious irrigation, followed by placement of SCAP in a Matrigel scaffold in the lumen of the disks. The bioengineered constructs were cultured for 7 days, followed by determination of cellular viability by using the CellTiter-Glo luminescence assay. Data were analyzed using 1-way analysis of variance with Bonferroni post hoc test. **Results:** Exposure of dentin to TAP or DAP at 1000 mg/mL resulted in no viable SCAP, whereas the use of these medicaments at 1 mg/mL had no adverse effect on cell viability. In contrast, $Ca(OH)_2$ treatment significantly increased SCAP survival and proliferation when compared with the control group. **Conclusions:** Dentin conditioning with TAP and DAP at commonly used clinical concentration (approximately 1000 mg/mL) alters dentin in such a way as to prevent SCAP survival. This lethal indirect effect of both TAP and DAP can be largely avoided if these medicaments are used at the 1 mg/mL concentration. Conversely, dentin conditioning with $Ca(OH)_2$ promotes SCAP survival and proliferation. *(J Endod 2014;40:521–525)*

Key Words

Dentin, double antibiotic paste, endodontics, regenerative, stem cells, toxicity, triple antibiotic paste

Pulpal necrosis of immature permanent teeth is a clinical challenge in endodontics. In this clinical presentation, continued root development is not expected, resulting in thin fragile dentinal walls, which make the tooth more susceptible to fractures (1–3). These teeth have been traditionally treated by application of intracanal calcium hydroxide for apexification procedures until a natural calcific barrier is induced, followed by obturation of the root canal system (4). The procedure was later modified to include placement of an artificial apical barrier of mineral trioxide aggregate, resulting in fewer dental visits and comparable favorable outcomes (5, 6). In the last 10 years, numerous published cases and case series described a new treatment protocol called either revascularization or regenerative endodontics that can result in continued root development and even positive responses to pulp vitality testing in certain treated immature teeth (7–14).

Regenerative endodontic treatment generally starts with chemical debridement of the root canal with minimal to no instrumentation, followed by application of an intracanal medicament. Reported cases used a triple antibiotic paste (TAP) (ciprofloxacin/metronidazole/minocycline), double antibiotic paste (DAP) (ciprofloxacin/metronidazole), or $Ca(OH)_2$ dressing. The recommended time of dressing varies depending on patient's signs and symptoms but usually takes between 2–4 weeks (14). In the second appointment, assuming absence of clinical signs and symptoms of infection, the treatment continues with the removal of the intracanal medicament, induction of intracanal bleeding, and coronal restoration. Resolution of signs and symptoms of pathosis and radiographic evidence of continued root development are often observed. These favorable outcomes are largely dependent on the adequate disinfection of the root canal system.

From the perspective of tissue engineering, regenerative endodontics relies on 3 important factors: stem cells, scaffold, and growth factors. Importantly, it has been demonstrated that regenerative endodontic procedures promote influx of an abundant amount of undifferentiated stem cells into the root canal system (15). These cells are believed to be originated largely from the apical papilla and inflamed periradicular tissues. Thus, maintaining stem cell survival as well as promoting their proliferation and differentiation must be a crucial goal in regenerative endodontics. This represents a significant shift in thinking from traditional root canal therapy that only focuses on disinfection as ways of favoring the prevention and healing of apical periodontitis. Thus, it is imperative to investigate the effect of various widely accepted disinfection procedures in stem cell fate, namely survival and terminal differentiation.

Prior studies have determined that irrigation protocols have a profound effect on both stem cells of the apical papilla (SCAP) (16) as well as the dental pulp stem cells (17). These studies focused on the enduring effects of irrigants on dentin as opposed to a direct toxic effect of chemicals on stem cell survival and differentiation. Another study evaluated whether antibiotic formulations widely used in regenerative endodontic procedures had a direct toxic effect in SCAP survival (18). Together, these studies have led

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to the conclusion that commonly used irrigants may negatively impact stem cells by both direct toxic mechanisms as well as by an indirect effect via altering dentin.

Parallel studies have focused on the effects of medicaments on stem cells. For example, antibiotic paste formulations used in regenerative procedures are directly toxic to stem cells even at concentrations as low as 1% of doses currently used in treating patients (18). Importantly, stem cells are released from their niche and delivered into root canal systems previously treated with these medicaments. Thus, besides the direct toxic effect on the stem cells in periapical tissues, the medicaments could still indirectly affect stem cells long after they are flushed out of the root canals. However, there is a gap in knowledge because to date, no study has evaluated whether these medicaments have an enduring indirect effect by altering dentin. Thus, this study aimed to evaluate the indirect effect of TAP, DAP, and $Ca(OH)_2$ on the survival of SCAP in a dentin disk model.

Materials and Methods

Teeth Collection and Dentin Disk Preparation

This study was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Extracted teeth were collected from the clinics of the University of Texas Health Science Center at San Antonio School of Dentistry and placed in 15 mmol/L sodium azide for 24 hours at 4°C, followed by copious irrigation with sterile saline, and used for preparation of the simulated immature roots with open apices.

Dentin disks were prepared as previously described (19, 20). Briefly, gingival and periodontal tissues were removed from the tooth surface with a sterile surgical blade. The teeth were then washed in cold Hank's balanced salt solution (HBSS) (Sigma Aldrich, St Louis, MO). An IsoMet 1000 precision saw (Buehler, Lake Bluff, IL) fitted with a diamond saw was used to generate dentin disks (1 mm in thickness) by axial cuts through the pulp chambers of molar teeth (19, 20). The dentin disk lumen was standardized to a diameter of 3.2 mm with the use of a bench-mounted press drill, with constant irrigation with cold HBSS. The tooth slices were then washed twice with cold sterile HBSS, blotted dry, gas sterilized, and stored at 4°C until used.

Irrigation Protocol and Medicament Treatment

Dentin disks were rehydrated in HBSS for 5 minutes, washed in NaOCl (1.5%) for 2 minutes, saline for 5 minutes, and EDTA (17%) for 2 minutes, followed by saline for 5 minutes. After the washes, intracanal medicaments were applied at the lumen of the disks for the experimental groups. The antibiotic mixture drugs were United States Pharmacopeia grade and consisted of TAP (Champs Pharmacy, San Antonio, TX) containing metronidazole, ciprofloxacin, and minocycline in a 1:1:1 ratio and DAP (Champs Pharmacy) containing metronidazole and ciprofloxacin in a 1:1 ratio mixed with water to generate TAP and DAP at concentrations of either 1 mg/mL (a watery consistency) or 1000 mg/mL (a paste consistency). Calcium hydroxide was used in a commercially available form (Ultracal; Ultradent, South Jordan, UT). In addition, control dentin disks were treated with HBSS. Disks were then incubated at humidified 37°C for 7 or 28 days. After the incubation period, dentin disks were thoroughly washed with the same initial protocol that consisted of NaOCl (1.5%), EDTA (17%), and sterile saline.

SCAP Culture, Scaffold, and 3-dimensional Culture System

A previously characterized SCAP cell line (RP-89) was used in this study (21). RP-89 cells from the 5th to 7th passages were cultured in medium composed of alpha-minimum essential medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine, 1X L-glutamine (Gibco), penicillin (100 U/mL; Gibco), and streptomycin (100 mg/mL; Gibco) and grown to 70%–80% confluency as described previously (16). At the day of the experiment, cells were detached, centrifuged, resuspended in α-minimum essential medium, and mixed in a 1:1 ratio with ice-cold growth factor–reduced BD Matrigel (BD Biosciences, Bedford, MA) to yield a concentration of 2.5×10^6 SCAP/mL. Dentin disks were placed on 24-well plates coated with a 1-mm to 2-mm layer of growth factor–reduced BD Matrigel, followed by placement of 200 μL cell/scaffold mixture in the dentin disk lumen. Then, plates containing the bioengineering constructs were incubated for 30 minutes at 37°C to allow for polymerization of the scaffold, followed by placement of culture medium, and incubation for 7 days at 37°C and 5% CO_2. In addition, SCAP in Matrigel were cultured directly in the plastic wells in the absence of treated dentin disks to serve as a control for cell survival and proliferation in a 3-dimensional culture system.

Cell Counting

After the culture period of 7 days, all groups were washed twice with HBSS at 37°C, followed by depolymerization of the BD Matrigel scaffold by adding 200 μL ice-cold BD cell recovery solution (BD Biosciences) to each well and incubating on ice for 10 minutes. Next, 400 μL CellTiter-Glo (Promega, Madison, WI) was added to each well and incubated at room temperature for 10 minutes. To determine background luminescence, depolymerized Matrigel without cells was also mixed with CellTiter-Glo reagent. Relative luminescence for each group was detected in a FlexStation 3 Benchtop Multimode Microplate Reader (Molecular Devices, Sunnyvale, CA).

Data Analysis

The mean relative luminescence units (RLU) were calculated for each group after background luminescence subtraction. All experiments were performed in triplicates of $n = 4$, resulting in final $n = 12$ per group. Data were analyzed with 1-way analysis of variance with Bonferroni post hoc test. For all tests, significance was set at $P < .05$ by using Prism 6 (Graph Pad, La Jolla, CA).

Results

Effect of Dentin Conditioning for 7 Days

Treatment of dentin with either TAP or DAP at the concentration of 1000 mg/mL for 7 days resulted in no SCAP survival when cultured in the conditioned dentin ($P < .001$) (Fig. 1). However, when used at a concentration of 1 mg/mL, both TAP and DAP showed no effect on SCAP survival when compared with the control group. On the other hand, $Ca(OH)_2$ resulted in significant increase in SCAP survival and proliferation compared with control ($P < .001$) and with TAP and DAP groups ($P < .001$).

Effect of Dentin Conditioning for 28 Days

Because the concentration of 1000 mg/mL of either TAP or DAP was completely lethal to SCAP after 7 days, they were not tested for the longer treatment of 28 days. For the 1 mg/mL concentration of either TAP or DAP, there was no significant difference in SCAP survival compared with the control group (Fig. 2). On the other hand, $Ca(OH)_2$ once again produced significantly greater SCAP survival and proliferation when compared with TAP, DAP, or the control group.

Discussion

The survival and differentiation of mesenchymal stem cells, such as SCAP, delivered into the root canal space in a regenerative procedure