Effects of Novel 3-dimensional Antibiotic-containing Electrospun Scaffolds on Dentin Discoloration

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Abstract

Introduction: Although intracanal application of the triple antibiotic paste (TAP) may offer advantages (eg, disinfection), this practice has been associated with significant drawbacks, including tooth discoloration. In this study, the color change of dentin was monitored during treatment with distinct TAP pastes and novel tubular-shaped 3-dimensional electrospun scaffolds containing minocycline (MINO) or doxycycline (DOX).

Methods: Two TAP pastes (TAP\textsubscript{MINO} [MINO, metronidazole, and ciprofloxacin] and TAP\textsubscript{DOX} [DOX, metronidazole, and ciprofloxacin]), 4 scaffold-based groups containing MINO or DOX at distinct concentrations, 1 antibiotic-free scaffold, and 1 untreated group (control) were investigated. Human canines were sectioned at the cementoenamel junction and tubular-shaped scaffolds or paste were placed into the root canals and sealed. Color measurements (CIE\textsuperscript{L*}a*\textsuperscript{b*} parameters) were performed at baseline and after 1, 3, 7, 14, 21, and 28 days. Color changes were expressed as $\Delta E^*$ values. In addition, scanning electron microscopy and energy-dispersive X-ray spectroscopy were also performed on the specimens after treatment. Data were analyzed using repeated measures analysis of variance (alpha = 0.05).

Results: All antibiotic-containing groups led to greater discoloration than the antibiotic-free groups. A severe discoloration occurred after 1 day. At the end of the experiment, antibiotic-treated samples exhibited crusts/agglomerates over the dentin surface, which totally or partially obliterated the dentinal tubules. The presence of MINO resulted in a greater color change than DOX.

Conclusions: Scaffolds containing MINO or DOX produced similar color change to dentin when compared with their respective TAP systems, although DOX-related discoloration was less pronounced. (J Endod 2016;42:106–112)

Key Words
Antibiotic, discoloration, disinfection, electrospinning, regeneration, scaffold

Dental caries and/or trauma in immature permanent teeth can lead to infection of the dental pulp, with a potential negative impact on tooth development. In the past decade, regenerative-based endodontic therapy (ie, root disinfection with the so-called triple antibiotic paste [TAP] followed by evoked bleeding) has been shown to be a viable treatment option for immature teeth with pulpal necrosis (1) because this technique allows for the replacement of damaged pulp by living tissue and tooth development (2).

An essential stage of regenerative endodontics is disinfection of the root canal via intracanal application of medicaments (eg, calcium hydroxide or the combination of antibiotics) in the form of a paste (3–5). Although intracanal treatment with TAP may offer advantages such as effective disinfection and lower probability of allergic reactions compared with systemic antibiotics, this practice has been associated with stem cell toxicity (6), development of bacterial resistance (7), and significant tooth discoloration (8–12). It has been shown that TAP produces substantial color change on dentin, especially because of minocycline (MINO) use because it can bind to the calcium ions found in dentin via chelation (13).

Several clinical reports have also confirmed that crown discoloration may appear within 24 hours of MINO application (8) and possibly after 1 hour of smear layer removal and placement of the TAP paste (12). Interestingly, a case report on a novel approach to eliminating tooth discoloration suggested that sealing the dentinal tubules of the pulp chamber with a bonding agent would be a viable alternative (14) although still not completely efficacious. Furthermore, antibiotics other than MINO (eg, doxycycline [DOX], cefadroxil, etc.) were also shown to produce tooth discoloration when incorporated with a paste vehicle (9), thus raising an important concern regarding the use of these antibiotic-containing materials.

From a clinical perspective, the major consequence related to the discoloration produced on anterior teeth treated with TAP is esthetically driven. Additional treatments such as tooth bleaching and restorative procedures are necessary to improve visual appeal after endodontic therapy, which may increase chair time and costs for the patient. Thus, alternative therapies producing a lesser degree of tooth discoloration yet preserving the antimicrobial efficacy are paramount. In this way, polymer-based antibiotic-containing scaffolds recently have been shown to be a promising method for attaining effective disinfection (4, 15–17). Notably, the antibiotic concentration incorporated

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into these scaffolds is considerably lower when compared with the concentration used in conventional TAP (4, 18). Therefore, the following hypotheses were tested:

1. The use of novel tubular-shaped 3-dimensional (3D) antibiotic-containing electrospun scaffolds would produce less or no discoloration on dentin when compared with the clinically advocated TAP paste.

2. The presence of MINO would produce greater color change when compared with an alternative antibiotic (ie, DOX).

Materials and Methods

Study Design

This study evaluated the effects of antibiotic-based delivery systems (ie, TAP pastes containing different combinations of antibiotics or tubular-shaped 3D antibiotic-containing scaffolds) on the color change of human dentin. Eight groups were investigated according to the material placed into the root canal: 2 TAP paste groups (TAPMINO [MINO, metronidazole (MET), and ciprofloxacin (CIP)] and TAPDOX [DOX, MET, and CIP]); 4 groups of antibiotic-containing scaffolds (MINO or DOX); 1 group of an antibiotic-free scaffold (Scaffold) to serve as the control for the novel scaffolds; and 1 group to act as the control for the TAP paste groups (saline, 0.9% sodium chloride irrigation; Baxter Healthcare Corporation, Deerfield, IL). Forty specimens were tested in total, which were evaluated with a chroma meter at different time intervals up to 28 days and have color change (ΔE*) as the study's outcome.

Synthesis of Tubular-shaped 3D Antibiotic-containing Electrospun Scaffolds

Polydioxanone polymer solutions (PDS II; Ethicon, Somerville, NJ) were prepared by dissolving PDS in 1,1,1,3,3,3-hexafluoro-2-propanol at a concentration of 10 wt%. Each solution was then incorporated with MINO (Sigma-Aldrich, St Louis, MO) or DOX (Sigma-Aldrich) at 25 or 50 wt% (respective to the total polymer weight). Antibiotic-free PDS solution was also prepared to serve as the control. Each solution was spun (ie, flow rate of 2 mL/h, 18-cm distance, and electrical voltage between 15 and 19 kV) into nanofibers using a conventional electrospinning system and collected on a custom-made grounded Teflon-coated stainless steel mandrel (KECO Coatings, Inc, Indianapolis, IN) (2.0 ± 0.02 mm) connected to a high-speed stirrer (BDC6015; Caframo, Wiarton, ON) at 120 revolutions per minute. After processing, the scaffolds (Fig. 14 and B) were cut into tubular-shaped specimens (6.0-mm height ± 0.1 mm) and dried under a vacuum to remove any remaining solvent (15, 16).

Tooth Sample Preparation and Initial Color Measurements (Baseline)

Forty human canines were sectioned 3 mm above and 5 mm below the cementoenamel junction to obtain 8.0-mm-thick (±0.1 mm) enamel/dentin samples (8). The smear layer was removed by sample immersion in 2.5% sodium hypochlorite (NaOCl) solution for 3 minutes followed by 3 baths of distilled water (DI) and then immersion in 1% EDTA (Inter-Med, Inc, Racine, WI) for 3 minutes. The apical foramen of all tooth samples was closed using a bonding system (Adper Scotchbond Multi-Purpose; 3M ESPE, St Paul, MN) and resin composite (Z100, 3M ESPE). After that, each specimen was then stored in DI until use. The color of all specimens was evaluated under a chroma meter (Minolta CR-241; Konica Minolta Sensing Americas, Inc, Ramsey, NJ) considering the aperture size of 0.3 mm (smaller measurable circle, Fig. 1E); the 3 most anterior areas of the tooth were measured using the CIEL*a*b* color system, and the 3 measurements were then averaged. As shown in Figure 1F–H, the wall of the root canal of each sample was used as the initial parameter to determine the location of the bigger measurable circle (1.8 mm), thus facilitating the measurement of the same area at each time point investigated.

Application of Testing Materials and Final Color Measurements

The 3D tubular-shaped electrospun scaffolds were weighed (S + DOX25% = 9.48 ± 2.36 mg [DOX = 2.37 ± 0.59 mg], S + DOX50% = 7.54 ± 0.91 mg [DOX = 3.77 ± 0.46 mg], S + MINO25% = 6.60 ± 0.86 mg [MINO = 1.65 ± 0.22 mg], and S + MINO50% = 9.62 ± 0.52 mg [MINO = 4.76 ± 0.26 mg]) and directly placed into the root canal, sized to match the diameter, and perfectly (no gaps) adapted to the canal walls (Fig. 1C). Standardized preparation of the root canal was performed by instrumentation up to K-file #40 and Gates Glidden #6 and enlargement with a round bur (φ = 2.7 mm). Meanwhile, the antibiotic pastes were prepared by mixing the respective antibiotics MET (Sigma-Aldrich), CIP, and MINO (TAPMINO) or DOX (TAPDOX) with saline at the clinically used concentration (1 g/mL) and then placed into the root canal with a sterile Lentulo spiral filler. One group of specimens was filled with saline (control). The root canals were then temporary sealed (Cavit, 3M ESPE). All samples were stored inside 24-well plates at 37°C. A wet environment was created to mimic the root canal condition. Briefly, non-sticky wax was placed on the bottom surface of the plate to stabilize the tooth sample. All samples were stored for 28 days and kept in direct contact with the testing materials. Color measurements were taken on days 1, 3, 7, 14, 21, and 28 (Fig. 1D and E).

Color Change Analysis

The color change (ΔE*) of each sample (ie, an average of the 3 preselected tested areas, Fig. 1F–H) and at each time interval was assessed using the following formula:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa*, and Δb* corresponded, respectively, to the difference between the test days and baseline $L^*$, $a^*$, and $b^*$ values.

Scanning Electron Microscopy and Energy-dispersive X-ray Spectroscopy

After 28 days, 2 samples from each group were randomly allocated for scanning electron microscopic analysis. The teeth were mesiodistally cleaved with a size 5 LeCron instrument (Hu-Friedy Mfg Co, LLC, Chicago, IL) in 2 halves. Although 1 of the halves was cleaned with DI water to evaluate the dentin surface, the other half was kept untouched to analyze the overall aspects of the material placed inside the root canal (pastes or scaffolds). The specimens were sputter coated with Au-Pd before imaging (FE-SEM, Model JSM-6701F; JEOL Ltd, Tokyo, Japan). Energy-dispersive X-ray spectroscopic (EDS) analysis was done to study the chemical composition of the dentin surface and materials immediately after their removal from the canal.

Statistical Analysis

The quantitative color change (ΔE*) data were statistically analyzed (SigmaPlot version 12; Systat Software Inc, San Jose, CA, USA) using repeated measures analysis of variance. Time was repeated within each sample, and group was a between-sample factor. The Sidak
Figure 1. (A) Image showing the Teflon-coated stainless steel rod used to prepare the 3D tubular-shaped electrospun scaffolds. (B) and (C) Images showing the appearance of the 6-mm-long scaffolds and placement into the root canal. (D) The chroma meter used in the present study. (E) A schematic diagram showing the 3 preselected anterior areas of each specimen used to measure the color of dentin. (F–H) An actual experimental view of the preselected areas at 0.3 mm. (I) Graph showing the progression of ΔE* values with increased days of storage. (J–L) Graphs showing changes in the L*, a*, and b* color parameters of each group at each time point tested.
method was used to control the overall significance level of the pairwise group comparisons at 5% for each time point and location.

Results
The color change results obtained in the study are shown in Figures 1 and 2. According to the statistical analysis, both factors “material” and “storage period” were significant (P ≤ .001) although their interaction was not (P = .462). Overall, groups containing MINO exhibited considerably higher color change than groups containing DOX; additionally, all antibiotic-based groups resulted in greater discoloration compared with the antibiotic-free groups (P ≤ .001) (Fig. 2A). A severe discoloration (beyond the human perceptibility threshold, ie, \( \Delta E^* = 3.7 \)) occurred after the first day of placement of all antibiotic-containing materials (Fig. 1I). Although the discoloration effect clearly increased with greater periods of storage, specimens treated with S + DOX25%, S + MINO25%, and S + MINO50% showed similar \( \Delta E^* \) values after 28 days (Fig. 2A) when compared with 1 day of storage (P ≥ .098). Related to the individual color parameters tested (Fig. 1J–L), all groups became darker (reduction in the \( L^- \) axis); however, it was more intensive for groups treated with antibiotics. Specimens treated with MINO-based materials exhibited reduction of the \( a^+ \) axis (ie, they became greenish), especially after day 1, whereas specimens treated with DOX-based materials slightly increased the \( b^- \) axis (Fig. 1K). The \( b^- \) axis was not pointedly affected by the tested materials (Fig. 1L).

<table>
<thead>
<tr>
<th>A</th>
<th>Period of storage (in days)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>Scaffold (S)</td>
<td>3.0±(0.5)*</td>
</tr>
<tr>
<td>TAP DOX</td>
<td>3.8±(0.1)*</td>
</tr>
<tr>
<td>TAP MINO</td>
<td>8.3±(2.6)*</td>
</tr>
<tr>
<td>S+DOX25%</td>
<td>8.9±(2.9)*</td>
</tr>
<tr>
<td>S+DOX50%</td>
<td>10.2±(1.5)*</td>
</tr>
<tr>
<td>S+MINO25%</td>
<td>13.2±(3.6)*</td>
</tr>
<tr>
<td>S+MINO50%</td>
<td>13.8±(3.9)*</td>
</tr>
</tbody>
</table>

Distinct superscript uppercase letters before means (in a same column) and lowercase letters after ±SD (in a same row) indicate statistically significant differences among groups and periods of storage, respectively (p<0.05).

<table>
<thead>
<tr>
<th>B</th>
<th>Color change</th>
<th>Root canal surfaces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1 day</td>
<td>7 days</td>
</tr>
<tr>
<td>Control</td>
<td>scaffold (S)</td>
<td>TAP DOX</td>
</tr>
</tbody>
</table>

Figure 2. (A) Table showing the color change (\( \Delta E^* \)) means (±standard deviation) for all groups investigated including control (saline), scaffold (S) based on polydioxanone/PDS, triple antibiotic paste/TAP prepared with doxycycline/DOX or minocycline/MINO, and scaffolds containing 25 or 50 wt% of DOX or MINO at different periods of storage. (B) Representative macrophotographs showing the color stability/change of specimens after 1, 7, 14, and 28 days of storage.
Representative macrophotographs of tooth samples treated with the distinct antibiotic(s) delivery methods at each time point investigated, except after 3 and 21 days of storage, are presented in Figure 2B. Although untreated specimens (control) and those treated with the antibiotic-free scaffold (S) exhibited no dentin discoloration, specimens treated with antibiotic-containing materials suffered a visually circumferential discoloration. The images also show that samples treated with MINO-based materials became greenish, whereas those treated with DOX became brownish. With regard to the root canal surfaces at the end of the experiment, surfaces treated with TAPMINO showed the most dentin discoloration.

Figure 3 shows the SEM micrographs and EDS spectra of the dentin surface after direct contact with the tested materials for 28 days as well as the surface of the materials used. Untreated specimens presented completely open dentinal tubules (no obliteration) and showed intense detection of calcium and phosphorus. In contrast, specimens treated with scaffolds and TAPDOX showed partial obliteration of the tubes and a vast amount of organic residues (carbon-based compounds). Specimens treated with TAPMINO exhibited nearly complete obliteration of the tubules and the presence of elements, such as zinc, calcium, phosphorus, and tellurium. Other chemical elements were also observed on the S + MINO50%–treated specimens. Thick crusts could be seen attached to the dentin surface of all specimens treated with antibiotics. Regarding the material's surface, all scaffolds appeared to be moderately intact after 28 days of storage. Within the TAPDOX paste, furule-shaped powder composed of carbon and oxygen was observed, whereas rodlike powder composed of carbon, oxygen, and chlorine was noted within the TAPMINO paste.

**Discussion**

The first hypothesis of this study was to determine whether the use of novel tubular-shaped 3D antibiotic-containing scaffolds would produce less or no discoloration on dentin when compared with the traditionally used TAP paste (ie, TAP prepared with MINO). Based on the reported findings, this hypothesis was rejected. Surprisingly, both antibiotic vehicles (ie, scaffold or paste) led to similar reported findings, this hypothesis was rejected. Surprisingly, both anti-
tibiotic paste (ie, TAP prepared with MINO). Based on the antimicrobial potential of novel TAP-mimic scaffolds has recently been shown against an *Actinomyces naeslundii* (4) biofilm, a very prevalent microorganism found in necrotic permanent immature teeth (25). The formation of calcium-enriched agglomerates over the dentin surface was also seen in a former study causing staining of the substrate, a phenomenon that was attributed to the presence of MINO (4). It can be speculated that the aforementioned agglomerates would affect not only the tooth color but also the structural and morphologic characteristics of dentin. These characteristics could have implications in the regenerative outcome. In this study, all specimens treated with antibiotic(s) exhibited the presence of crusts/agglomerates partially covering the dentin surface (Fig. 3). These agglomerates showed a heterogeneous composition (EDS analyses) although calcium elements could also be detected, corroborating the results of a previous study (4). Once again, it can be noticed that even the less concentrated scaffold groups resulted in the formation of these crusts, denoting that the contact of the antibiotic(s) with the substrate, regardless of the concentration or vehicle used, is sufficient to form these mineralized agglomerates. Considering that MINO and DOX have a very small structure (please note the SEM micrographs showing the shape and size of the antibiotic powders), it can be speculated that antibiotics can interact easily with the dentin components, forming complex agglomerates that may serve as an antibiotic(s) reservoir. Although one might view this reservoir ability as an advantage as it pertains to the antibacterial potential of the treatment, the application of antibiotics into the root canal is known to negatively affect the survival of stem cells (6), and it changes the morphologic characteristics of radicular dentin (26), possibly limiting their use. It is shown in the SEM micrographs that specimens treated with antibiotics revealed the dentinal tubules as obliterated by these crusts, considerably reducing dentin permeability. Conversely, the specimens that did not receive any antibiotic treatment exhibited no tubule obliteration (control) or only slight obliteration (scaffold) (Fig. 3).

The use of antibiotic-containing scaffolds during regenerative endodontic therapy is quite new in dentistry and should be investigated further in order to clarify the effects of these biomaterials on dentin properties. Although the experimental scaffolds containing MINO or DOX did not prevent dentin discoloration, this strategy carries clinical
Figure 3. SEM micrographs and EDS spectra showing the morphology and superficial chemical composition of dentin (cleaned surface) and the tested materials (material’s surface) after 28 days of experiment. Untreated specimens (control) did not show dentinal tubule obliteration; calcium and phosphorus were intensively detected. Specimens treated with scaffolds and TAPDOX showed partial dentinal tubule obliteration and a vast amount of organic residues (carbon-based compounds). Specimens treated with TAPMINO exhibited nearly complete dentinal tubules obliteration and the presence of elements, such as zinc, calcium, phosphorus, and tellurium. Thick crusts could be seen attached to the dentin surface of all specimens treated with antibiotics. Regarding the material’s surface, all scaffolds appeared to be moderately intact after 28 days of storage.
potential for the treatment of necrotic teeth. The replacement of MINO by other antibiotics, such as amoxicillin and clavulanic acid (27), is under investigation as a potential discoloration-free strategy. The method used in this study to measure color change was based on the CIEL*a*b* color system, which is a device-independent system. Although it has been considered the universal method to assess color changes that are easily perceptible to the human eye, one should bear in mind that the use of an alternative system such as the RGB color space might have produced different results. Nonetheless, the combination of the quantitative (ΔE) and qualitative (macrophotographs) data allowed for a satisfactory collection of important information regarding the dentin discoloration potential of antibiotics used for regenerative endodontics.

Taken together, scaffolds containing MINO or DOX produced similar color changes to dentin when compared with their respective TAP paste systems. Materials containing DOX suffered less discoloration than materials prepared with MINO. Both drugs were associated with the formation of crusts/agglomerates over the radicular dentin surface.

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The authors deny any conflicts of interest related to this study.

References


Regenerative Endodontics