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ORIGINAL ARTICLE

Prevention of coronal discoloration induced by regenerative endodontic treatment in an ex vivo model

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Abstract
Objectives The aim of this study was to assess the effect of sealing the pulp chamber walls with a dentin-bonding agent (DBA) on prevention of discoloration induced by regenerative endodontic procedures (REPs) in an ex vivo model.
Materials and methods Ninety-six bovine incisors were prepared and randomly divided into two groups. In one group, the pulp chamber walls were sealed with DBA before placement of triple antibiotic paste (TAP) containing minocycline inside the root canals, but in the other group, DBA was not applied. After 4 weeks, the root canals were filled with human blood and each group was then randomly divided into four subgroups ($n = 12$) according to the endodontic cements placed over the blood clot (ProRoot MTA, OrthoMTA, RetroMTA, or Biodentine). The color changes (ΔE) were measured at different steps. The data were analyzed using *t* test and two-way ANOVA.
Results The specimens in which dentinal walls of pulp chamber were sealed with DBA showed significantly less coronal discoloration at each step of regenerative treatment ($p < 0.001$). However, application of DBA did not completely prevent the clinically perceptible coronal color change. Sealing the blood clot with different endodontic cements did not result in significant difference in coronal discoloration ($p > 0.05$).
Conclusions Sealing the pulp chamber walls before insertion of TAP decreased coronal discoloration following REP using different endodontic cements but did not prevent it.
Clinical relevance Discoloration of teeth undergoing REPs is an unfavorable outcome. Considering the significant contribution of TAP containing minocycline to the coronal tooth discoloration even after sealing the pulp chamber walls, the revision of current guidelines in relation to the use of TAP with minocycline might need to be revised.

Keywords Biodentine · Calcium silicate-based materials · Coronal discoloration · Dentin-bonding agent · Mineral trioxide aggregate · Regenerative endodontic treatment

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Introduction

Regenerative endodontic procedures (REPs) generally involve disinfection of the root canal followed by introduction of a blood clot or platelet-rich plasma/platelet-rich fibrin, which is sealed with a bioactive material such as white mineral trioxide aggregate (MTA) [1]. These procedures result in thickening of the root canal walls and continuing root development [2–5]. However, coronal discoloration of teeth that have undergone REPs is an unfavorable outcome [6–8].

A broad range of differences in clinical protocols is seen in the studies related to REPs. The "Regenerative Endodontics Committee" founded by the American Association of Endodontists (AAE) has recommended some considerations for a regenerative procedure [9] which has been lately

revised in 2016 [1]. Furthermore, the European Society of Endodontology (ESE) has developed a position statement regarding revitalization procedures in 2016 [10].

It has been shown that the most popular medicament in REPs is triple antibiotic paste (TAP) composed of ciprofloxacin, metronidazole, and minocycline [11]. Although ESE position statement suggests a nondiscoloring calcium hydroxide as an intracanal medicament [10], TAP has been still recommended by AAE clinical considerations [1]. The ability of antibiotic combinations to eliminate bacteria within dentinal tubules [2, 12] has been the main reason for the widespread use of TAP in REPs. On the other hand, TAP has been associated with significant tooth discoloration [6, 13].

Although antibiotic compounds, especially those containing minocycline, have been attributed as the major cause of discoloration, entrance of evoked bleeding into the root canal and placement of MTA just below the cementoenamel junction (CEJ) for sealing the blood clot can be considered as the other factors that underline the crown discoloration. In a data analysis of clinical protocols, it has been reported that MTA was used as the intracanal coronal barrier in 85% of the clinical articles. Several studies have reported undesirable tooth discoloration after the use of MTA [14–18]. Other calcium silicate-based materials such as Biodentine (Septodont, Saint Maur des Fossés, France), OrthoMTA, and RetroMTA (BioMTA, Seoul, Korea) have been introduced to overcome the shortcomings of MTA including coronal discoloration.

In order to minimize the risk of crown discoloration, AAE considerations for REPs suggest the sealing of pulp chamber in case of using TAP, as well as covering the blood clot with bioceramics or other calcium silicate-based materials (e.g., Biodentine) as alternatives to MTA [1]. Therefore, this study aimed to assess the preventive role of a dentin-bonding agent on the coronal discoloration via sealing the pulp chamber walls, in an ex vivo model of regenerative endodontic procedure using triple antibiotic paste as the intracanal medicament and different endodontic cements (ProRoot MTA, OrthoMTA, RetroMTA, and Biodentine) for coronal sealing of blood clot.

Materials and methods

Ninety-six bovine mandibular incisors having almost identical crown and root size from animals of approximately the same age were selected. Teeth were disinfected by immersion in 0.5% chloramine T solution for 48 h and then stored in normal saline solution until use.

Experimental setup

The apical portion of each specimen was resected to obtain a standardized root length of 15 mm below the labial CEJ. In order to seal the apical openings with composite resin material, the apical 4 mm of the root canals and total surrounding dentin of cross-sectional surface were etched for 15 s with 35% phosphoric acid (Vococid, Voco, Germany) and rinsed. A dentin-bonding agent (Solobond M, Voco, Germany) was applied to the etched surfaces and cured for 20 s. Composite resin material (Grandio, Voco, Germany) was then placed incrementally and cured using a LED curing light (Valo, Ultradent, Products Inc., South Jordan, USA) for 40 s. After that, endodontic access cavities were prepared and the root canals were shaped using #3 to 6 Gates-Glidden drills. The root canals were irrigated with 20 mL 1.5% NaOCl followed by 20 mL 17% EDTA each for 5 min. After the root canal space was dried using paper points, the specimens were randomly divided into two groups (n = 48) as follows:

Group 1: *DBA*. The inner surfaces of the pulp chamber in this group were etched for 15 s with 35% phosphoric acid (Vococid, Voco, Germany) and rinsed. The dentin-bonding agent (Solobond M, Voco, Germany) was applied to the etched surfaces and cured for 20 s.
Group 2: *No DBA*. The inner surfaces of the pulp chamber were not sealed with DBA.

In the next step, triple antibiotic mixture was prepared by mixing equal proportions of ciprofloxacin, metronidazole, and minocycline (1:1:1) with sterile water to a final concentration of 0.1 mg/mL and was delivered into the root canals via a syringe to ensure that the solution was confined to the root canal below the labial CEJ. After that, the access cavities were sealed with a temporary filling material (Zonalin, Kemdent, UK). The specimens were then incubated at 37 °C in fully saturated humidity for 4 weeks. Then, temporary coronal seal was removed and the root canals were irrigated with 20 mL 17% EDTA for 5 min and dried with paper points. In group 1, in order to deal with the possibility of previous DBA layer impairment during temporary filling material removal, DBA was reapplied on the access cavity walls in the same manner described for the first application of DBA. In both groups, the root canals were then filled with human blood using a syringe until 4 mm below the facial CEJ. Whole fresh human blood was collected from healthy consented volunteers by a trained individual and approved by a panel from the Tehran University of Medical Sciences Ethical Committee (Ethics code: IR.TUMS.VCR.REC.1395.649). A blood clot was allowed to form for 15 min. After that, a resorbable collagen matrix (Bone Protect Fleece, Dentegris, Germany) was placed over the blood clot. The specimens of each group were then randomly assigned to one of four experimental subgroups (n = 12) and labeled according to the applied endodontic cements: ProRoot MTA, OrthoMTA, RetroMTA, and Biodentine. Each material was prepared according to the manufacturers' instructions and placed on the top of the collagen

matrix in a homogeneous layer of about 3 mm underneath the facial CEJ. A cotton pellet wetted with saline was then placed over the endodontic materials and the cavity was temporarily sealed with Coltosol (Coltene, Altstätten, Switzerland). The specimens were incubated at 37 °C in fully saturated humidity for 72 h. Subsequently, the access cavities were filled with composite resin material, A1 shade (Grandio, Voco, Germany). The shade of the composite was matched to the coronal tooth structure measured with a spectrophotometer (Vita Easyshad; VITA Zahnfabrik, Bad Säckingen, Germany).

Tooth color assessment

For reproducible color assessment, a window measuring 3 × 3 mm was created on the cervical and middle thirds of the crowns using a needle-shaped diamond bur in such a way that two thirds of its height was in the cervical and one third was in the middle third of the tooth crown [18]. A spectrophotometer (Vita Easyshad; VITA Zahnfabrik, Bad Säckingen, Germany) was used to measure the L^*, a^*, and b^* values. L^* indicates the value of lightness-darkness, a^* indicates greenness-redness, and b^* indicates blueness-yellowness. The device was calibrated before use for each specimen. The color measurements were taken at the following five steps:

S0: prior to placement of antibiotic mixture as the baseline color
S1: 4 weeks after placement of antibiotic mixture
S2: immediately after placement of endodontic cements
S3: immediately after permanent filling of the access cavity
S4: 4 months after permanent filling of the access cavity

Color measurements were performed three times inside the marked window on each for every reading, and the mean value of three measurements was calculated. The color change (ΔE) between the baseline (S0) and the other steps (S1, S2, S3, and S4) was calculated using the following formula:

$$\Delta E = \left[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\right]^{1/2}$$

Photographs were taken of the specimens at each treatment step using a digital camera for informal visual comparison.

Statistical analysis

Data were evaluated using SPSS software (PASW Statistics 18; SPSS Inc., Chicago, IL). The effect of bonding agent on the color changes at the end of antibiotic therapy (S1) was analyzed using t test. The effect of dentin-bonding agent and the type of endodontic cement on color change at the end of S2, S3, and S4 compared to baseline color (S0) was analyzed using two-way ANOVA. The level of statistically significant difference was set at $p < 0.05$.

Results

The mean values for the color changes in each subgroup are shown in Fig. 1. The results of this study showed that sealing the dentinal walls of the access cavity with DBA significantly decreased crown discoloration at any step ($p < 0.001$). Antibiotic therapy made the most contribution to coronal discoloration in both groups with or without DBA compared to the other treatment steps. Considering the type of endodontic cements, there was no significant difference between the coronal discoloration *in* subgroups of four endodontic cements neither at the end of treatment (S3) nor after the 4-month follow-up (S4) ($p > 0.05$).

Although sealing the access cavity walls with DBA significantly decreased the coronal discoloration, all specimens exhibited a clinically perceptible discoloration ($\Delta E \geq 3.3$) at the end of each step. Photos of a specimen from each group with or without the application of DBA are shown in Fig. 2.

Discussion

Crown discoloration has been reported as an unfavorable outcome following REPs. This is the first study to assess the role of sealing the dentinal walls of the pulp chamber using a dentin-bonding agent (DBA) before the initiation of regenerative endodontic procedures in preventing coronal discoloration using an ex vivo model for regeneration. In the present study, bovine incisors were selected to evaluate coronal discoloration. It has been shown that bovine teeth are suitable substitute for human teeth in assessing the crown discoloration [13, 19, 20]. The use of human teeth in ex vivo studies is limited due to ethical issues, difficulty to obtain adequate restorations or caries-free specimens, and insufficient flat labial surface for color measurement [21]. Furthermore, it is easy to create a model for immature teeth with thin dentinal walls and wide canal lumens with bovine teeth.

In the current study, sealing the dentinal tubules with DBA before placement of TAP into the root canals significantly decreased the coronal discoloration at the end of the antibiotic therapy as well as at the completion of treatment. But it is important to mention that DBA did not completely prevent the clinically perceptible color change of the crown induced by TAP. This result is in accordance with Kim et al. [6] who showed that the use of an adhesive dentin-bonding (AdheSE; Ivoclar Vivadent, Schaan, Liechtenstein) on the dentinal walls of the root canals before insertion of TAP reduced the overall

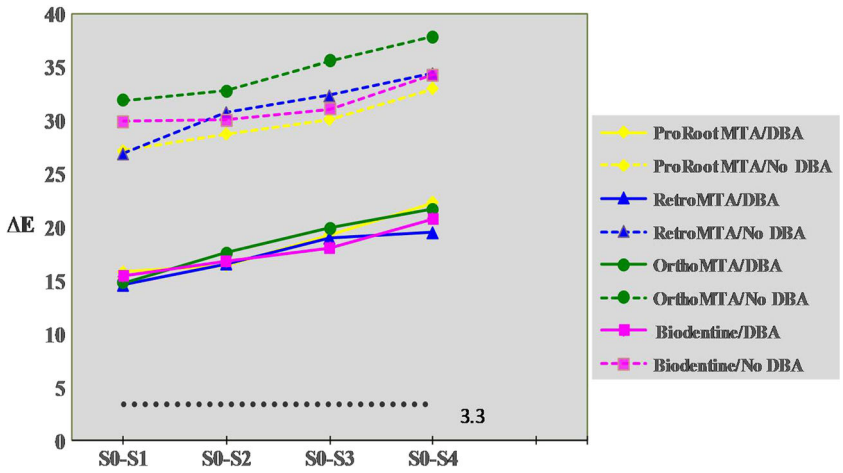

Fig. 1 Mean ΔE value changes at each treatment step. The dotted line represents clinically perceptible discoloration of $\Delta E \geq 3.3$

color change of the crown but did not completely prevent it. However, it seems to be more reasonable to apply DBA on the access cavity walls rather than into the root canals in REPs because of the handling problems associated with using DBA within the root canals. Moreover, covering the dentin which surrounds the canal might compromise the outcome of regenerative endodontic treatment.

TAP containing minocycline is the most popular of all intracanal medicaments in endodontic regenerative treatments. It has been shown that minocycline and TAP containing minocycline are associated with substantial tooth staining [6, 13]. Minocycline chelates the calcium ions and stains the dentin [22]. It has been found that even after irrigation using different techniques; about 88% of the TAP remained in the root dentinal walls to a depth greater than 350 μm [23]. In the present study, the root canals were medicated with TAP for 4 weeks. The technique for revascularization introduced by Banchs and Trope [24] included TAP dressing for 4 weeks. Since then, a large number of clinical studies, case reports, and case series have been published concerning regenerative endodontic treatments, revealing a broad range of variety in treatment protocol. In a data analysis of clinical protocols used in REPs, Kontakiotis et al. [11] showed that the root canals were medicated with minocycline-containing TAP for ≥ 3 weeks in most studies. Furthermore, AAE considerations for endodontic regenerative procedure recommend a low concentration of TAP for 1–4 weeks. In the current study, the clinical observation of specimens which were not sealed with DBA revealed severe coronal discoloration within the first 2 weeks of antibiotic therapy. In an in vitro study on dentin discoloration induced by TAP and other antibiotics, Porter et al. [25] showed no significant difference between ΔE values after 28 days of storage when compared with 21 and 14 days. Kim et al. [6] also showed no significant difference between mean changes in E^*, L^*, a^*, and b^* values after 7 days compared with 14 days of storage. In the present study, coronal discoloration was clinically visible 24 h after filling the root canals with TAP in the specimens in which the access cavity walls did not seal with DBA. This is in accordance with the finding of studies that showed discoloration began to appear 24 h after insertion of TAP [6, 25]. Therefore, reducing the duration of antibiotic therapy from 21–28 days to 1–2 weeks or even 24–48 h does not seem to be completely effective in preventing coronal discoloration [6, 25].

Another step in regenerative endodontic procedure is to induce bleeding and formation of a blood clot in the root canal

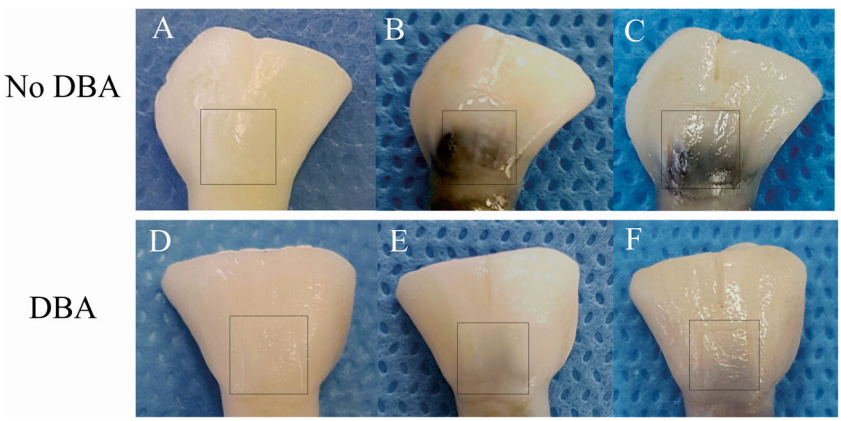

Fig. 2 Photographs of teeth in which pulp chamber was not sealed (no DBA) or sealed (DBA) with dentin-bonding agent. **a**, **d** At baseline. **b**, **e** At the end of the 4-week antibiotic therapy. **c**, **f** At the 4-month follow-up. The color changes were measured within the defined area

followed by placement of endodontic cements below the CEJ for sealing the blood clot. In this study, the blood was inserted into the canals using a syringe to the limit of 4 mm below the labial CEJ. However, in some clinical situations, it may be difficult to control the level of blood in the root canal and confine it to the ideal level, which is 3–4 mm below the gingival margin. Therefore, the dentinal walls of pulp chamber might be contaminated with blood. Discoloration of tooth structure induced by blood has been shown by many studies [14, 18, 26, 27]. Penetration of erythrocytes into the tooth structure [14] and accumulation of hemoglobin and hematin molecules within dentinal tubules following hemolysis of erythrocytes [28] are among the possible mechanisms of tooth discoloration in REPs.

Several studies have revealed the unfavorable discoloration of tooth structure induced by calcium silicate-based materials [14–18]. The composition of a material is of great importance in its discoloration potential. The resultant discoloration depends on metal constituents such as bismuth, iron, aluminum, and magnesium. Possible mechanisms of tooth discoloration are related to the oxidation of the iron content [14], oxidation of bismuth oxide [29, 30], and interference of bismuth oxide with dentin collagen [21]. Calcium silicate-based materials with alternative radiopacifiers such as calcium zirconia complex, zirconium oxide, or tantalum oxide are available clinically. It has been shown that discoloration potential of materials which do not include bismuth oxide in their formulation (e.g., RetroMTA, Biodentine, and Neo MTA Plus) was significantly lower than that of materials containing bismuth oxide (e.g., ProRoot MTA) [15, 27, 31–33]. However, those mentioned studies did not evaluate the discoloration potential of materials in the presence of blood contamination. The results of the present study showed no significant difference between the discoloration potential of ProRoot MTA, OrthoMTA, RetroMTA, and Biodentine neither in DBA nor in no DBA groups at the end of the treatment and at a 4-month evaluation. In the present study, although endodontic cements were placed over a collagen matrix to avoid apical displacement of the material, the cements were approximately in contact with blood. The results of the current study are in agreement with those of previous study that showed no significant difference between the coronal discoloration induced by ProRoot MTA, OrthoMTA, and Biodentine in the presence of blood contamination [18]. It has been shown that blood contamination significantly exacerbated discoloration associated with calcium silicate-based materials containing bismuth oxide or other radiopacifiers [14, 18, 26]. One hypothesis put forward to explain the exacerbation of tooth discoloration in the presence of blood would be the absorption and hemolysis of erythrocytes to calcium silicate-based materials which can cause the discoloration of the material and tooth structure [14, 26]. In the recent study, another explanation for similar findings with ProRoot MTA and its recommended alternatives by the AAE clinical considerations might be the extreme discoloration potential of TAP which may mask the effect of different endodontic cements on the color change.

The application of DBA on the dentinal walls of the pulp chamber would decrease the interaction between minocycline/dentin and bismuth oxide/collagen as well as the entrance of erythrocytes into dentinal tubules, leading to less coronal discoloration following regenerative endodontic treatment. Furthermore, coverage of access cavity walls with DBA makes them to be more easily cleaned in case of contamination with TAP or blood.

It is worth mentioning that in endodontic regenerative treatments, the color changes associated to each step of treatment could not be measured per se because of the presence of the color change induced in the previous treatment steps. Therefore, the results of this study cannot be simply compared with those of some other studies which have evaluated the tooth discoloration caused by calcium silicate-based materials or intracanal medicaments.

Based on the results of this study, application of DBAs may be recommended to seal the dentinal tubules of the pulp chamber; especially when minocycline-containing TAP is used as the intracanal antimicrobial agent in endodontic regenerative treatments. However, there may be concerns about the toxic effect of different bonding systems on the stem cells, especially in unpolymerized forms [34, 35]. Given that in REPs, the blood clot as a rich source of stem cells is attempted to be placed below the CEJ, the possible risk of direct contact between stem cells and dentin-bonding agents, which are applied on the pulp chamber walls and already completely cured before the stage of blood entry into the root canal system, would be the least. Thus, in order to prevent the contact between DBAs and stem cells, along with providing enough space for endodontic cements to be placed over the blood clot, it recommends that the blood clot be limited to a few millimeters below CEJ level. Moreover, further researches would be advisable to access DBAs with the least toxic effect on the stem cells used in regenerative treatments.

Conclusions

Taken together, sealing the dentinal tubules of pulp chamber before application of TAP containing minocycline into the root canal led to a significantly less tooth discoloration. However, application of DBA did not completely prevent the clinically perceptible color change. Sealing the blood clot even with bioceramics and nonbismuth oxide containing calcium silicate-based materials in teeth treated with TAP containing minocycline did not resolve the problem of discoloration. Considering the significant contribution of minocycline-containing TAP to the coronal tooth discoloration, the current guidelines in relation to the use of TAP with

minocycline as an intracanal medicament might need to be revised.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by a panel from the Tehran University of Medical Sciences Ethical Committee (Ethics code: IR.TUMS.VCR.REC.1395.649).

Informed consent Informed consent was obtained from all individual participants included in the study.

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