The Effects of EDTA on Blood Clot in Regenerative Endodontic Procedures

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Abstract

Introduction: In regenerative endodontic procedures (REPs), a blood clot acts as a natural scaffold for regenerating dental pulp tissue. In current protocols, 17% EDTA is recommended for liberating growth factors from root dentin. Although EDTA affects clot formation in periodontal studies, the anticoagulant effect of EDTA has not been shown in REPs. Therefore, this study aimed to evaluate the effects of 17% EDTA on the characteristics and fiber density of blood clots using in vitro dentin blocks. Methods: The roots of 35 human mandibular premolars were prepared to simulate open apices and irrigated with the following protocols: (1) normal saline solution (NSS), (2) EDTA (1 minute) + NSS (E1N), (3) EDTA (5 minutes) + NSS (E5N), (4) EDTA (1 minute) + NSS (E1), and (5) EDTA (5 minutes) (E5). The roots were split, and human blood was placed. The characteristics and fibrin density of clots were observed using a scanning electron microscope. Fibrin densities in all irrigation groups were evaluated using image software (National Institutes of Health, Bethesda, MD) and statistically analyzed using the Friedman test and the Kruskal-Wallis test with Bonferroni adjustment. Results: Samples in the NSS, E1N, and E5N groups revealed denser fibers with an abundance of erythrocytes when compared with those in the E1 and E5 groups. Fiber densities in the E1 and E5 groups showed significantly lower values than those in the NSS, E1N, and E5N groups in all regions of roots (P < .05). No statistically significant difference at all levels was observed in all irrigation groups. Conclusions: A decrease in clot formation was affected by EDTA irrigation for 1 and 5 minutes. Final flushing with NSS could improve fibrin formation. (J Endod 2019;45:281–286)

Key Words
Blood coagulation, EDTA, fibrin, regenerative endodontics

Significance
Clinicians are confronted with problems creating blood clot during REPs, especially with a prolonged clotting time. EDTA has high affinity to chelate calcium ions from blood. This study showed EDTA affects blood cells and fibrin formation in root canals.

The treatment of necrotic immature teeth is challenging because of their short and thin root canal wall. One of the best types of treatment, which attempts to preserve root dentin structure and enhances root development, is regenerative endodontic procedures (REPs) (1). This procedure is based on the concept of tissue engineering, consisting of stem cells, scaffolds, and essential growth factors (2).

Blood is usually created from periapical tissues and influxes into a root canal space. A clot naturally occurs after tissue injury by activation of thrombin and fibrinogen to form a cross-linked fibrin network scaffold. Blood clots have been broadly used in the biomedical field as a scaffold for stem cell homing (3) because they consist of essential growth factors to support stem cell proliferation and differentiation (1, 4–7). However, an ideal natural scaffold should show

1. an adequate porosity for cell seeding;
2. effectiveness to transport the nutrients, oxygen, and waste;
3. appropriate physical and mechanical strength;
4. a minimal degree of an inflammatory response; and
5. a similar biodegradable ability compared with the tissue regeneration process (2).

The American Association of Endodontists and the European Society of Endodontology recommend clinicians use 17% EDTA solution as a final irrigation (8, 9). The primary use of EDTA is as an irreversible chelating agent. It binds calcium ions and liberates the growth factor from root dentin. In medicine, EDTA is also used as an anticoagulant in blood collection tubes (10). However, the effects of EDTA on blood clots inside an EDTA-treated root canal have not yet been shown. The present study aimed to investigate the effects of residual EDTA on the microscopic feature of blood clot in root canal including characteristics and fiber density. The null hypothesis was that there is no difference in blood clot characteristics among irrigation groups using scanning electron microscopy.

Materials and Methods

The study protocol was approved by the Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University Institutional Review Board for ethics approval (COE no. MU-DT/PY-IRB 2017/024.1807). Thirty-five human single-rooted mandibular...
premolars extracted for orthodontic reasons were included in this study. Periodontal tissues were removed with a scalpel blade. Then, the selected teeth were stored in a 0.1% thymol solution at 4°C until use.

**Sample Preparation**

The teeth were cut perpendicularly to the long axis of the root at the cementoenamel junction level using a slow-speed diamond saw (Isomet; Buehler Limited, Lake Bluff, IL). The apical end of the sectioned root was further trimmed until obtaining a uniform length of 9 mm. The root segment was then prepared using no. 1—4 Gates Glidden drills (Kerr, Kerr Corporation, Orange, CA) through each canal to achieve an open apex of 1 mm in diameter under 20 mL 1.5% sodium hypochlorite irrigation (11).

After that, 2 longitudinal grooves were made on the external surface along the specimen at the buccal and lingual aspects using a high-speed cylindrical diamond bur under copious irrigation without hitting the root canal. Then, 2 parallel horizontal grooves were made beneath the cementoenamel junction for 3 and 6 mm at the longitudinal grooves to separate the specimen into coronal, middle, and apical portions, respectively.

**Irrigation Protocols**

The specimens were randomly divided equally into 5 experimental groups. Before irrigation, the specimens were settled in plastic drawers with impression material (Silagum-Putty; DMG Chemisch-Pharmazeutische, Hamburg, Germany). A 20-mL disposable plastic syringe (Nipro Hypodermic Syringe; Nipro Corporation, Osaka, Japan) with a 25-G irrigation needle (Nipro Hypodermic Needle, Nipro Corporation) was used and positioned approximately 1 mm from the root end to reproduce the clinical protocol. Each experimental group was irrigated as follows:

1. The NSS group: 20 mL normal saline solution (NSS) for 5 minutes
2. The E1N group: 20 mL 17% EDTA for 1 minute followed by 20 mL NSS for 5 minutes
3. The E5N group: 20 mL 17% EDTA for 5 minutes followed by 20 mL NSS for 5 minutes
4. The E1 group: 20 mL 17% EDTA for 1 minute
5. The E5 group: 20 mL 17% EDTA for 5 minutes

After that, the sectioned roots were dried with sterile paper points. The specimen was split vertically in half using a cutting instrument (Chisels; Hu-Friedy, Chicago, IL), and 1 of the dentin blocks was selected to test.

**Blood Sample Collection**

The blood sample was collected from a healthy volunteer who did not smoke; have anemia, acute infection, liver disease, jaundice, or coronary syndrome; or take any ongoing anticoagulant medication (12). The blood sample was collected from a healthy volunteer who did not smoke; have anemia, acute infection, liver disease, jaundice, or coronary syndrome; or take any ongoing anticoagulant medication (12).

**Specimen Preparation for Scanning Electron Microscopy**

The specimens were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 1 hour. The sample was washed 3 times in distilled water for 10 minutes. Subsequently, the samples were dehydrated in a series of ethanol dilutions (30%, 50%, 70%, 90%, and 95%) and 3 times at 100% with an equilibration step of 5 minutes each). After fixation, the samples were dried using hexamethyldisilazane (Sigma-Aldrich, St Louis, MO) for 2 hours under an analog steel fume hood (GTech; PromotionSci, Nonthaburi, Thailand). All processes were conducted at room temperature. The samples were sputter coated with 12.5 nm platinum-palladium at 2.2 kV and 20 mA for 5 minutes using an E-102 Ion Sputter (Hitachi, Tokyo, Japan). Areas at the coronal, middle, and apical portions of the root were visualized with a JSM 6610LV scanning electron microscope (JEOL, Tokyo, Japan) at 500× and 2500× magnifications.

**Blood Clot Characterization**

The characteristics of blood clots were observed by direct visual examination at 2500× magnification in 5 random areas at each level of the sample. Low-magnification images were taken at 500× magnification in the center of all levels to validate and evaluate the homogeneity of the specimen in a larger area.

**Fiber Density of Blood Clots**

Micrographs at 2500× magnification were evaluated using ImageJ software (National Institutes of Health Bethesda, Bethesda, MD). Fiber density was determined using modified methods proposed by Hugenholtz et al (15). A 5 × 6 line grid was placed on individual images, and the number of fibers intersecting the lines were counted using ImageJ software. The total number of fibers crossing the line per 10 μm at each level of the sample from the 3 micrographs was averaged (15).

**Statistical Analysis**

Before evaluation, intraobserver reliability of fiber density was assessed with the intraclass correlation coefficient. Comparison of fibers per 10 μm among 5 experimental groups at each level was performed using the Kruskal-Wallis test, and pair-wise comparisons were then undertaken with Bonferroni adjustment. A comparison of fibers per 10 μm among the levels for each group was performed using the Friedman test. All statistical data analyses were performed using SPSS Version 18.0 (SPSS Inc, Chicago, IL). A P value < .05 was considered statistically significant.

**Results**

**Characteristics of Blood Clots**

The clot surface in the NSS group was composed of a dense meshwork of fibrins with abundant biconcave erythrocytes at all levels of the root canal surfaces (Figs. 1A, 2C, and 3A). The biconcave erythrocytes were the most commonly formed elements trapped inside the fibrin network. The surfaces of clots in the E1N (Figs. 1B, 2B, and 3B) and E5N groups (Figs. 1C, 2C, and 3C) were also composed of a dense meshwork of fibrins with abundant biconcave erythrocytes at all levels of the root canals. Occasionally, stacks of shrinkage erythrocytes were found in E5N group (Fig. 2C arrows).

The number of fibrin networks in E1 (Figs. 1D, 2D, and 3D) and E5 specimens (Figs. 1E, 2E, and 3E) at all levels of the root canal surfaces were dramatically lower and shorter than that observed in NSS specimens (Figs. 1A, 2A, and 3A). Occasionally, inactivated platelets clumping with a few fibrin fibers were observed in E1 (Figs. 1D and 2D arrows) and E5 specimens (Fig. 1E arrows).

**Fiber Density**

Before evaluation, the intraclass correlation coefficient was 0.99. Fiber density in the NSS group showed the greatest values of 11.60, 12.38, and 13.33 fibers per 10 μm at the coronal, middle, and apical portions, respectively. The median values of fiber density in E1N and E5N groups were lower than in the NSS group at all levels (P > .05).
E1 and E5 specimens showed statistically significantly lower fiber density values than NSS, E1N, and E5N specimens at all portions of the root (P < .05). Nevertheless, the number of fibrins at each level did not statistically significantly differ in all groups (P > .05) (Table 1).

**Discussion**

Blood clotting is the process of forming a natural clot wherein blood changes from a liquid to a gel. It has many advantages over other alternative scaffolds, such as no allergic reaction, reduced costs and visiting time, convenience, and comfort for patients (2). The clotting process involves many blood cells and clotting factors. One of the clotting factors is calcium ions. Calcium ions play an important role in the clotting process as a cofactor. They are required for the activation of factors II, VII, IX, X, and platelets (16). In an EDTA-treated canal, the calcium ions in blood are chelated by residual EDTA and result in interruption of the clotting process.

Previous research has shown that fibrin and platelets were primarily on the outside of the clots. In contrast, inside the clot was composed mostly of erythrocytes with little fibrin and few platelets (17). Therefore, the present study aimed to investigate the chelating effect of residual EDTA on the microscopic features of dentin-contacted blood clots in root canal dentin. Scanning electron microscopy was used to analyze blood clots because it can visualize the microscopic structure of fibrin network density and is able to determine the characteristic of blood components on the blood-dentin contacted surface, which was affected by the residual chelating agent.

In our study, a red blood cell deformity could be observed in the EDTA-treated groups (ie, shrinkage and crenation caused by water diffusing out of the cells) (10, 18). Stacks or aggregations of erythrocytes, which are called rouleaux formation, were also observed in the EDTA-treated groups. This formation may be associated with many factors, such as increased plasma proteins, especially...
fibrinogen levels, in low shear stress conditions (19). This phenomenon was consistent with a related study that observed rouleaux formation in the laboratory when EDTA was added to patient’s serum; however, the phenomenon was rare, and its mechanism remained uncertain (20). Additionally, our results exhibited platelet clumping in the EDTA groups. The previous study has shown that EDTA affected a receptor in platelet membranes and caused the loss of fibrinogen binding function, resulting in the clumping of platelets (21, 22).

In addition, our results showed that fiber density in the coronal, middle, and apical portions of the root canal did not significantly differ in each group of the irrigation protocols. We ruled out the possibility of technical error by exploiting a model with Gates Glidden drills (size 1–4) to prepare a parallel wall canal space with a constant 1.1-mm diameter. Therefore, the irrigation needle was accessible to penetrate into all of the root canals.

When EDTA irrigation time was reduced from 5 minutes to 1 minute, fiber density did not show any differences in the results. This may be attributed to the small amount of residual EDTA creating an extensive effect on fibrin formation. After EDTA irrigation, flushing with NSS could enhance fibrin formation because it reduced residual EDTA in the root canal. Using EDTA is recommended in regenerative endodontics because it liberates bioactive molecules from dentin, and these molecules modulate the biological activities of cells recruited from the periapical tissues. Based on the results of this study, using EDTA followed by NSS did not affect fiber density, but it might possibly affect the amount of growth factor released from root dentin. Widbiller et al (23) showed that the amount of transforming growth factor beta 1 significantly decreased from 535 to 197 pg/mL after the EDTA irrigation time was reduced from 3 minutes to 1 minute. They also showed that the amount of growth factor released when irrigated with EDTA followed by phosphate-

Figure 2. A root canal treated with various irrigation protocols at the middle portion of the root at a magnification of 2500×. (A) The NSS group: 20 mL NSS for 5 minutes; (B) the E1N group: 20 mL 17% EDTA for 1 minute followed by 20 mL NSS for 5 minutes; (C) the E5N group: 20 mL 17% EDTA for 5 minutes followed by 20 mL NSS for 5 minutes; (D) the E1 group: 20 mL 17% EDTA for 1 minute; and (E) the E5 group: 20 mL 17% EDTA for 5 minutes. Occasionally, (C) stacks of shrinkage erythrocytes (arrows) were shown, and (D) inactivated platelets clumping with a few fibrin fibers were observed (arrows).
buffered saline was lower than that when irrigating with EDTA alone. Therefore, the amount of growth factor released after the 2-step irrigation protocols, which was not taken into consideration in this experiment, should be studied further.

To date, clinicians must confront prolonged clotting times and the fact that blood clots have broken down after a calcium silicate–based material is placed over them in a clinical situation (24). It has been shown that a calcium silicate–based material will be supported by a

**Figure 3.** A root canal treated with various irrigation protocols at the apical portion of the root at a magnification of 2500 ×. (A) The NSS group: 20 mL NSS for 5 minutes; (B) the E1N group: 20 mL 17% EDTA for 1 minute followed by 20 mL NSS for 5 minutes; (C) the E5N group: 20 mL 17% EDTA for 5 minutes followed by 20 mL NSS for 5 minutes; (D) the E1 group: 20 mL 17% EDTA for 1 minute; and (E) the E5 group: 20 mL 17% EDTA for 5 minutes.

**TABLE 1.** A Statistical Comparison of the Median Number of Fibers per 10 μm in Different Groups after Irrigating with Each Protocol (N = 7)

<table>
<thead>
<tr>
<th></th>
<th>Number of fibers per 10 μm</th>
<th>P value (Friedman test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coronal</td>
<td>Middle</td>
</tr>
<tr>
<td>NSS</td>
<td>11.60a (8.31, 17.48)</td>
<td>12.38a (9.25, 18.03)</td>
</tr>
<tr>
<td>E1N</td>
<td>8.99a (6.50, 14.43)</td>
<td>9.51a (7.29, 14.59)</td>
</tr>
<tr>
<td>E5N</td>
<td>8.34ab (0, 11.09)</td>
<td>9.13ab (3.27, 10.55)</td>
</tr>
<tr>
<td>E1</td>
<td>0.22ab (0, 0.38)</td>
<td>0.22ab (0.03, 0.57)</td>
</tr>
<tr>
<td>E5</td>
<td>0.17ab (0.07, 0.40)</td>
<td>0.23ab (0.11, 0.38)</td>
</tr>
<tr>
<td>P value (Kruskal-Wallis test)</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
</tbody>
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NSS, irrigated with 20 mL normal saline solution (NSS) for 5 minutes; E1N, irrigated with 20 mL 17% EDTA for 1 minute followed by 20 mL NSS for 5 minutes; E5N, irrigated with 20 mL 17% EDTA for 5 minutes followed by 20 mL NSS for 5 minutes; E1, irrigated with 20 mL 17% EDTA for 1 minute; E5, irrigated with 20 mL 17% EDTA for 5 minutes.

Different lowercase letters indicate a statistically significant difference (P < .05) among irrigation protocols at each level of the root.
stable blood clot in REPs. Otherwise, the root development might interfere with the material displacement, and the tight coronal seal will be interrupted. In addition, tissues formed following the current procedures do not completely form the pulp-dentin complex in histologic studies (25, 26). The range of scaffold pore sizes that is optimal to support adhesion, proliferation, and differentiation of mesenchymal stem cells has not been reported. This should be determined to support clinical regenerative procedures and histologic features in further study.

Conclusion

The blood morphologic characteristics were affected by EDTA as well as fibrin formation in a time-independent manner. Our study has shown that a final flush with 20 mL NSS for 5 minutes revealed a major effect on improving blood clot formation. However, the levels of the root canal, which are the coronal, middle, and apical portions, did not affect clot formation regarding REPs.

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

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