



EFFECT OF PASSIVE ULTRASONIC ACTIVATION OF ETHYLENEDIAMINETETRAACETIC ACID ON CLOT-COVERED AREA AND FIBRIN NETWORK ORGANIZATION IN A REGENERATIVE ENDODONTIC MODEL

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ABSTRACT

Objective: In regenerative endodontic procedures, the intracanal blood clot serves as a natural scaffold, and its interaction with conditioned dentin may influence the early regenerative environment. Although ethylenediaminetetraacetic acid (EDTA) is widely used for dentin conditioning, its calcium-chelating properties may affect clot formation. This ex vivo scanning electron microscopic study evaluated the effect of 17% EDTA, with or without passive ultrasonic irrigation (PUI), on ImageJ-derived clot-covered area and semiquantitative fibrin network organization in an immature root canal model.

Methods: Thirty extracted human maxillary central incisors were prepared and standardized to simulate immature open-apex canals. The specimens were assigned to three irrigation protocols: distilled water with syringe-and-needle irrigation (DW-SNI), 17% EDTA with syringe-and-needle irrigation (EDTA-SNI), and 17% EDTA with passive ultrasonic irrigation (EDTA-PUI). Fresh venous blood was applied to the intraradicular dentin surfaces, and clot formation was allowed for 10 minutes at 37°C. Scanning Electron Microscopy (SEM) images obtained from the coronal, middle, and apical thirds were analyzed using ImageJ to quantify fibrin/clot-covered area. Fibrin-like network organization was scored using a 4-point semiquantitative scale.

Results: ImageJ-derived fibrin/clot-covered area differed significantly among irrigation protocols ($P = .022$) and canal thirds ($P = .001$), whereas the interaction between irrigation protocol and canal third was not significant ($P = .092$). The EDTA-PUI group showed significantly greater fibrin/clot-covered area than the DW-SNI group in all canal thirds. In the middle third, EDTA-SNI also showed higher values than DW-SNI, and EDTA-PUI showed higher values than EDTA-SNI. Semiquantitative fibrin-like network organization scores did not differ significantly among irrigation protocols or canal thirds.

Conclusions: Within the limitations of this ex vivo study, passive ultrasonic activation of 17% EDTA was associated with greater ImageJ-derived clot-covered area without producing a detectable change in semiquantitative fibrin-like network organization. These findings suggest that EDTA-PUI may increase clot-associated surface coverage on intraradicular dentin under the tested conditions, but this should not be interpreted as direct evidence of improved fibrin density or clot homogeneity.

Keywords: Regenerative endodontics; Ethylenediaminetetraacetic acid; Passive ultrasonic irrigation; Blood clot; Fibrin-like network; Scanning electron microscopy; ImageJ

1 INTRODUCTION

Pulp necrosis and apical periodontitis in immature permanent teeth remain clinically challenging conditions because incomplete root development is accompanied by thin dentinal walls and increased susceptibility to fracture [1-3]. Although apexification procedures have long been used to manage such cases, these approaches are primarily intended to induce apical barrier formation and do not reliably restore pulp vitality or support continued physiologic root maturation [4-6]. In contrast, regenerative endodontic treatment (RET) has emerged as a biologically based alternative that aims to create an environment conducive to tissue repair or regeneration within the root canal system through the coordinated contribution of stem cells, signaling molecules, and a suitable scaffold [1, 2]. By promoting periapical healing together with continued root development, RET may improve the structural prognosis of immature teeth [7, 8].

A central prerequisite for successful RET is the establishment of a biologically functional intracanal scaffold [4, 9]. The induced blood clot is the most widely used natural scaffold because it provides a fibrin-rich three-dimensional matrix that may support cell homing, adhesion, migration, and tissue ingrowth [5, 10]. In addition to scaffold formation, RET outcomes are also influenced by the chemical conditioning of root canal dentin [11, 12]. In this context, EDTA plays an important role because it exposes dentin matrix components and may enhance the release of bioactive molecules, including transforming growth factor beta, thereby facilitating cell-related events relevant to regeneration [11].

Despite these favorable biological effects, EDTA may also adversely affect blood clot formation [13, 14]. As a calcium-chelating agent, EDTA can interfere with the coagulation cascade by reducing the availability of calcium ions required for thrombin generation and fibrin formation [15]. Accordingly, residual EDTA within the canal space may compromise fibrin-like network density and alter the ultrastructural quality of the blood clot [13]. Because the structural organization of the clot is directly related to its function as a scaffold, the potential trade-off between dentin conditioning and preservation of clot architecture deserves further investigation [16, 17].

Another important factor in RET is the method used to deliver irrigants within the canal system [1, 2]. SNI provides limited irrigant exchange beyond the needle tip and within anatomical irregularities [3, 4]. PUI, by contrast, improves irrigant movement and enhances contact between the solution and canal walls through acoustic streaming and cavitation-related effects [18]. Although this may increase the effectiveness of dentin conditioning [19], it remains unclear whether ultrasonic activation may also intensify the interaction between EDTA and the subsequently induced blood clot [20]. Thus, comparison of nonactivated and ultrasonically activated EDTA with a distilled water control may help clarify whether EDTA conditioning and ultrasonic activation influence clot-covered area and fibrin network organization differently [20, 21].

Therefore, the aim of the present study was to evaluate the effect of passive ultrasonic activation of 17% EDTA on ImageJ-derived clot-covered area and semiquantitative fibrin network organization in intracanal blood clots formed in a regenerative endodontic model. The null hypothesis was that the irrigation protocol would not significantly affect either outcome.

2 MATERIALS AND METHODS

The study protocol was approved by the Health Science University ethics committee (approval number: 2025-101). Thirty extracted human maxillary central incisors were included in this study. Teeth extracted for periodontal reasons were cleaned of adherent soft tissue remnants using a scalpel and stored in distilled water at 4°C until use.

Only teeth with a single canal, complete root development, comparable root dimensions, and no previous endodontic treatment were included. Teeth with root curvature greater than 10° according to Schneider's method, root caries, cracks, fractures, external or internal resorption, calcification, restorations, or other structural defects were excluded. Canal patency was confirmed with a size 15 K-file (Perfect, Shenzhen, China). The crowns were removed perpendicular to the long axis at

the cemento-enamel junction using a slow-speed diamond saw (Sunshine Diamonds, Langenhagen, Germany) under water cooling, and the roots were further trimmed to obtain a standardized length of 7 ± 1 mm. Root canal preparation was performed with Reciproc R40 instrument (VDW, Mühli, Germany) under irrigation with 1.5% sodium hypochlorite (Microvem, Istanbul, Türkiye) and distilled water. An immature open-apex model was then created by enlarging the apical foramen to 1.3 mm with Gates-Glidden (Proud, Londra, England) drills sizes 1 to 5. Reference grooves were subsequently created on the external root surface of each specimen to separate the coronal, middle, and apical thirds [14].

After root canal preparation and open-apex simulation, 2 longitudinal grooves were created on the external root surface at the buccal and lingual aspects using a high-speed cylindrical diamond bur under copious water cooling, without penetrating the root canal space. Subsequently, 2 parallel horizontal grooves were made perpendicular to the longitudinal grooves to demarcate the coronal, middle, and apical thirds of each specimen.

2.1 Irrigation Protocols

The specimens were randomly assigned to 3 experimental groups ($n = 8$). Before irrigation, each specimen was embedded in plastic droppers using impression material (Silagum-Putty; DMG Chemisch-Pharmazeutische, Hamburg, Germany) to simulate a closed irrigation environment. 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 2 mm from the root end to reproduce the clinical protocol.

The specimens were irrigated according to the following protocols:

- **Distilled water + SNI Group (DW-SNI):** The canals were irrigated with 20 mL distilled water over 5 minutes using needle irrigation.
- **17% EDTA + SNI Group (EDTA-SNI):** The canals were irrigated with 20 mL of 17% EDTA solution (Microvem, Turkey) over 5 minutes using needle irrigation.
- **17% EDTA + PUI Group (EDTA-PUI):** The canals were initially irrigated with 18.5 mL of 17% EDTA. The final 1.5 mL was delivered in 3 cycles of 0.5 mL, each followed by 20 seconds of PUI using [Irrisonic E1, Helse, Santa Rosa de Viterbo, Brazil], positioned 2 mm short of the working length without intentional contact with the canal walls.

In all groups, irrigation was performed with gentle in-and-out approximately 4–5 mm amplitude, while maintaining the irrigation needle or ultrasonic tip 2 mm short of the working length. No additional final rinse was performed after completion of the assigned irrigation protocol.

2.2 Blood Sample Collection and Clot Formation

Following irrigation, the canals were dried with sterile paper points. Each root was then split longitudinally with a chisel, and a dentin block from each specimen was selected for further analysis. Fresh venous blood was collected from a healthy adult researcher with no systemic disease or anticoagulant medication use. A 3- μ L blood sample was applied to the root canal surface of each specimen, and clot formation was allowed to occur in a humidified chamber at 37°C for 10 minutes.

2.3 SEM Analysis

Following clot formation, the specimens were fixed in 2.5% glutaraldehyde for 1 hour, rinsed with phosphate-buffered saline, and dehydrated in ascending ethanol concentrations from 30% to 100%. The dehydrated specimens were mounted on metal stubs, sputter-coated with gold/palladium, and examined using a SEM (Zeiss Evo15; Carl Zeiss, Wetzlar, Germany) operating at 3 kV.

SEM images were obtained from the coronal, middle, and apical thirds of each specimen. Images acquired at $\times 1000$ magnification were used to document the general distribution of the blood clot along the intraradicular dentin surface. Higher-magnification images acquired at $\times 2500$ were obtained from representative areas at each canal third and were used for semiquantitative evaluation of the fibrin-like network.

Fibrin-like network organization was evaluated using a 4-point semiquantitative scoring system by an examiner blinded to the experimental groups. The scoring system was based on the apparent density, continuity, interlacing, and overall organization of fibrin-like network structures within the clot. Score 1 indicated sparse or discontinuous fibrin-like strands with poor network organization; score 2 indicated a limited and loosely organized fibrin-like network; score 3 indicated a moderately dense and relatively continuous fibrin-like network; and score 4 indicated a dense, continuous, and well-organized fibrin-like network. Higher scores therefore represented greater fibrin-like network organization.

2.4 Quantitative Image Analysis of Fibrin/Clot-Covered Area

SEM images obtained at $\times 2500$ magnification were further analyzed quantitatively using ImageJ software (National Institutes of Health, Bethesda, MD, USA). After exclusion of the SEM information bar and non-relevant background areas, images were converted to 8-bit grayscale and processed using a standardized thresholding procedure to distinguish fibrin/clot-covered areas from the background. The threshold-positive area corresponding to clot-associated structures, including fibrin-like network components and associated cellular elements, was quantified and recorded as the ImageJ-derived fibrin/clot-covered area. The same image-processing parameters were applied consistently to all images. All image selection and quantitative measurements were performed by an examiner blinded to the experimental groups.

2.5 Statistical Analysis

Fibrin-like network organization scores were analyzed as ordinal data. Differences between the scores attributed to the images of the specimens in the study groups at each canal level were analyzed using the Kruskal-Wallis test followed by Dunn pairwise comparison test with Bonferroni correction. The Friedman test was used for intragroup comparisons of the coronal, middle, and apical thirds within each group. ImageJ-derived blood clot covered area values were analyzed separately using a mixed-effects model after $\log_{10}$ transformation, with irrigation protocol, canal region, and their interaction as fixed effects and specimen as a random effect. Pairwise comparisons were adjusted for multiple testing. P values $< .05$ were considered statistically significant. All analyses were conducted using R software version 4.3.1 for Windows (R Foundation for Statistical Computing, Vienna, Austria). Graphs were generated using the ggplot2 package.

3 RESULTS

The ImageJ-derived fibrin/clot-covered area and semiquantitative fibrin-like network organization scores were evaluated at the coronal, middle, and apical thirds of the root canals. Representative SEM images demonstrated clot-associated structures on the intraradicular dentin surface in all experimental groups. Descriptive evaluation indicated that the EDTA-PUI group had higher and more consistent fibrin/clot-covered area values, whereas the EDTA-SNI group showed an intermediate pattern and the DW-SNI group exhibited greater variability among canal thirds.

The descriptive statistics of the ImageJ-derived fibrin/clot-covered area are shown in Table 1. The fibrin/clot-covered area differed significantly among the irrigation protocols ($P = .022$) and among the canal thirds ($P = .001$). The interaction between irrigation protocol and canal third was not statistically significant ($P = .092$). In pairwise comparisons stratified by canal third, the EDTA-PUI group showed significantly higher fibrin/clot-covered area values than the DW-SNI group in the coronal, middle, and apical thirds ($P < .05$). In the middle third, the EDTA-SNI group also showed significantly higher values than the DW-SNI group ($P = .005$) and the EDTA-PUI group showed significantly higher values than the EDTA-SNI group ($P = .048$). No statistically significant differences were found in the remaining pairwise comparisons ($P > .05$).

Intragroup comparisons showed significant differences among canal thirds only in the DW-SNI group. In this group, the middle third had significantly lower ImageJ-derived fibrin/clot-covered area values than the coronal and apical thirds ($P = .001$ and $P = .013$, respectively). No significant differences among the coronal, middle, and apical thirds were detected in the EDTA-PUI or EDTA-SNI groups ($P > .05$).

The distribution of semiquantitative fibrin-like network organization scores is shown in Table 2. No significant differences in semiquantitative fibrin-like network organization scores were detected among the irrigation protocols in the coronal, middle, or apical thirds ($P = .428$, $P = .115$, and $P = .841$, respectively). Furthermore, semiquantitative fibrin-like network organization scores did not differ significantly among the coronal, middle, and apical thirds within the DW-SNI, EDTA-PUI, or EDTA-SNI groups ($P = .499$, $P = .483$, and $P = .296$, respectively).

4 DISCUSSION

This study evaluated the effect of EDTA, with or without passive ultrasonic irrigation, on ImageJ-derived fibrin/clot-covered area and semiquantitative fibrin-like network organization in intracanal blood clots formed on intraradicular dentin surfaces. The main finding was that the EDTA-PUI group showed greater ImageJ-derived fibrin/clot-covered area values than the DW-SNI group, whereas semiquantitative fibrin-like network organization scores did not differ significantly among the irrigation protocols or canal thirds. In addition, the fibrin/clot-covered area was significantly affected by both irrigation protocol and canal third, but the interaction between these factors was not statistically significant. Therefore, the null hypothesis was partially rejected for the ImageJ-derived fibrin/clot-covered area analysis but not for the semiquantitative fibrin-like network organization score.

The increase in ImageJ-derived fibrin/clot-covered area in the EDTA-PUI group was not accompanied by a parallel increase in semiquantitative fibrin-like network organization scores. This finding suggests that passive ultrasonic activation of EDTA mainly affected the extent of clot-associated surface coverage rather than producing a detectable change in the morphologic organization of the fibrin-like network. Accordingly, the ImageJ-derived area should be interpreted as a surface-coverage outcome within the selected SEM fields and not as direct evidence of increased fibrin density, improved clot homogeneity, or superior scaffold quality.

When considered in relation to previous studies evaluating the effect of EDTA and its activation on intracanal clot structure, the present findings show both concordant and divergent aspects [22-24]. Vieira et al. investigated blood clot formation on dentin surfaces after different EDTA activation protocols, including passive ultrasonic activation, in an ex vivo immature tooth model [25]. They reported clot formation with high cellular density in all groups; however, EDTA-treated canals showed significantly lower fibrin network density scores than saline-irrigated canals, whereas no significant differences were found among the different EDTA activation protocols, including ultrasonic activation. In the present study, neither EDTA-SNI nor EDTA-PUI resulted in significantly lower semiquantitative fibrin-like network organization scores compared with DW-SNI. In contrast, EDTA-PUI increased the ImageJ-derived fibrin/clot-covered area compared with DW-SNI. Nevertheless, the absence of a significant difference between EDTA-SNI and EDTA-PUI in semiquantitative fibrin-like network organization scores is consistent with previous SEM-based studies reporting that ultrasonic activation of EDTA did not significantly alter fibrin network scores compared with nonactivated EDTA [23, 25].

The apparent discrepancy between the reduced fibrin network density reported in previous SEM-based studies after EDTA irrigation and the absence of a significant effect on semiquantitative fibrin-like network organization scores in the present study may be explained, at least in part, by methodological differences [18, 26, 27]. Previous work used saline as the reference irrigant and evaluated fibrin network density using a 4-point scale focused primarily on density [13, 22, 28]. In contrast, the present study compared EDTA-SNI and EDTA-PUI with DW-SNI and used a composite semiquantitative score that included density, continuity, interlacing, and overall organization of the fibrin-like network. Differences in the control irrigant, endpoint definition, blood volume, clot formation time, image magnification, and image-selection criteria may influence the SEM-based appearance of clot-associated structures [18, 29, 30]. In addition, the ImageJ-derived fibrin/clot-covered area in the present study represented the proportion of the selected field occupied by clot-associated structures, including fibrin-like network components and associated cellular elements, whereas previous studies evaluated fibrin network density as a separate scoring outcome [18, 22, 31]. These methodological differences indicate that SEM-based clot assessment may vary according to the selected outcome parameter and should not be interpreted as a single uniform measure of fibrin network quality [23, 32].

The broader biological literature supports a complex role for EDTA in regenerative endodontics [23-25, 33]. EDTA is widely used as a final irrigant for smear layer removal and dentin conditioning [24, 34]; however, as a calcium-chelating agent, it may also interfere with calcium-dependent steps of coagulation, including thrombin generation and fibrin formation [31, 35]. This dual effect has raised concern that residual EDTA within the canal space may influence blood clot formation or stability [14, 36]. In a systematic review, dos Reis-Prado et al. reported that EDTA-treated dentin positively influenced TGF- β release and cell-related events, including migration, attachment, and differentiation, although the available evidence was mainly derived from in vitro studies and well-designed animal and clinical studies were still limited [24]. In the context of the present findings, the potential anticoagulant effect of EDTA did not translate into lower semiquantitative fibrin-like network organization scores compared with DW-SNI. Moreover, EDTA-PUI was associated with a greater ImageJ-derived fibrin/clot-covered area. These findings suggest that, under the present experimental conditions, the dentin-conditioning effect of EDTA, particularly when combined with PUI, may have contributed to increased clot-associated surface coverage without producing a detectable deterioration in fibrin-like network organization [13, 23].

The distribution of ImageJ-derived fibrin/clot-covered area among canal thirds may also have methodological and biological relevance. In the DW-SNI group, the middle third showed significantly lower fibrin/clot-covered area values than the coronal and apical thirds, indicating greater regional variation in clot-associated surface coverage. In contrast, no significant differences among the coronal, middle, and apical thirds were detected in the EDTA-SNI or EDTA-PUI groups. This finding may suggest that EDTA-based dentin conditioning, with or without passive ultrasonic irrigation, was associated with a more consistent pattern of clot-associated coverage along the canal surface. However, this interpretation should be made cautiously because the interaction between irrigation protocol and canal third was not statistically significant. From a regenerative endodontic perspective, more consistent clot coverage along the canal wall may be relevant because the blood clot acts as a natural scaffold for cell migration and tissue ingrowth [37-40]; nevertheless, the present findings cannot be interpreted as direct evidence of improved scaffold quality or clinical regenerative potential [41, 42].

Several limitations of this study should be acknowledged. First, the ex vivo design, using standardized dentin blocks from extracted teeth and a simulated open-apex model, does not fully replicate the complex anatomy, dynamic bleeding, or host immune and inflammatory responses present in clinical regenerative endodontic cases. Second, blood was obtained from a single healthy donor; therefore, interindividual variations in coagulation factors, platelet function, or hematologic status were not considered. Third, only one EDTA concentration (17%), exposure time, and PUI protocol were investigated. Different EDTA concentrations, activation durations, or irrigation sequences, such as EDTA followed by a final saline rinse, could produce different effects on ImageJ-derived fibrin/clot-covered area and fibrin-like network organization. Fourth, the semiquantitative scoring system, although applied by a blinded examiner, is inherently subjective and may not detect subtle morphologic differences that could be captured by more advanced image analysis or three-dimensional approaches. Finally, the evaluation was limited to early clot formation after 10 minutes of incubation; therefore, no information was obtained regarding clot maturation, resistance to mechanical disturbance, stem cell behavior, or regenerative outcomes.

Future research should integrate SEM-based assessment of ImageJ-derived fibrin/clot-covered area and fibrin-like network organization with functional and biological endpoints, such as growth factor release, stem cell attachment and migration, and histological patterns of tissue formation in immature root models. Studies directly combining EDTA-PUI protocols with biologically oriented outcomes could help clarify whether the increased fibrin/clot-covered area observed in the present study is associated with improved regenerative responses. Animal and clinical studies systematically comparing different irrigation regimens, activation methods, and final rinse strategies are also needed to strengthen the evidence base for regenerative endodontic protocols.

5 CONCLUSION

Within the limitations of this ex vivo study, EDTA-PUI resulted in greater ImageJ-derived fibrin/clot-covered area on intraradicular dentin surfaces than DW-SNI, whereas semiquantitative fibrin-like network organization scores were not significantly affected by the irrigation protocol. These findings indicate that passive ultrasonic activation of EDTA may increase early clot-associated surface coverage without causing a measurable alteration in fibrin-like network organization under the present experimental conditions.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

The author declares no conflict of interest.

Statement of ethical approval

The study protocol was approved by the Health Science University ethics committee (approval number: 2025-101).

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