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Original article

# Comparative evaluation of two chelating agents in collagen fiber network modification over dentinal tubules: An in vitro analysis

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ABSTRACT

*Objective:* To compare effectiveness of ethylenediaminetetraacetic acid (EDTA) and citric acid in removing collagen fiber network covering dentinal tubules of human teeth.
*Materials and Methods:* Eighteen dentin discs were divided in three groups; Gp 1: discs received no treatment (control), Gp 2: discs etched with 17% EDTA (pH = 7.1), and Gp 3: discs etched with 6 wt% citric acid (pH = 4.0). Scanning electron microscopy (SEM) was performed to assess collagen fiber removal and X-ray diffraction (XRD) was implemented to analyse crystal peaks of discs.
*Results:* The SEM analysis demonstrated more collagen removal with EDTA treatment compared to citric acid treated specimens. Grade 6 (81% to 100% fiber removal) was mostly achieved for Gp 2 samples whereas grade 2 (1% to 20% fiber removal) was mostly achieved for Gp 3 samples and inter-group comparisons between these groups were statistically significant ($p < 0.05$). X-ray diffractogram of control and experimental samples demonstrated absence of calcite phase in experimental groups. The change in peak shapes and intensities were observed and citric acid treated samples revealed more intense peaks than EDTA group.
*Conclusion:* Our study found 17% EDTA to be more effective in removing collagen fibers when matched with 6% citric acid.
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## 1. Introduction

The human tooth structure is composed of three calcified tissues, of which enamel is irreparable whereas dentin and cementum possess partial capability to regenerate (Huang, 2011). Dentin forms majority of tooth structure in the anatomical crown and in the root (Ali and Farooq, 2013). Dentin contains millions of tiny tubules with fluids and movement of this fluid (when dentin is exposed to a stimuli) is thought to be responsible for dentin

hypersensitivity (DH) (Farooq et al., 2015). Dentin also contains a network of collagen fibers coming from intertubular and peritubular dentin which cover these tubules (Khan et al., 2019). This collagen fiber network is more dense and prominent in intertubular dentin and is mostly Type-I in nature with little traces of Type-III and V are also found in dentin strucutre (Goldberg et al., 2011).

One of the most effective way to stop DH is to occlude the open tubules (Goldberg et al., 2011). Over the last few decades, researchers have performed many in-vitro experiments to test the potential of various agents in blocking dentinal tubules (Farooq et al., 2015). Prior to performing these experiments, removal of collagen fibers is essential as this network can increase shear bond strength of dentin (Cederlund et al., 2002). Thus interfering with the results and consequently, real efficiency of tubule blocking agents could not be assessed accurately. For the removal of collagen fibers, two most common chelating agents currently being used are ethylenediaminetetraacetic acid (EDTA) and citric acid. The EDTA exerts its chelation effects by demineralizing divalent cations (Blomlöf et al., 1997 Aug) whereas citric acid is a decalcifying and cleansing agent (Scelza et al., 2003). The EDTA is also most commonly used

during endodontic treatment to remove inorganic components from the canals and citric acid between the concentrations of 1% to 50%, is used commonly to remove smear layer and intratubular smear plugs (Gandolfi et al., 2018).

There is a deficit of studies in the literature where efficacy of these chelating agents has been compared in terms of collagen modification over dentinal tubules. Therefore, the aim of this study was to compare the competence of EDTA and citric acid in removing peritubular and intertubular collagen fiber network covering dentinal tubules. The null hypothesis ($H_0$) of our study was that both EDTA and citric acid perform equally in removing collagen fiber network over dentinal tubules of dentin discs which were obtained from freshly extracted human teeth.

## 2. Materials and methods

Ethical approval was obtained before commencing the study from the review board of institute and all protocols and ethical standards mentioned in Helsinki declaration (1964) and its later modifications were strictly followed.

### 2.1. Dentin disc preparation

Eighteen human extracted third molar teeth were obtained from oral surgery department of the institute. The teeth were stored in 10% formalin at room temperature. Eighteen dentin discs (N = 18) of 1.5 mm ± 0.2 mm were developed by cutting the teeth mesio-distally over cementoenamel junction utilizing a water-cooled diamond saw (Isomet ® 5000 Linear Precision Saw, Buehler Ltd, IL, USA) at a speed of 3000 rpm. Coronal enamel was also detached in the same fashion. The top surfaces of the discs were marked and lower unmarked surfaces were then used for experiments.

### 2.2. Grouping of specimens and experiments

The dentin discs were randomly divided in three groups based on the surface treatment they received and each group received six discs; Gp 1: dentin discs received no treatment post-preparation and were stored in distilled water (control), Gp 2: dentin discs were etched with 17% EDTA (pH = 7.1, Biodynamics. Ibiporã, PR, Brazil) for 1 min, and Gp 3 (dentin discs were etched with 6 wt% citric acid (pH = 4.0) for 1 min. The citric acid was freshly prepared by adding 6 g of citric acid powder (FunFresh Foods®, CA, USA) in 100 mL of deionized water. One mL of EDTA and citric acid were applied once to the unmarked surfaces of respective discs with the help of a 0 sized paint brush (hobbycraft®, Dorset, UK). Post-surface treatment, distilled water was used to wash the discs for 30 s and they were then left to air dry before further characterization.

### 2.3. Scanning electron microscopy (SEM) analysis

The treated and non-treated discs were mounted on stubs, gold coated and then the analysis was performed utilizing an SEM (FEI, Inspect F50, The Netherlands) with an electron mode of 10 kV. The micrographs were taken at various magnifications. The percentage (%) of removal of collagen fibers in a single disc was calculated by the following formula;

The % of removal of collagen fibers

$$= \frac{\text{Number of tubules with collagen fibers} \times 100}{\text{Total number of tubules in a single micrograph}}$$

The tubules which were not completely seen in the micrographs were not considered for analysis. The SEM micrographs were assessed after slightly modifying the following criteria proposed earlier by Chockattu et al. (2017). The criteria and grading was as follows;

- Grade 1: Collagen fibers not removed at all = 0%
- Grade 2: Collagen fibers slightly removed = 1–20%
- Grade 3: Collagen fibers partially removed = 21–40%
- Grade 4: Collagen fibers moderately removed = 41%–60%
- Grade 5: Collagen fibers mostly removed = 61%–80%
- Grade 6: Collagen fibers completely removed = 81%–100%.

### 2.4. X-ray diffraction

Phase analysis of control and experimental samples were performed using X-ray diffraction (XRD) equipment (Rigaku MiniFlex X-ray diffractometer, Tokyo, Japan). The diffractometer was operated at 0.15416 nm wavelength, 10 mA current, and 30 kV voltage. Each sample was scanned from $2\theta \sim 10° - 70°$ with a 0.02° step size. The crystal size (D) evaluation was done in the long dimension and the mean size in the plane of cross-section. The D was calculated using Scherrer equation (Islam et al., 2017): $D = K \lambda / \beta_{\frac{1}{2}} \cos\theta$

Where $\lambda$ is the X-ray wavelength, $\theta$ is the diffraction angle and K is a constant.

### 2.5. Statistical analysis

Data analysis was performed using SPSS-20.0 (IBM product, Chicago-USA). Mann Whitney test for two independent samples was used to compare percentage and average of collagen fiber removal between groups according to the grading criteria. Mann Whitney test for two dependent samples was used to perform pairwise comparison of percentages for each grading criteria within a group. P-value $\leq 0.05$ was considered statistically significant difference of means.

## 3. Results

### 3.1. SEM results

The SEM analysis demonstrated no collagen fiber network removal for control group (Fig. 1), however, more collagen fiber removal was achieved for EDTA etched samples (Fig. 2) as compared with citric acid treated specimens (Fig. 3). Since Gp 1 achieved grade 1 for all the samples, it was excluded from further statistical analysis. On the basis of percentage of collagen fiber removal, grade 6 (81% to 100% removal) was mostly achieved for Gp 2 samples whereas grade 2 (1% to 20% removal) was mostly achieved for Gp 3 samples and inter-group comparisons between the EDTA and citric acid groups for these grades were statistically
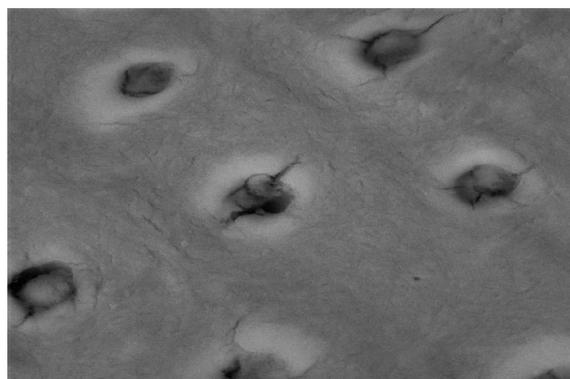


**Fig. 1.** SEM micrograph of a dentin disc from control group which remained untreated (10,000× magnification, scale bar. 10 μm).
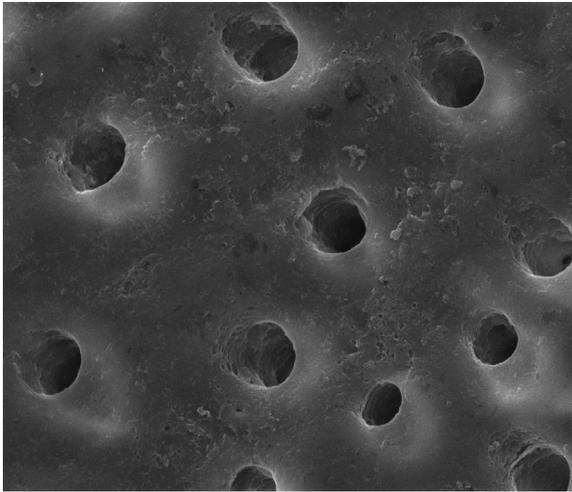
**Fig. 2.** SEM micrograph of a dentin disc treated with EDTA (10,000× magnification, scale bar: 10 μm).
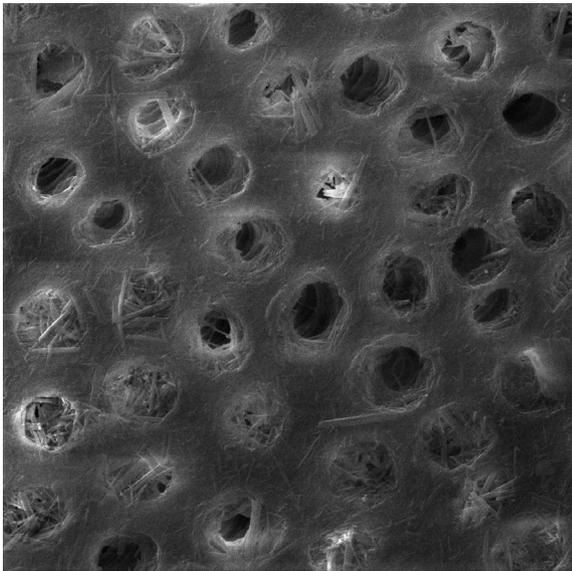


**Fig. 3.** SEM micrograph of a dentin disc treated with citric acid (10,000× magnification, scale bar: 5 μm).

significant ($p < 0.05$) (Table 1). Pairwise comparison of percentages of each grade present within a group revealed statistically significant differences ($p < 0.05$) for EDTA group whereas only grade 2 versus grade 6 comparison for the citric acid group showed statistically significant result ($p < 0.05$) (Table 2).

### 3.2. X-ray diffraction results

The comparative X-ray diffractogram of control and experimental samples are shown in Fig. 4. The phase is similar to hydroxyla-

**Table 2**
Showing pairwise comparison of percentage of each grade within a group.

|  | EDTA | Citric Acid |
|---|---|---|
| Grade 2 vs Grade 3 | 0.046* | 0.465 |
| Grade 2 vs Grade 4 | 0.028* | 0.893 |
| Grade 2 vs Grade 5 | 0.028* | 0.465 |
| Grade 2 vs Grade 6 | 0.028* | 0.028* |

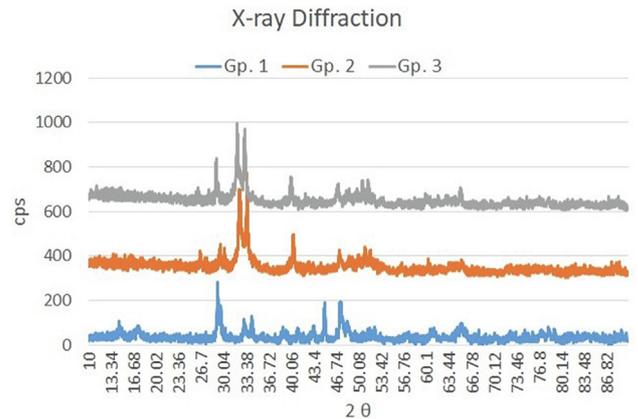\* Statistically significant at 0.05 level of significance.



**Fig. 4.** Showing selection of XRD diffractograms of untreated sound disc, EDTA treated disc, and citric acid treated disc.

patite (HAP), carbonated apatite, and apatite as per JCPDS files (009–0432, 96–900-3550, 96–900-3551, 96–900-3552). The peak at $2\theta \approx 29.18$ (2 1 0) in control group was attributed to whitlockites (i.e., calcite). The calcite phase was present besides HAP phases i.e., 211, 112, and 300 planes. The change in peak shapes and intensities were observed in experimental group, where citric acid treated samples revealed more intense peaks than EDTA treated samples. The 310 plane was not observed in control sample, however, after surface treatment with EDTA and citric acid, this plane appeared. In contrast, 222 plane was intense in control group and became less intense in both experimental groups. No change in d-spacing was found, however, the crystal size decreased with the increase in planes (Table 3).

## 4. Discussion

Based on the results of this study, the $H_0$ that EDTA and citric acid perform equally in removing collagen fibers was rejected. Our study demonstrated that EDTA etched samples were able to remove collagen fibers more effectively as opposed to citric acid when the dentin discs were exposed to these two chelating agents. Although no exactly similar studies are present in the literature, researchers have compared EDTA and citric acid in head to head trials for different other areas related to dentistry. In an earlier study a comparison was made between EDTA and citric acid to analyze their efficiency in removing intracanal medicament Ca

**Table 1**
Showing mean percentages of each grade present within a group and statistical comparisons were made between the groups.

|  | Mean (SD) | | | | |
|---|---|---|---|---|---|
|  | Grade 2 | Grade 3 | Grade 4 | Grade 5 | Grade 6 |
| EDTA | 0 (0) | 1.4 (3.4) | 4.7 (5.4) | 22.1 (22.2) | 71.9 (22.6) |
| Citric Acid | 78.7 (16.6) | 2.9 (4.6) | 7.1 (11.4) | 6.7 (6.9) | 4.6 (3.1) |
| P-value | 0.002* | 0.528 | 0.864 | 0.147 | 0.004* |

\* Statistically significant at 0.05 level of significance.

**Table 3**
Showing graph peaks and crystal size of sound dentin, EDTA treated dentin, citric acid treated dentin.

| Sound dentin | | EDTA treated dentin | | Citric acid treated dentin | |
|---|---|---|---|---|---|
| Graph Peaks | Crystal Size | Graph Peaks | Crystal Size | Graph Peaks | Crystal Size |
| **29.18** | 215 | **26.54** | 257.915 | **26.2** | 171.337 |
| **29.56** | 215.093 | **29.52** | 242.753 | **28.94** | 172.44 |
| **33.08** | 202.998 | **30.14** | 233.057 | **32.04** | 173.826 |
| **34.22** | 200.027 | **32.88** | 232.073 | **33.54** | 174.12 |
| **41.02** | 183.004 | **32.78** | 232.703 | **32.38** | 173.826 |
| **43.24** | 182.461 | **34.1** | 229.117 | **33.38** | 174.12 |
| **39.2** | 188.319 | **40.38** | 211.158 | **40.08** | 178.008 |
| **45.04** | 179.008 | **47.22** | 198.633 | **46.98** | 182 |
| **47.42** | 175.539 | **51.14** | 192.599 | **50.56** | 184.747 |
| **48.26** | 173.605 | **51.8** | 191.449 | **51.46** | 185.347 |
| **47.62** | 175.539 | **60.32** | 183.012 | **65.26** | 198.327 |
| **65.3** | 158.032 | **65.54** | 177.916 | | |

(OH)$_2$ (Chockattu et al., 2017). It was demonstrated from the results of that study that EDTA performed better than citric acid in cleaning Ca(OH)$_2$ from canal walls (Chockattu et al., 2017). Concerning smear layer elimination, a previous study reported that the smear layer deduction capability of 17% EDTA was considerably superior to 20% citric acid (Wu et al., 2012). Eldeniz et al. (2005) investigated the consequence of EDTA and citric acid on the hardness and surface roughness of dentin (Eldeniz et al., 2005). Their results demonstrated that citric acid was found to be the least hardest agent among the test groups. Similar efficiency of EDTA was observed from our results where EDTA removed collagen fiber network of peritubular and intertubular dentin (which was present around and inside dentinal tubules respectively) more efficiently as compared with citric acid.

EDTA is the most effective chelator available which is used in endodontics (Ulusoy and Görgül, 2013). The EDTA acts by combining with the calcium ions of dentin and then demineralizes it (Von der Fehr and Nygaard-Ostby, 1963). 1n late 1950's, Nygaard-Ostby first proposed that a lyophobic substance such as dentin, can be soluble in water (Hülsmann et al., 2003). Ostby (1957) was also the first to describe demineralization of dentin by EDTA and its salt through the principle of constant solubility product (Nygaard-Ostby, 1957). It was proposed that an equilibrium is developed between the salt solution and the amalgamated precipitate as the ions from the precipitate go in the solution, whereas ions in the solution are then de-precipitated as solids (Nygaard-Ostby, 1957). When the disodium salts of EDTA are released to this balance, calcium is eradicated from this solution. This action promotes further ion discharge from dentin in order to maintain a constant solubility product (Hülsmann et al., 2003). In this manner, EDTA causes effective chelation of dentin and this could be the reason for EDTA to perform better in our study compared with citric acid. On the other hand, citric acid is a weak chelator which is less toxic on tissues (Bhatnagar and Kumar, 2004) which also caused less collagen fiber removal in our study.

Calcite is a mineral which together with aragonite forms calcium carbonate in dentin (Kulak et al., 2007 Mar 28). Unlike sea urchin's tooth which is entirely composed of calcite, (Märkel and Titschack, 1969). human dentin contains some percentage of calcite in its composition. In our study, it was detected on XRD analysis that calcite peak at $2\theta \approx 29.18$ (210) was only observed in control group and not in experimental groups. The probable reason for this is the removal of superficial layer of dentin which contained calcite for both experimental groups, therefore the intensity was reduced. Although EDTA removed more collagen fibers, calcite peak in XRD analysis was also reduced, whereas the intensity of calcite peak in citric acid group was higher than EDTA. Marshall et al. (1998) in an earlier study reported that peritubular dentin

is rapidly etched following application of citric acid and this etching rate is inversely proportional to the pH of citric acid and increases linearly with application time Marshall et al. (1998). It has been reported in a previous study that citric acid is ineffective in removing calcium ions at neutral pH but at lower pH values, it works better as a chelator (Sousa and Silva, 2005). On the other hand, EDTA even at neutral or near neutral pH, is still more potent as a chelating agent (Sousa and Silva, 2005). These results are in agreement with our study where even though pH value of EDTA was more than citric acid, still it was able to remove more collagen fibers compared with its counterpart. The demineralization caused by EDTA could be interpreted by explaining the concept of "*solubility product constant principle*". When EDTA is used, it captures calcium ions which dissolve in the solution, consequently, other dentin ions dissolve in order to keep the solubility product constant (Ostby, 1957). Every single calcium ion can bind to a single EDTA molecule and when equilibrium is achieved, no further dissolution occurs (Martinelli et al., 2019).

Our study had few limitations. The first limitation was the in-vitro nature of the current study. In-vivo setting can offer various other dynamic challenges such as interaction with saliva and intra-oral temperature variation that could be different from in to vitro experiments. Another limitation of our study was using only one commercial brand of EDTA and citric acid. Contrarily, it was important to utilize one brand of chelating agents to standardize the experiments. Future studies with other brands of chelating agents with different concentrations should be carried out to obtain more accurate findings. Studies with longer exposure times are also recommended.

## 5. Conclusion

Our study found 17% EDTA to be more effective in removing peritubular and intertubular dentin covering the tubules of dentin discs when matched with 6% citric acid. Although pH value of EDTA was less than citric acid, still it was able to eliminate more collagen fibers validating its role as a more potent chelator.

## Declaration of Competing Interest

None.

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