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Glycolic acid as the final irrigant in endodontics: Mechanical and cytotoxic effects

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ABSTRACT

The aim of this study was to investigate the effects of glycolic acid (GA) on the microhardness, roughness, dentin mineral content distribution; smear layer removal and cytotoxicity. One hundred human teeth were randomly divided into six groups: distilled water (control group), 17% EDTA, 10% citric acid (CA), 5% GA, 10% GA, and, 17% GA. Microhardness and roughness were measured in the canal lumen. Scanning electron microscopy (SEM) images (2000×) for smear layer removal evaluation; energy dispersive X-ray spectroscopy (EDS) for chemical analysis. Cell viability assay was made on fibroblast cells. The lowest microhardness and higher roughness were observed for 17% GA. GA showed the ability to remove the smear layer to a similar level as EDTA and CA, with no statistical difference between the concentrations used. GA and CA were cytotoxic in a dose-dependent manner. GA showed potential as an endodontic agent for final irrigation in root canal terapies.

1. Introduction

Preparation of the root canal aims to reduce the population of microorganisms from the root canal and create a final shape that allows adequate filling of the canal space [1]. Irrigation solutions used in endodontics, such as sodium hypochlorite (NaOCl) and chlorhexidine, are extremely important for the root canal preparation comprising mechanical procedures. However, there is no single solution with known capability to remove both the organic and inorganic compounds of the smear layer [2].

The smear layer created during root canal preparation has approximately 2 to 5-μm thickness and two separate parts: the first superficial layer that is poorly adherent and the second layer that comprises plugs located within the dentinal tubules [3]. Removal of the smear layer is recommended to increase the permeability of the dentinal tubules and enable adaptation of the root-canal filling material [4] as well as improve the antimicrobial effect of irrigating solutions [5].

Ethylenediaminetetraacetic acid (EDTA) and citric acid (CA) are the most commonly used irrigation solution for removal of the smear layer [6,7]. However, both solutions demonstrate negative characteristics such as erosion of both inter and peri-tubular dentin [8,9], which may increase the risk of the vertical tooth fracture [10,11]. Also, EDTA is considered an organic pollutant in water and forms complexes with metals that remain in the environment for many years since they are not easily biodegraded [7,12]. Therefore, there is need to identify an effective agent for removal of the smear layer without harmful effects, previously pointed out.

Glycolic acid (GA) or hydroxyacetic acid belongs to the group of alpha hydroxyl acids that also includes CA. It is used in the pharmaceutical industry as an organic component, especially in skin cosmetics [13] and as a monomer in the preparation of biocompatible polymers, such as PLGA (poly(lactic-*co*-glycolic acid)), which are used in tissue engineering [14,15]. It is a colorless, odorless, and hygroscopic crystalline solid, with high solubility in water [16]. *In vitro* and *in vivo* studies demonstrated that GA has the ability to induce collagen synthesis and fibroblast proliferation [16–18]. GA's low pKa, low molecular weight, and organic nature makes it excellent choice for performance on mineral surfaces as dental structures. Recently, its use to replace phosphoric acid as a surface etchant of enamel and dentin was proposed [19]. Furthermore, GA is readily biodegradable [15]; therefore, unlike EDTA, its waste disposal is not a problem. These characteristics indicate the potential of GA for use in dental applications such as removal of the smear layer in endodontic therapy. However, there are no studies in the literature showing the ability of GA as a

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final irrigant agent during root canal preparation.

Therefore, the aim of this study was to determine the effect of GA in terms of the microhardness, roughness, and mineral content distribution of dentin, removal of the smear layer, and cytotoxicity when used as a final irrigant during root canal preparation. The hypothesis of this study is that GA is suitable as final irrigation solution during the root-canal preparation.

2. Materials and methods

This study was approved by the institutional review board of a local university (#1.886.128). Mandibular single rooted human extracted teeth were selected for this study. The tooth selection was based on the dimension, similarity in morphology, absence of caries, cracks and endodontic treatment. The adherent soft tissues were cleaned and all the teeth were stored in saline solution at 4 °C until further tests.

2.1. Sample size calculation

To calculate the sample size and power of the tests, the Minitab (Mininc, State College, PA, USA) software was used and the parameters were determined from a pilot study. Minimum detectable difference between the mean and standard deviation of the mean was 12 and 4.3 for microhardness; 0.16 and 0.04 for roughness; 3.0 and 0.52 for removal of the smear layer. Thus, the sample size for each group to achieve the power of 0.8 and significance level of 0.05, was a minimum of five for microhardness, three for roughness, and three for the smear layer. Cytotoxic screening was performed with samples number as suggested by the ISO 10993-5 [20].

2.2. Microhardness and roughness

Sixty single-rooted human teeth were used in these tests. The crowns were removed at the cementum-enamel junction using a high-speed bur under water-cooling refrigeration. The working length (WL) was established using a size #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) that was introduced into each root canal until its tip was visualized at the apex and then reduced by 1 mm. In order to standardize the diameter of the root canal and remove the pulp, the roots were prepared using hand files up to the #45 K-file (Dentsply, Maillefer, Ballaigues, Switzerland). Irrigation was performed with 5 mL of 2.5% NaOCl between each file with a needle (25 × 4 mm) placed at 2-mm distance from the WL and final irrigation with 5-mL distilled water (DW) was performed to remove any residue.

The roots were sectioned longitudinally into the buccal and lingual segments using a water-cooled diamond disc at low speed; the resulting 120 segments were divided into the microhardness and roughness groups of 60 each. The dentin between the canal lumen and cementum was abraded with diamond burs (2135 FF, KG Sorensen, SP, Brazil) to facilitate polishing of the dentin at the root-canal lumen and embedded in autopolymerizing acrylic resin, leaving the dentin surface and the canal lumen exposed. The lumen of the root canal was polished with felt discs embedded in aluminum oxide paste.

The samples were randomly divided in six groups and immersed for 1 min in 50 mL of one of the following irrigation solutions: DW (pH 6.80); 17% EDTA (pH 7.17); 10% CA (pH 1.58); 5% GA (pH 2.17); 10% GA (pH 2.10); 17% GA (pH 2.09). After immersion, the specimens were rinsed with 5-mL DW to remove any residual test solution. Each group was then divided in two subgroups for analysis of microhardness (n = 10) and roughness (n = 10) (Fig. 1A).

Dentin microhardness was measured with a Knoop indenter at magnification of 40× (Shimadzu HMV-2000; Shimadzu Corporation, Kyoto, Japan) under a 25-g load for 15 s. Three indentations were made in each specimen: the first indentation was made at 1.000-μm distance from the entrance of the root canal, and the two other indentations were made at a distance of 200 μm from each other. The hardness value for each specimen was obtained as the average of those for the three indentations, according to a previously described protocol [21]. Normal distribution of the data of each experimental group was confirmed using Andersom Darling test ($p > 0.05$). Knoop microhardness was statistically analyzed using one-way analysis of variance (ANOVA) and Tukey test ($p < 0.05$).

The surface roughness (Ra, μm) of the canal lumen was measured in each specimen maintained at horizontal position in a rugosimeter (Mitutoyo SJ-410, Kanagawa, Japan); the mean value of Ra was determined as the average of those at three randomly selected areas ($1 \times 1\ mm^2$) with the first at 2-mm distance from the entrance of the root canal, and the other two at a distance of 2-mm from each other. The mean and standard deviation of Ra was determined for the entire surface roughness which is defined as the arithmetic mean of all absolute distances of the roughness profile from the centerline within the measured length [22]. The data were not normally distributed ($p < 0.05$) and hence, transformed into the square root and analyzed using ANOVA and Tukey test ($p < 0.05$).

2.3. Smear layer removal by scanning electron microscopy examination (SEM)

Thirty mandibular extracted human incisors were selected for this test. The crowns were removed at the cemento-enamel junction using a high-speed bur under a water-cooled diamond disc. Two longitudinal grooves were prepared on both the buccal and lingual surfaces with a diamond disc without penetrating the canal. The samples were cleaned ultrasonically for 5 min in DW and the apices were sealed with composite resin (Filtek Z250, 3M, MN, USA). The canal preparation was performed with a reciprocating file (R25 Reciproc, VDW, Munich, Germany) and manual instrumentation up to #40 K-file (Dentsply, Maillefer, Ballaigues, Switzerland) and irrigation with 5 mL of 2.5% NaOCl between each file with a needle (25 × 4 mm) placed at 2-mm distance from the WL and final irrigation with 5-mL DW was performed to remove any residue.

Samples were randomly divided in six groups (n = 5) by irrigation substance and protocols presented previously. Irrigation was performed with 5-mL test substance for 1 min with a needle (25 × 4 mm) placed at 2-mm distance from the WL. Final irrigation was performed with 10-mL DW and dried with paper tips. The roots were then split into two halves with a hammer and a microtome blade. For each root, the half containing the most visible part of the apex was used in the study. Dehydration was performed in a desiccator at 60 °C for 72 h and the specimens were then mounted on an aluminum stub and coated with gold. Scanning electron microscopy (SEM) images at magnification of 2000× (Shimadzu, SSX-550A, Tokyo, Japan) were obtained and analyzed following the protocol of Hülsmann et al. [23] as follows: score 1, no smear layer and the dentinal tubules open; score 2, small amounts of the smear layer- and the dentinal tubules open; score 3, thin smear layer and the dentinal tubules partially open; score 4, partial covering with thick smear layer; score 5, full covering with thick smear layer. Each group included 15 images for the three thirds (Fig. 1B).

Blind evaluation was performed independently by two observers; if there were conflicting results, a third examiner determined the score. The inter-examiner's reliability was determined using the coefficient of Kappa test. The data were not normally distributed ($p < 0.05$); hence, the between-group differences were compared nonparametrically using Kruskal-Wallis and Dunn tests ($p < 0.05$).

2.4. Mineral content distribution by energy dispersive X-ray spectroscopy (EDS)

During image acquisition to evaluate removal of the smear layer, an image of the middle third of each group was selected for energy dispersive X-ray spectroscopy (EDS) analysis (Fig. 1) to determine the atomic ratio (at, %) of calcium (Ca), phosphorus (P), sodium (Na),

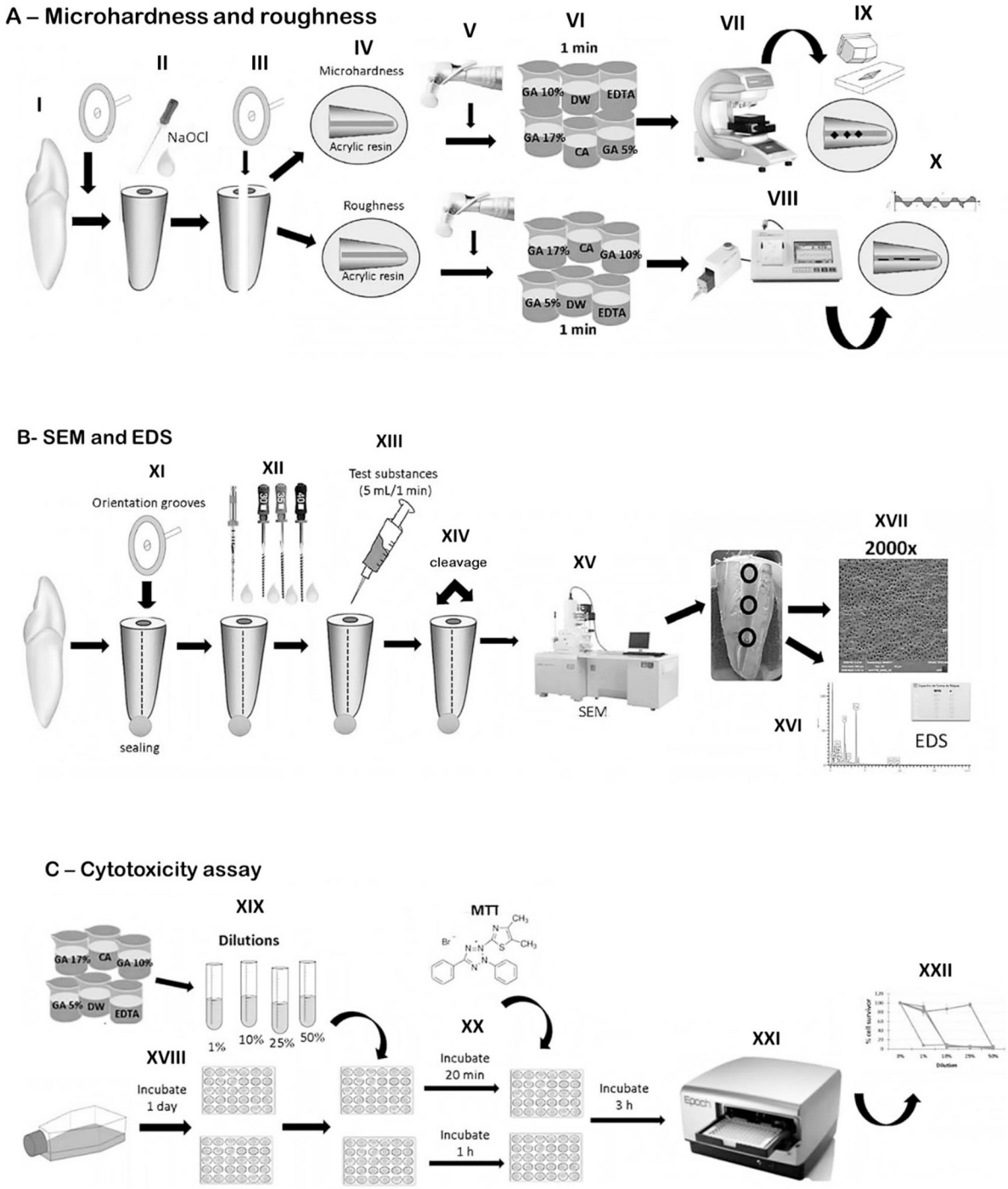

Fig. 1. A: Microhardness and roughness (I) crowns were removed from 60 root canals (II) roots instrumentation up to the instrument #45 K-file; (III) roots clivage; (IV) acrylic resin immersion; (V) root canal lumen was polished; (VI) test solution; (VII) Knoop indenter; (VIII) rugosimeter; (IX) Knoop microhardness measurement; (X) roughness measurement; B - SEM and EDS analysis - (XI) thirty mandibular incisors were prepared with external orientation grooves; (XII) instrumentation up to the instrument #40 K-file; (XIII) final rinse with test solutions for 1 min; (XIV) roots clivage; (XV) and (XVI) scanning electron microscope and images obtation; (XVII) EDS analysis; C - Cytotoxicity assay - (XVIII) cell culture; (XIX) dilutions of test solutions; (XX) time incubation; (XXI) multiwell spectrophotometer; (XXII) cytotoxicity results.

chlorine (Cl), magnesium (Mg), and zinc (Zn). EDS technique was used for chemical analysis of the surface to determine the presence of precipitate in the canal walls, and the number and energy of the X-rays emitted from the specimen were measured. The energy of the X-rays is characteristic of the difference in energy between the two shells, and the atomic structure of the chemical element from which they are emitted, which allows evaluation of the specimens' elemental composition (Fig. 1B).

2.5. Cytotoxic assay by fibroblasts cell culture

Fibroblast cells (lineage, 3T3) were obtained from the American Type Culture Collection and cultured in DMEM (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Inc. Corporarate, MA, USA), 100 μg mL^{-1} of streptomycin, and 100 mg/mL penicillin at 37 °C in a humidified incubator in an atmosphere containing 5% CO_2. Confluent cells were detached with

Table 1
Mean and standard deviation (SD) values of the microhardness and roughness in the groups.

Groups	Knoop microhardness		Roughness	
	Means (SD)	Statistical Tukey category	Means (SD)	Statistical Tukey category
DW	30.3 (4.8)	A	0.054 (0.009)	C
EDTA 17%	27.9 (3.3)	AB	0.112 (0.04)	B
CA 10%	24.5 (4.5)	B	0.116 (0.03)	B
GA 5%	24.7 (6.3)	AB	0.119 (0.02)	AB
GA 10%	24.8 (3.0)	AB	0.135 (0.03)	AB
GA 17%	18.1 (2.6)	C	0.155 (0.01)	A

DW, distilled water; EDTA, ethylenediaminetetraacetic acid. CA citric acid; GA glycolic acid; Different letters represent significant statistically differences ($p < 0.05$).

Table 2
Mean and standard deviation (SD) values of the smear-layer scores.

Groups	Cervical		Middle		Apical	
	Means (SD)	Statistical Kruskal Wallis category	Means (SD)	Statistical Kruskal Wallis category	Means (SD)	Statistical Kruskal Wallis category
DW	4.8 (0.4)	C,a	4.6 (0.4)	B,a	5.0 (0.0)	C,a
EDTA 17%	2.2 (0.5)	AB,a	2.7 (0.9)	A,a	3.9 (0.7)	BC,b
CA 10%	2.2 (0.7)	AB,a	1.8 (0.9)	A,a	2.5 (1.1)	A,a
GA 5%	2.4 (0.5)	AB,ab	1.8 (0.8)	A,a	3.0 (0.7)	AB,b
GA 10%	1.7 (0.4)	A,a	2.2 (0.6)	A,a	3.1 (0.7)	AB,b
GA 17%	2.6 (0.5)	B,ab	2.4 (0.5)	A,a	3.0 (0.0)	AB,b

Medians followed by different lowercase letters in the same row are statistically different ($p < 0.05$).
Medians followed by different uppercase letters in the same column are statistically different ($p < 0.05$).

0.25% trypsin and 0.05% EDTA (Gibco) for 5 min, and aliquots were subcultured. Cells were seeded in 96-well plates (1×10^4 cells/well). After 24 h, the culture medium was removed and cells were treated with 100-μL aliquots of 17% EDTA, 10% CA and 17% GA at different dilutions with DMEM (1/2, 1/4, 1/10 and 1/100) for 20-minutes and 1-hour time-periods.

Cell viability was determined through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assays. MTT (Sigma, St Louis, MO, USA) was prepared as a working solution of 0.5 mg/mL in complete medium just before use. After the tested time-periods, culture media was removed and 100-μL MTT-solution was added to each well. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 4 h. After incubation, the MTT was removed, and 100-μL DMSO (Sigma, St Louis, MO, USA) was placed in each well to dissolve the formazan crystals produced within the cells (Fig. 1C).

The plates were shaken for 5 min, the blue solution was transferred to a 96-well plate, and the optical density of the solution contained in each well was read at 540 nm wave length in an automatic microplate reader (EPOCH; Biosystems, Curitiba, PR, Brazil). Each experiment was performed using 3 cultures for each group and repeated 3 times. The formazan content of each well was computed as a percentage of the control group (cell treated with DMEM, without any irrigant).

Kolmogorov-Smirnov's test was used to evaluate the normal distribution, and the data were analyzed using ANOVA and Tukey's test ($p < 0.05$).

3. Results and discussion

The mean Knoop microhardness and roughness values and standard deviation are summarized in Table 1. Statistically significant difference was detected among the final irrigation solutions ($p < 0.0001$). GA 17% showed greatest reduction in the microhardness of all groups ($p < 0.05$). DW, EDTA, GA 5%, and GA 10% showed similar values with no statistical between-group difference ($p > 0.05$); group-wise comparison revealed that CA also decreased the microhardness of dentin, however, the reduction was lower than that achieved by GA 17% ($p < 0.05$).

Microhardness reduction may lead to reduction in modulus of elasticity and flexural strength of dentine [24]. Based on this, GA 5 and 10%, CA and EDTA showed better results compared to GA 17%. On the other hand, it was reported that superficialmicrohardness reduction in dentin of root canal lumen is desired because facilitates the access and action of endodontic instruments in narrow calcified root canals [21]. In the present study, Knoop indentation method was used according reported by Cruz-Filho et al. [21]; Knoop indentation method is more sensitive to surface change and textures than the Vickers method that penetrates about twice as far into the specimen as the Knoop indenter, being, therefore, more appropriate to evaluate superficial microhardness.

GA 17% achieved greatest reduction in the surface microhardness compared with the other groups, suggesting capability to generate mineral changes in the root canal dentin [25]. GA 5 and 10% showed no significant difference as compared to EDTA, CA, and DW suggesting that GA microhardness reduction is associated with increasing concentrations. Similar results are presented in a research where GA 35% was to effective than phosphoric acid to reduce dentin microhardness [19]. In this context and based in the methods used, the present study showed that GA 17% are effective in reducing the microhardness of the most superficial dentin layer which facilitates the biomechanical preparation considerably under clinical conditions, nevertheless, these results should be related to other evaluations as dentin flexural strength and action in dentin integrity to prove this assumption.

The roughness for all groups was increased significantly as compared with that of DW ($p < 0.0001$). GA 17% showed a high mean roughness with significant difference as compared to those of EDTA and CA ($p < 0.05$) but similar to those of GA 5% and 10% ($p > 0.05$). EDTA, CA, and GA 5%, and 10% showed no statistically significant difference ($p > 0.05$) (Table 1).

In the present study, all final irrigation solutions showed an increase in the surface roughness compared to DW. Among the solutions tested, GA 17% presented higher value of the surface roughness with no statistically difference from GA 5% and 10%. Therefore, GA concentration was not associated with increasing roughness. No significant differences were observed among the other groups. Rough surfaces enable a clinical

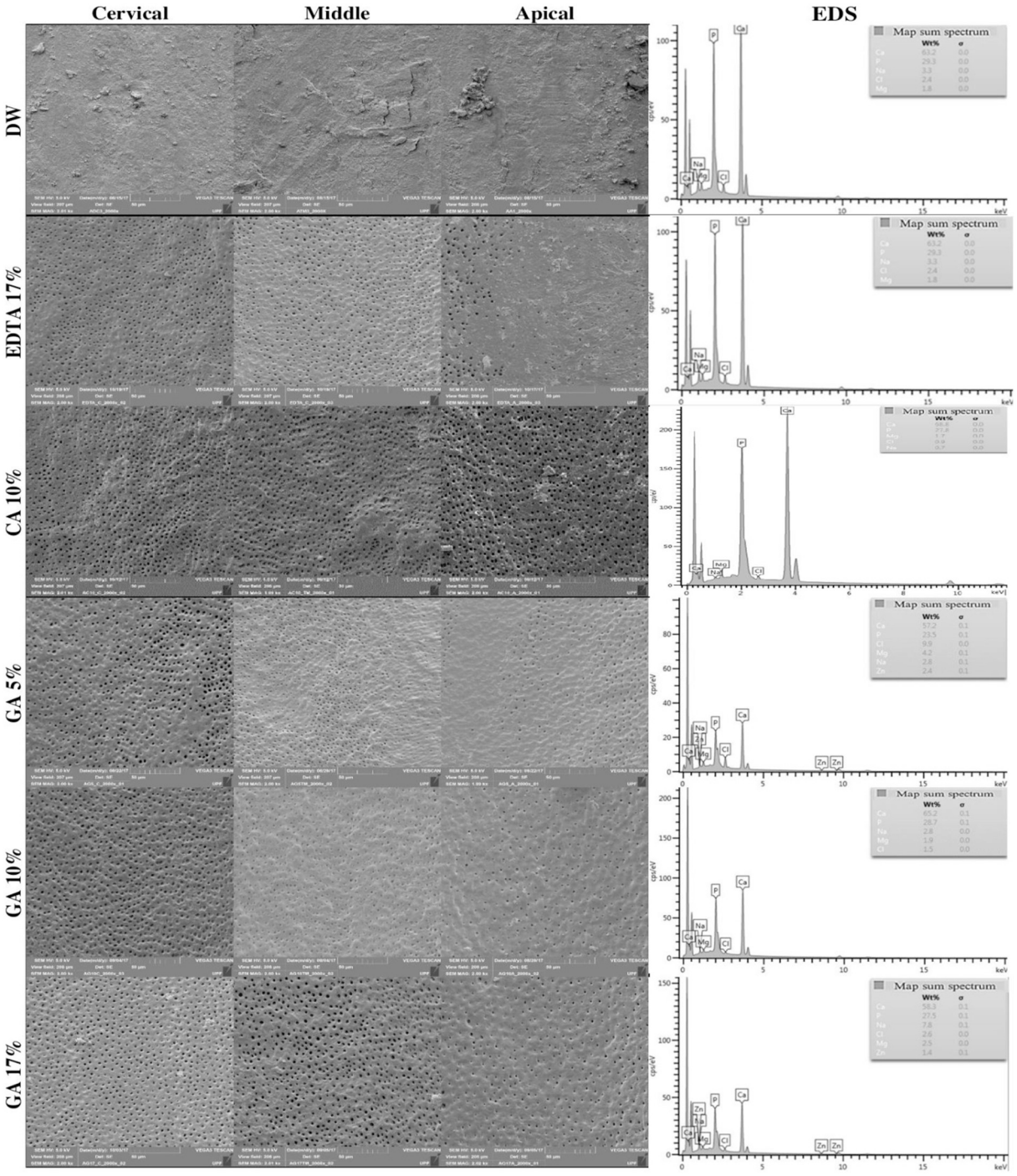

Fig. 2. Representative scanning electron microscopy (SEM) images of the root-canal walls irrigated with DW, EDTA 17%, and GA 5, 10 and 17% at the cervical, middle, and apical third of the canal walls. The EDS results with the respective atomic ratios (at, %) of calcium (Ca), phosphorus (P), sodium (Na), chlorine (Cl), and magnesium (Mg) and Zinc (Zn).

benefit in micromechanical bonding of the adhesive materials that requires the presence of surface irregularities of the adherent into which the adhesive can penetrate [22].

The results presented by GA can be explained by the demineralization of dentin caused by its acidic pH, which may lead to reduction of the microhardness and increase of the surface roughness [22], as well as the small size of its molecules [13]. Kataoka et al. [13] reported that when GA is applied for the purpose of skin exfoliation, it has easy penetration compared to other alpha hydroxyl acids like CA and malic acid, due to the small size of its molecule. Therefore, softening effects on the dentinal walls with microhardness reduction and increasing roughness, can be advantageous in the clinic however, the degree of softening and demineralization may have an influence on the physical and chemical properties of this structure [26,27].

The evaluation results of the smear layer are shown in Table 2 and Fig. 2. The Kappa coefficient test indicated high agreement between the raters for interpretation of scores related to removal of the smear layer (Kappa = 0.903). There was significant difference between DW and the other final-irrigation groups. GA groups presented similar results as compared with those of EDTA and CA at all thirds ($p > 0.05$). However, no final irrigation solution was able to completely remove the smear layer mainly at the apical third.

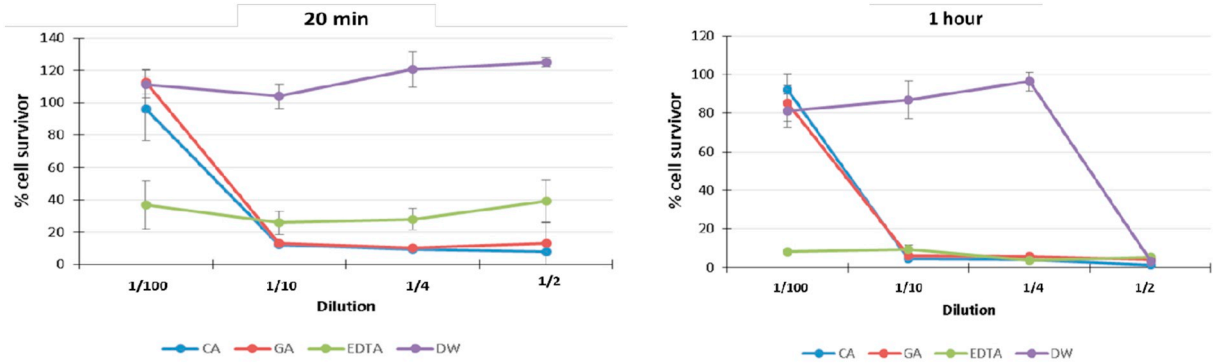

Fig. 3. Cell viability was determined and the means and standard deviations were compared. $p < 0.05$. Results are indicated as % of the control group.

No erosion areas were observed through analyses using SEM images and EDS (Fig. 2). Ca, P, and Na elements which are the components of dentin were detected in stable amounts, indicating no chemical alteration in the analyzed samples; Cl was detected in all groups, possibly as NaOCl residue.

Removal of the smear layer is indicated to improve the adaptation of the root filling material [4] and antimicrobial action of the irrigating solutions [5]. Nevertheless, NaOCl and chlorhexidine solutions, commonly used as endodontic treatment, do not have this capacity [2]. The results of present study are in agreement with the above mentioned studies as the group treated with NaOCl and final irrigation with DW revealed a large amount of the smear layer covering the dentinal tubules (Fig. 2). GA had effect to remove the smear layer at all used concentrations with no statistical differences compared to those of EDTA and CA and no differences between other GA concentrations at middle and apical third demonstrating that GA actually exhibits smear layer removal ability even in 5% concentration. No erosion areas were observed in SEM images for all groups and EDS (Fig. 2) results demonstrated no chemical alterations and no precipitate formation. According to mentioned, results demonstrates that GA at 5% and 10% were to effective than GA 17% in smear layer remotion, therefore, these concentrations should be taken into account in future researches.

Nevertheless, no final irrigation solution had capability to completely remove the smear layer mainly at the apical third, which may be due to the reduction of the diameter and increase of the depth of the root canal [28]. To overcome this disadvantage, irrigant agitation devices, such as sonic or ultrasonic irrigation, can be applied to improve removal of the smear layer [29].

EDTA in 1/100 dilution was more cytotoxic than the other substances at both evaluation time-points ($p < 0.05$); whereas, all irrigation solution groups at > 1/10 dilution, with the exception of DW showed similar high cytotoxic results (Fig. 3). At the 1-hour time-point, in all irrigation solution groups at > 1/10 dilution, the percentage cell viability was reduced to nearly 0%; 17% GA and 10% CA showed similar results at all dilutions evaluated.

In the present study cytotoxicity was performed using 3T3 cell lines, as established cell lines had reproducibility of the results, besides their rapidly multiplication and unlimited life span. For these reason, these types of cell lines are indicated by ISO 10993-5 [20]. The cytotoxic results indicated that 17% EDTA has more cytotoxic effects when compared to 10% CA and 17% GA. Previous studies also demonstrated higher cytotoxic effects of EDTA when compared to CA [30,31]. Moreover, Oh et al. [32] also demonstrated that EDTA is more cytotoxic when compared to CA and GA.

4. Conclusions

Based on our results, the study's hypothesis was confirmed. GA has potential for use as the final rinse agent in endodontics, however, data to evaluate additional GA characteristics such as pH level, surface tension and effects on the collagen fibrils of dentin, flexural strength, and promoting adhesion of endodontic cements, as well as sonic and ultrasonic activation methods are necessary to confirm these applications.

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