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Evaluation of Dentin Micro-hardness and Antibacterial Efficiency against E.faecalis after Irrigation with Sodium Hypochlorite Followed by either Grape Seed Extract or EDTA: A Randomized In-Vitro Study

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Abstract

Objectives: Evaluation of dentin micro-hardness, dentinal erosion and antibacterial efficiency against E.faecalis after irrigation with sodium hypochlorite (NaOCl) followed by grape seed extract (GSE)solution versus irrigation with NaOCl followed by EDTA.

Materials and Methods: Regarding dentin micro-hardness test; 16 teeth were divided into 2 groups; intervention (5.25%NaOCl+13%GSE) and comparator (5.25%NaOCl+17%EDTA) groups. Each tooth was decoronated and sectioned vertically. Dentin microhardness was evaluated before and after irrigation at the coronal, middle and apical thirds. Representative specimens from the micro-hardness test were used for dentin surface examination using scanning electron microscope (SEM). For the evaluation of the antibacterial efficiency, 18 teeth were decoronated and inoculated with E. faecalis. Microbial analysis for the specimens was performed before and after chemicomechanical preparation to obtain the pre-and post-treatment number of colony forming unit (CFU). The data obtained from each test were statistically analyzed.

Results: Results revealed that comparator irrigation protocol caused significantly higher percentage reduction (34.75 %) compared to the intervention irrigation protocol (17.48 %, p= 0.021). SEM of dentin in the intervention group showed no or mild erosion. While the comparator group showed moderate to severe dentinal erosion. For antibacterial efficiency test there was significant difference (p=0.008) in bacterial CFU/mL before (3167.78 ±959.83 CFU/mL) and after (21.11±13.64 CFU/mL) irrigation in the intervention group. Moreover, in the comparator group there was significant difference (p=0.008) in bacterial CFU/mL before (2684±806.63 CFU/mL) and after (22.22±12.02 CFU/mL) irrigation.

Conclusions: Based on the limits of the present study, it could be concluded that using GSE following NaOCl irrigation protocol caused less adverse effects regarding; dentin micro-hardness and dentinal erosion compared to using EDTA following NaOCl. Using NaOCl followed by GSE irrigation protocol has a comparable effect to NaOCl followed by EDTA regarding the antibacterial activity. GSE represents a natural alternative irrigant to EDTA.

1. Introduction:

Endodntic irrigation is an important step during root canal treatment. Root canal irrigation allows for cleaning areas that cannot be reached by mechanical instruments alone such as; dentinal tubules, isthmus and ramification areas. Moreover, irrigants remove the smear layer from the radicular wall, lubricate the root canal and flush out debris from the root canal system.(1) For a long time, sodium hypochlorite (NaOCl) has been

considered the most commonly used irrigant due to its ability to dissolve organic tissues, its antimicrobial efficiency, as well as to being cheap with reasonable shelf life.[2] However, NaOCl induces structural changes in the organic components of dentin mainly the collagen, leading to reduction in the mechanical properties of root dentin. In addition, NaOCl cannot dissolve inorganic dentin remnants from the root canal.[3,4] In a trial to solve these problems, irrigation with 5.25% NaOCl alternated with 17% ethylenediaminetetraacetic acid (EDTA) was proposed. Nowadays, this is the most commonly used irrigation protocol especially in narrow and calcified root canals. EDTA is a chelating agent used to remove smear layer and is able to remove calcium ions from dentin. However, prolonged exposure to EDTA leads to excessive removal of the dentin inorganic part and may cause dentin erosion.[3,4]

The combined action of NaOCl and EDTA causes alteration in the collagen matrix and decalcification of dentin which decrease the dentin microhardness.[5] For this reason, natural alternatives such as proanthocyanidins (PAs) have been studied to be used as endodontic irrigants in an attempt to overcome the detrimental effects of synthetic irrigants. PAs are a complex subgroup of the flavonoid compounds that are found in different fruits, vegetables, seeds and barks.[6] It was found that grape seed extract (GSE) contains 74-78% proanthocyanidins.[7] PAs are naturally occuring antioxidants that have also antimicrobial, anticarcinogenic and vasodilatory actions.[8] Moreover, PAs were found to enhance the mechanical properties of dentin because they inhibit collagen breakdown and reduce its enzymatic degradation by matrix metalloprotease enzyme.[9] It was also found that PAs increase the biodegradation resistance of dentin by formation of covalent cross links within dentin collagen.[10]

Therefor, the aim of the present study was to evaluate and compare the dentin microhardness, dentinal erosion and antibacterial efficiency against E.faecalis after irrigation with NaOCl followed by GSE solution versus irrigation with NaOCl followed by EDTA. The null hypothesis of the present study was; there is no difference between irrigation with NaOCl followed by grape seed extract and irrigation with NaOCl followed by EDTA regarding; dentin micro-hardness, dentinal erosion and antibacterial efficiency against E. faecalis.

2. Materials and Methods:

A total of 34 single human permanent incisors or premolars with single root canals were obtained from patients with ages from 25-50 years old and included in the present study; 16 teeth for the dentin microhardness test and 18 teeth for the antibacterial efficency test. For micro-hardness test; based on a previous study by Saha et al., 2017[11] the difference in dentin micro-hardness between the 2 groups was 3±2 VHN. Using power 80% and 5% significance level, 8 teeth were needed in each group (16 teeth total). For antibacterial efficiency test; based on a previous study by Zare et al., 2017[12] the difference in bacterial counts between the 2 groups was 43.2±30. Using power 80% and 5% significance level, 9 teeth were in each group (18 teeth total). Sample size calculation was achieved using PS: Power and Sample Size Calculation Software Version 3.1.2 [Vanderbilt University, Nashville, Tennessee, USA]. The teeth were collected from the dental extraction clinic, Oral and Maxillofacial Surgery Department, Faculty of Dentistry, Cairo University, Egypt. The present study was approved by the Research Ethics Committee (Approval number 18714), Faculty of Dentistry, Cairo University, Egypt.

Teeth with straight roots extracted due to orthodontic or periodontal problems were included in the present study. Teeth with previous endodontic treatment, cracks, root caries or root resorption were excluded from the present study. The teeth were cleaned from hard and soft debris on the root surface using hand scaler (Zoll-Dental, USA) and were stored in distilled water at room tempreture until time of use. The randomized assignment of the teeth into groups was done using www.random.org website. The included teeth were inserted by the investigator in sequentially numbered, opaque sealed envelopes. The outcome assessors and statistician were blinded.

Preparation of the GSE solution:

The 13% GSE solution was obtained by dissolving 13g of GSE powder (Puritans Pride INC, USA) in 100ml distilled water.[13] The GSE solution was then stirred for 5 min using a magnetic stirrer (Stuart UC125D, UK).[14] The pH of the GSE solution was measured by a pH meter (Hanna precision pH meter, model pH211, Romania). The pH of the prepared GSE solution was 5.7.

2.1. Dentin micro-hardness test:

A total of 16 single rooted teeth were decoronated at the level of the cementoenamel junction using a high-speed disk (Kavo EWL K10, Germany). The roots were bisected longitudinally in a buccolingual direction into two halves using an automated diamond saw machine (Isomet 4000, Buehler Ltd., Lake Bluff, IL, USA) under copious water irrigation. A total of 32 root halves were obtained and placed horizontally with radicular dentin surface facing upwards in a new freshly mixed auto-polymerizing acrylic resin (Acrostone, Acrostone Dental Manufacture, Egypt) in a uniform plastic rings with internal diameter of 2cm as shown in Figure 1.[15]

Each specimen was individually mounted on the stage of Vickers micro-hardness tester (NEXUS 4000TM, Innovatest, model no 4503, Netherland). Micro-hardness indentations were marked with a Vickers diamond indenter at 300 g load and dwell time of 20 seconds with magnification 20x for measuring baseline micro-hardness values (M_i)for the three radicular thirds (coronal, middle and apical thirds).[11,15] The intervention (5.25%NaOCl + 13%GSE) group specimens were immersed in 5.25% NaOCl solution (Research lab fine chem industries, India) for 15 minutes followed by immersion in 13% GSE solution for another 15 minutes. The comparator (5.25%NaOCl + 17%EDTA) group specimens were immersed in 5.25% NaOCl solution for 15 minutes followed by immersion in 17% EDTA solution (Prevest DenPro, India) for another 15 minutes. This was done to maintain uniformity and standardization between the groups.[11,16]

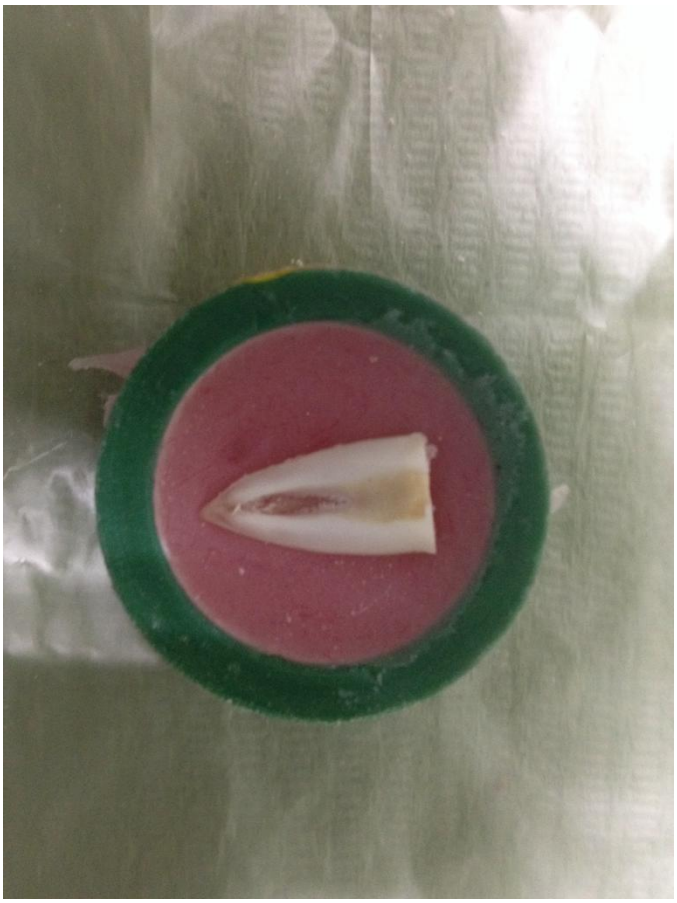

Figure 1: Root half placed in freshly mixed acrylic resin.

Post-treatment micro-hardness values (M_p) were recorded afterwards as mentioned above. For each specimen, the percentage reduction in radicular dentin micro-hardness values were calculated according to the following equation:

$\frac{Mi- Mp}{Mi} \times 100$ [%],

where M_i is the initial micro-hardness value and M_p is the post-treatment micro-hardness value.[15]

2.2. Dentin surface examination for dentinal erosion using Scanning Electron Microscope (SEM) :

Four representative specimens from the micro-hardness test; two specimens from each group, were used for dentin surface examination using scanning electron microscope (SEM) (Model Quanta 250 FEG, FEI Company, Netherlands). The root specimens were mounted, and gold sputter coated (EMITEC, K550X sputter coater, England). Scanning Electron Micrographs were taken

at 2000x.[17]

2.3. Antibacterial efficiency against Enterococcus faecalis (E faecalis) :

A total of 18 teeth were decoronated at the level of the cementoenamel junction using a high-speed disk (Kavo EWL K10, Germany). To ensure canal patency, a stainless-steel K- file size 15 (MANI, INC, Japan) was inserted in each root canal until the tip of the file was visualized from the apical foramen, then the working length were calculated by deducting 1mm. To enable easy handling and to mimic the in vivo conditions, the roots were fixed vertically in polypropylene Eppendorf tubes by the means of putty silicone rubber impression material (Silaxil, Lascod Spa, Italy). The root blocks were then steam autoclaved (Sturdy Autoclave, class N autoclave, SA-232) at 122° C for 33 min under pressure 1.7kg/cm^2.[18] To ensure absence of any microorganisms, paper points (Diadent Co, Korea) of a suitable size were inserted inside each root canal and then transferred to blood agar plates to ensure effectiveness of sterilization. The plates were then incubated in an incubator (Precision TM Dual program illuminated incubator, USA) at 37°C for 24 hours and observed for growth of any microorganisms.

The antibacterial efficiency against E.faecalis was evaluated using agar dilution method. Each root canal was contaminated (inoculated) with E. faecalis with a standardized strain ATCC 29212 {0.5 McFarland [1.5 x10^8 colony forming units] standard} suspension using insulin syringe inserted throughout the depth of the canal.[19] Then 0.5 ml of bacterial suspension was injected inside each root canal. Then, the root blocks were incubated at 37°C for 24 hours. Three paper points size 20 were used to obtain specimens from the depth of each root canal and were transferred to polypropylene Eppendorf tube containing sterile Brain Heart Infusion (BHI) broth (Himedia, India). BHI is characterized by presence of abundant nutrients available for the bacteria.[20,21]

Then 0.1ml (100 μL) of the broth was transferred using a micropipette to 0.9ml of freshly prepared (BHI) broth to prepare 1ml of BHI broth with 1/9 concentration.[20,22] The total 1ml was aspirated from the tube by a micropipette and transferred to BHI agar (Himedia, India) plate. The 1ml broth was spread over each plate using L-shaped glass tube and the 18 BHI agar plates (9 plates/ group) were placed in an incubator at 37°C for 24 hours. After 24 hours the bacterial colonies in each plate were counted visually and were represented as the number of colony-forming units (CFU). For each plate colony per milliliter (CFU/mL) was calculated by multiplying the number of colonies per plate by the reciprocal of the dilution (the number of CFU in each plate was multiplied by 10) to get the pre-treatment colony per milliliter (CFU/mL) S_1.[23]

Standard mechanical instrumentation was performed for all roots using the step-back technique. The root canals were prepared using stainless steel K-files (MANI, INC, Japan) up to master apical file size 45. Coronal 2/3 of all root canals were prepared by hand stainless steel H-file (MANI, INC, Japan) size 50 up to size 80. The mechanical preparation was accompanied with application of 2ml 5.25% (NaOCl) after each file size in all root canals. The irrigant was introduced inside each root canal using disposable plastic syringes. Finally, the root canals of the intervention group were rinsed with 5ml GSE solution and for the comparator group, the root canals were rinsed with 5ml EDTA solution. The root canals were flushed with 5ml distilled water and were then dried using paper points of the same size as the corresponding master apical file.

Microbial analysis for the specimens was performed after chemicomechanical preparation to obtain the final number of colony forming unit. The specimens were obtained from the depth of each root canal and cultured as mentioned above.[23] The post-treatment colony per milliliter (CFU/mL) S_2 were calculated and the efficiency of each irrigation protocol was measured by calculating the percentage reduction in the colony count using the following equation:

$$\frac{[[CFU/mL\ S1 - CFU/mL\ S2]}{CFU/mL\ S1} \text{ x } 100\ (\%).$$[24]

2.4. Statistical methods:

Data were coded and entered using the statistical package SPSS version 25. Comparisons be-

tween groups were done using unpaired t-test in normally distributed quantitative variables while non-parametric Mann-Whitney test was used for non-normally distributed quantitative variables. For comparison of serial measurements within each group paired t test was used. P-values less than 0.05 were considered as statistically significant.[25]

3. Results:

3.1. Results of dentin micro-hardness:

The median and range values of percentage reduction in radicular dentin micro-hardness are listed in Table 1. Results showed that irrigation with the comparator irrigation protocol (34.75%) caused significantly higher percentage reduction compared to the irrigation with the intervention irrigation protocol (17.48%).

Table 1: Percentage reduction [%] in radicular dentin micro-hardness in the intervention and comparator groups.

	Intervention group 5.25% NaOCl + 13%GSE	Comparator group 5.25% NaOCl + 17% EDTA	P-value
Median (%)	17.48	34.75	0.021*
Range (minimum value (%)-maximum value (%)	[2.53-40.95]	[1.61-50]	

*; significant ($p<0.05$)

3.2. Dentin surface examination for dentinal erosion using Scanning electron microscope (SEM) :

Scanning Electron Micrographs of dentin in the intervention group showed no or mild erosion and all tubules appeared normal in appearance and size, Figure (2). While dentin in the comparator group showed moderate to severe erosion, where the peritubular and intertubular dentin appeared destroyed and some dentinal tubules were connected to each other, Figure (3).

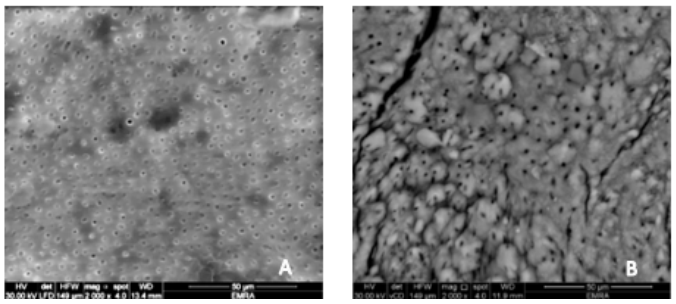

Figure 2: canning Electron Micrographs of the intervention group specimens showing; (A) no dentinal erosion and (B) mild dentinal erosion.

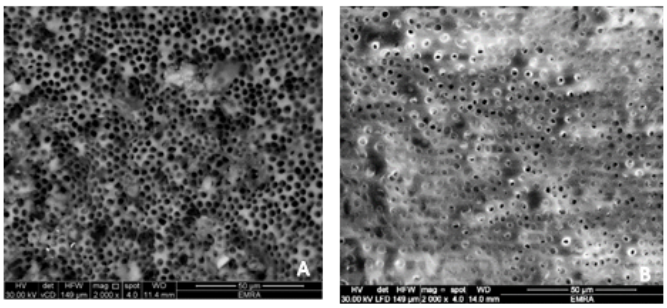

Figure 3: Scanning Electron Micrographs of the comparator group specimens showing; (A) severe dentinal erosion and (B) moderate dentinal erosion.

3.3. Results of Antibacterial efficiency test:

The results revealed no significant difference between the two groups before ($p=0.297$) and after ($p=0.796$) irrigation. In the intervention group, there was significant difference ($p=0.008$) in bacterial CFU/mL before (3167.78 $\pm$959.83 CFU/mL) and after (21.11$\pm$13.64 CFU/mL) irrigation. Moreover, in the comparator group, there was significant difference ($p=0.008$) in bacterial CFU/mL before (2684$\pm$806.63 CFU/mL) and after (22.22$\pm$12.02 CFU/mL) irrigation as shown in table 2.

*; significant ($p<0.05$).

The results of the percentage reduction (%) in the bacterial CFU/mL revealed no statistically significant difference ($p= 0.297$) between

Table 2: The mean and standard deviation (SD) values of the bacterial CFU/mL before and after irrigation for the intervention and comparator group.

Groups	Intervention group (5.25% NaOCl + 13% GSE)	Comparator group (5.25% NaOCl + 17% EDTA)	P-value
Before irrigation (CFU/mL)	3167.78 ±959.83	2684±806.63	0.297
After irrigation (CFU/mL)	21.11±13.64	22.22±12.02	0.796
P- value	0.008*	0.008*	

the intervention (99.17±0.85 %) and comparator (99.06±0.60 %) groups as shown in table 3.

Table 3: Table 3: The mean and standard deviation (SD) values of percentage reduction (%) in the bacterial CFU/mL.

Groups	Intervention group	Comparator group	P-value
Mean± SD	99.17±0.85 %	99.06±0.60 %	0.297

4. 4- Discussion:

4.1. Dentin micro-hardness:

The dentin micro-hardness was significantly decreased after irrigation with both the intervention and comparator irrigation protocols. This is contributed to the dissolving action of NaOCl on the dentin organic components mainly type I collagen by breaking down the bonds between the carbon atoms and fragmentation of the long peptide chains leading to the disintegration of dentin collagen and decreasing the dentin micro-hardness.[11,26] These results were in agreement with **Ari et al. in 2004**[16] who showed that treatment with either 2.5% or 5.25% NaOCl solutions reduced the radicular dentin micro-hardness.

Moreover, EDTA solution further decreased the radicular dentin micro-hardness, which could be explained by the ability of EDTA to bind to inorganic components especially divalent ions of dentin. This chelating effect of EDTA cause demineralization and softening of dentin.[27] **Bakr et al. in 2016**[28], **Nikhil et al. in 2016**[29] and **Saha et al. in 2017**[11] reported that treatment of root dentin with 17% EDTA significantly reduced the dentin micro-hardness. The intervention irrigation protocol was found to significantly decrease the dentin micro-hardness in the present study. This could be attributed to the fact that the prepared GSE solution has an acidic pH (5.7) which might have played a role in demineralizing radicular dentin.

Furthermore, PAs have the ability to bind divalent metals effectively such as iron and copper. The metal-chelating capacity of PAs is due to presence of hydroxyl groups especially o-dihydroxyl phenyl groups of the B ring which are essential sites for metal chelation.[30,31]

In the present study, irrigation with the comparator irrigation protocol caused significantly higher percentage reduction compared to the irrigation with the intervention irrigation protocol Table 1. Therefore, the null hypothesis regarding the dentin micro-hardness was rejected. This could be attributed to the deleterious effect of the combined use of NaOCl and EDTA which caused a pronounced negative effects on dentin compared to using EDTA or NaOCl alone. The results of the present study were in agreement with **Sayin et al. in 2007**[15], **Mishra et al. in 2012**[32] and **Das et al. in 2014**[33] who reported that the use of combined treatment [NaOCl and EDTA] significantly decreased the dentin micro hardness. These results were due to further exposure of the calcified material of the demineralized dentin to EDTA through removal of the organic matrix by NaOCl.[15]

4.2. Dentin surface examination for dentinal erosion using Scanning electron microscope (SEM) :

Erosion is the loss of tooth substance by a chemical process such as the dissolving action of an irrigant.[4] Radicular dentin erosion could be one of the destructive effects of using endodontic irrigants. This could be explained by the fact that the use of the endodontic irrigants may result in the degradation of the organic matrix of root dentin and eventually the reduction of the mineral content.[34]

There are three grade scoring system of dentinal erosion; score (1) where there is no, or minimal erosion and all dentinal tubules look normal in appearance and size. Score (2) moderate erosion which is characterized by eroded peritubular dentin, and score (3) severe erosion; in which the dentin surface is totally destroyed, with intertubular dentin destroyed and some tubules are connected to one another.[35]

In the present study the intervention group showed no or mild erosion, while dentin in the comparator group showed moderate to severe erosion, Figures (2) and (3). Therefore, the null hypothesis regarding the dentinal erosion was rejected. The mild dentinal erosive effect of the intervention irrigation protocol on the radicular dentin could be explained by the known collagen cross-linking potential of the GSE. This effect might have counteracted the damaging effect of NaOCl on the dentin organic component. The cross-linking potential of GSE could be explained by the formation of hydrogen bond between the protein amide carbonyl of collagen type I and the phenolic hydroxyl groups of PAs. This is in addition to the covalent, ionic bonds and the hydrophobic interactions between PAs and dentin collagen. Additionally, PAs enhance proline hydroxylase enzyme activity which catalyzes the hydroxylation of proline; an essential step in collagen biosynthesis and inhibit matrix metalloprotease enzyme.[36–38]

On the other hand, the severe dentinal erosive effect of the comparator irrigation protocol on the radicular dentin could be explained by the demineralization and excessive opening of the dentinal tubules by EDTA. These results agreed with those obtained by **Aksel et al. in 2016**[34] who reported that the treatment of dentin with 5% NaOCl followed by 17% EDTA leads to loss of large amount of calcium and more pronounced decalcification. The study explained this by the more rapid diffusion of EDTA into the peritubular and intertubular dentin due to the destruction of the organic matrix caused by initial application of NaOCl. Additionally, **Siso et al. in 2015**[39] and **Aranda-Garcia et al. in 2013**[40] reported that the application of EDTA followed by NaOCl caused peritubular and intertubular erosion. This could be explained by the hyper-decalcification effect of EDTA. This effect was more pronounced when NaOCl was used with EDTA as it accelerates dentinal erosion through organic matrix destruction.

4.3. Antibacterial efficiency against E feacalis:

In the present study, the root blocks used for the antibacterial efficiency test were autoclaved and their complete sterility was confirmed by absence of any microbial growth on the blood agar plates. The results showed that both the intervention (5.25% NaOCl + 13% GSE) and the comparator (5.25%NaOCl+ 17%EDTA) irrigation protocols showed no significant difference in the bacterial CFU/mL and percentage reduction in the bacterial CFU/mL. Therefore, the null hypothesis regarding the antibacterial efficiency against E.faecalis was accepted. Moreover, the bacterial CFU/mL was significantly decreased after irrigation with the intervention and the comparator irrigation protocols. This could be attributed primarily to the use of 5.25% NaOCl in both protocols. NaOCl is widely known for its wide spectrum antimicrobial effect. The antimicrobial effect of NaOCl is due to formation of hypochlorous acid (HOCl) when NaOCl ionizes in water. HOCl adversely affects the vital functions of the bacterial cell through oxidation of sulfhydryl groups of the bacterial enzymes leading to the formation of disulfide linkages and interruption of the bacterial metabolic reactions. Moreover, NaOCl cause disruption of bacterial DNA and chlorination of nucleotide bases.[23,24]

The results are in agreement with **Baca et al. in 2011**[21] who found that irrigation with 5.25% NaOCl followed by 17% EDTA inhibited the growth of E.faecalis. This was explained by the achievement of better access of NaOCl to the dentinal tubules especially in the apical third after removal of the smear layer by EDTA. In addition, **Johal et al. in 2007**[41] and **Mohammadi et al. in 2013**[42] reported that irrigation with NaOCl with EDTA significantly reduced the bacterial colonies within the root canal system. The antibacterial activity of EDTA could by due to the chelation action of cations from the bacterial outer membrane.[42]

On the other hand, GSE is a natural antioxidant which contains 74-78% PAs, a type of polyphenols. GSE is a weak acid that can be used in the removal of smear layer as reported by **Margono et al. in 2017**.[7] GSE polyphenols have antimicrobial properties against Gram negative and Gram positive bacteria. Polyphenols affect the selective permeability of the bacterial plasma membrane leading to the leakage of intracellular substances and damage of the bacterial cell.[24] Furthermore, **Albino Souza et al. 2017**[43] showed that GSE has comparable antimicrobial effect as clinically used irrigants such as CHX (Chlorhexidine) and QMix (QMix is a solution of bisbiguanide antimicrobial agent (2% CHX) and a polyaminocarboxylic acid calcium-chelating agent (17% EDTA)). [44] This effect is due to the antimicrobial effect of PAs and phenolic compounds in GSE which act on the bacterial cell wall of E.faecalis.

5. Conclusions:

Based on the limits of the current study, it could be concluded that:

1) Using 13% GSE following 5.25% NaOCl as an irrigation protocol caused less adverse effects on radicular dentin regarding; dentin microhardness and dentinal erosion compared to using 17% EDTA following 5.25% NaOCl.

2) Using 5.25% NaOCl+ 13% GSE irrigation protocol has a comparable effect to 5.25% NaOCl+ 17% EDTA irrigation protocol regarding the antibacterial activity against E. feacalis

3) GSE represents a better natural alternative irrigant to EDTA following NaOCl.

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