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Research Article

Effect of Different Chelating Agents on Calcium Ion Quantification and on Microhardness of Human Radicular Dentin-An *In Vitro* Study

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Abstract

Introduction: The purpose of this study was to evaluate the effects of 17% ethylenediaminetetraacetic acid, 1% Phytic acid, 0.2% Chitosan on Calcium ion quantification and microhardness of human root canal dentin using inductively coupled plasma optical emission spectrometry and Vickers microhardness tester.

Materials and Methods: A total of eighty extracted mandibular premolars were decoronated and root canal treatment was prepared. Samples were divided into two subgroups, one half for calcium ion quantification and other half for microhardness testing. Based on the type of chelating agent used in each subgroup, samples (N = 10) were divided Repetition (three test and one control). The calcium loss was evaluated using the inductively coupled plasma optical emission spectrometry and microhardness with Vickers microhardness tester.

Statistical analysis: Data were analyzed for calcium ion quantification using one-way ANOVA, Post hoc Tukey's test and for microhardness testing using Student's t test.

Results: Calcium loss along with resultant drop in microhardness were maximal in ethylenediaminetetraacetic acid (EDTA) group with a significant difference from Phytic acid and Chitosan group ($p < 0.05$). There was no statistically significant difference seen between the Phytic acid and Chitosan groups ($p > 0.05$) in both the tested parameters (Calcium ion quantification and Microhardness).

Conclusion: Use of 0.2% chitosan and 1% phytic acid can be thought of as alternatives to ethylenediaminetetraacetic acid when used as chelating agent when used along with sodium hypochlorite.

Keywords: *Calcium; Microhardness; EDTA; Phytic Acid; Chitosan; ICP-OES; Vickers Microhardness Tester*

Abbreviations

EDTA: Ethylenediaminetetraacetic Acid; ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

Introduction

The main goal of cleaning and shaping the root canal system is to eliminate the aggressive and irritating agents such as microorganisms, their products and vital and necrotic pulp tissue remnants [1]. Endodontic instrumentation, either manual or mechanized techniques, creates smear layer on the root canal walls and smear plugs into dentinal tubules. McComb and Smith in 1975 first reported the presence of smear layer on the surface of instrumented root canals [3]. A systemic review and meta-analysis of leakage studies concluded that the smear layer removal improves the fluid-tight seal of the root canal system [1,2].

However though myriad endodontic irrigating solutions have been proposed, a combination of sodium hypochlorite (5.25% NaOCl) and ethylenediaminetetraacetic acid (17% EDTA) have been recommended as standard irrigating protocol for smear layer removal due to their ability to remove both organic and inorganic components of the smear layer [4,5].

Evidence based studies suggest that when NaOCl is used alone, it acts only on the organic component of dentin and alters its physical and mechanical properties. If NaOCl is used consequently with chelating agents, such as EDTA, it is capable of dissolving the organic remnants of the smear layer and predentin in addition to demineralizing the inorganic mineralized portion of the root canal wall. NaOCl is the most ubiquitous irrigating solution in endodontics. It has numerous favourable qualities and NaOCl performs bactericidal cytotoxicity, benefits the dissolution of organic debris and enhances minor lubrication.

Various authors have reported that the use of NaOCl in combination with EDTA, causes the degradation of the desired properties of NaOCl, such as pH, free available chlorine, antimicrobial activity and tissue dissolution ability. Also this synergistic action results in severe erosion of root canals and dentinal tubules. Research claims that the use of EDTA for more than one minute causes dentinal tubule erosion [5,8].

EDTA is a synthetic, non-biodegradable material which is considered as pollutant in the root canal system and reported to be cytotoxic to macrophages [5,9]. Reviewing all these facts, a search for an alternative agent which is more biodegradable to replace EDTA continues.

One such material that has been evaluated in the study is chitosan, a naturally produced polysaccharide extracted from the exoskeleton of crustaceans by deacetylation of chitin from the shells of crabs and shrimps. It is empowered with properties like biocompatibility, biodegradability, bioadhesion and also proven to be non- toxic to human cells. It is second most abundant material in nature after cellulose [22]. Studies have reported that it has high chelating capacity for different metallic ions [1,8]. In a research conducted by Silva PV., *et al*. the authors found 0.2% chitosan to be equally effective to 15% EDTA and 10% citric acid in smear layer removal and in the reduction of dentin microhardness [11,12].

The other material which is used in the study is phytic acid (phytate or inositol hexakisphosphate, IP6-saturatedcycic acid) which is a naturally occurring product. It is extracted from whole grains, cereals, legumes, nuts and seeds, and is the primary energy source for the germinating plants [6,15]. They are also found in mammalian cells [7,15]. It is one of the most potent natural mineral chelator, which has the affinity to calcium ions because of its high negatively charged molecule which is pH dependent. The acidic and chelating function of phytic acid can make it an effective agent for smear layer removal. It also shows good biocompatibility with the periapical tissues. Microhardness reduction in dentin was found to be comparatively less with phytic acid when compared to EDTA [5,9].

Aim of the Study

The purpose of this present study was to evaluate the effects of 17% Ethylenediaminetetraacetic acid (EDTA), 1% Phytic acid and 0.2% chitosan on calcium ion quantification and microhardness of human root canal dentin using inductively coupled plasma optical emission

spectrometry (ICP-OES) and Vickers microhardness tester.

Materials and Methods

Preparation of samples

Eighty freshly extracted human mandibular premolars were collected from the out patient department of the Department of Oral and Maxillofacial Surgery, Panineeya Mahavidyalaya Institute of Dental Sciences and Research Centre, Hyderabad, India.

The teeth were cleaned and made free of debris and calculus and stored in saline solution.

Inclusion criteria: Non -carious, intact, single rooted mandibular human premolar teeth, with no surface defects, cracks or fillings.

Exclusion criteria: Carious teeth, fractured/cracked teeth, restored teeth, multi-rooted teeth.

All teeth were decoronated at the cementoenamel junction using a diamond disc with a water spray and the pulp was extirpated using a barbed broach (size 0), (MANI, Inc., Tochigi, Japan). Working length was verified with an ISO size #10 K-file (MANI, Inc., Tochigi, Japan). After establishing the working length, root canal preparation was initiated in a crown down fashion (Pro Taper Gold, Dentsply Maillefer, Ballaigues, Switzerland). Master apical preparation was done till finishing file F3. Care was taken to keep the apex patent. The canal was irrigated with 5 ml of 3% NaOCl solution between each instrument change. Finally, the canal was irrigated with 10 ml of deionized water. Then samples were divided into two groups: one half of the samples for calcium ion quantification from the root canal and the other half for microhardness testing.

Calcium ion quantification

Each tooth was placed in a 15 ml of falcon tubes with the lid perforated in such a way that the crown portion of the tooth remained outside the lid and the root portion was inside the tube. The gap between the tooth and the perforation on the lid was sealed with Filtek Z250 composite resin (3M, Universal Restorative, USA). Based on the type of chelating agent used, the samples (n = 10) were divided into four groups (three experimental and one control group).

For the preparation of 0.2% chitosan solution (pH 3.2), 0.2g of chitosan (Nanobiotech Pvt. Ltd, Chennai, India) with 90% degree of deacetylation was diluted with 100 mL of 1% acetic acid and the mixture was stirred for 2h using a magnetic stirrer.

For the preparation of 1% phytic acid (pH 3.2), 1g of phytic acid (Pioneer chemical industries, Hyderabad, India) in 100 mL of distilled water and the mixture was stirred for 2h using a magnetic stirrer.

Accordingly, Group I - 17% EDTA solution, Group II - 1% Phytic acid, Group III - 0.2% chitosan, Group IV -saline were categorized. Each sample was irrigated with 5ml of test irrigants for 5 minutes (1 ml/minute) using 27 gauge needle attached to a syringe. Irrigation was performed by introducing the needle as far apically as possible so that the irrigant flowed through the entire length of the canal and exited through the patent apical foramen into the collection tube below.

After collection of the solution in the tubes, the lids were removed and the teeth separated from the lids. The tubes were labeled with identical new caps without perforations for analysis by ICP-AES (BAARC, Hyderabad). Ten tubes were used for each test solution to determine the calcium ion concentration and a mean value was calculated for each group. EDTA and phytic acid was diluted using deionized water to prevent it from quenching the plasma flame of the spectrometer. While the chitosan solution was further diluted in a 0.1% lanthanum solution (by mass/volume) in order to avoid the interference of the chitosan polymeric matrix with calcium ion quantification. An ICP-AES was used to carry out the procedure and the calcium ion concentration values thus obtained were tabulated for statistical

analysis.

Microhardness testing

Teeth were longitudinally split into two parts, one half of the root specimens were taken and embedded horizontally in an auto polymerizing acrylic resin, so that their dentin surface was exposed. The dentin surfaces of the mounted specimens were grounded flat with a silicon grit paper under distilled water to remove any surface scratches.

Three separate indentations were made at the cervical, middle and apical levels of the root dentin in each sample with a Vickers diamond indenter (Mitutoyo Corporation, Yokohama, Kanagawa, Japan) microhardness tester at X 10 parallel to the edge of the root canal lumen at a depth of 0.5 mm from the pulp-dentin interface, each using a 200-g load and a 10-s dwell time and the pretreatment microhardness values were calculated.

Later, the samples were divided into 4 groups (three experimental and one control group). Group I - 17% EDTA solution, Group II - 1% phytic acid, Group III - 0.2% chitosan, Group IV- control. Samples were immersed in 5 ml of the test irrigants for 5 minutes. Specimens were rinsed in distilled water and blotted dry. Vickers hardness number was again recorded as described before for each specimen and the decrease in microhardness was calculated as the percentage (%) in microhardness values.

For calcium ion quantification, the concentrations of calcium ions of the four independent groups were compared by one-way analysis of variance (ANOVA) and significance of the mean difference between the groups were compared with Tukey's post-hoc test. For microhardness testing, paired t - test was performed. Data was summarized as a mean ± standard deviation.

Results

The mean concentration of calcium ions removed from the root canals by EDTA, phytic acid and chitosan is shown in table 1. ANOVA revealed the significant ($P < 0.05$) effect of each group (chelating agents) on mean concentration of calcium ions removed from the root canal ($F = 339.032$, $P < 0.05$). Tukey's post-hoc multiple comparison tests revealed the significantly ($P<0.05$) different removal of calcium

Descriptive								
Calcium								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
EDTA	10	186.100	28.4584	8.9993	165.742	206.458	149.0	229.0
Phytic acid	10	22.429	1.6549	.5233	21.245	23.613	19.7	24.2
Chitosan	10	25.161	5.3988	1.7072	21.299	29.023	22.0	40.4
Saline	10	5.170	2.2500	.7115	3.560	6.780	1.7	8.8
Total	40	59.715	75.6081	11.9547	35.534	83.896	1.7	229.0

ANOVA					
Calcium					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	215325.500	3	71775.167	339.032	0.000*
Within Groups	7621.429	36	211.706		
Total	222946.929	39			

***Table 1**: Comparative evaluation of mean concentration of calcium ions (µg/ml) removed by EDTA, phytic acid, chitosan by one-way ANOVA.*

**p < 0.05; statistically significant.*

Multiple Comparisons						
Dependent Variable: Calcium Tukey HSD						
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EDTA	Phytic acid	163.6710*	6.5070	.000	146.146	181.196
	Chitosan	160.9390*	6.5070	.000	143.414	178.464
	Saline	180.9300*	6.5070	.000	163.405	198.455
Phytic acid	EDTA	-163.6710*	6.5070	.000	-181.196	-146.146
	Chitosan	-2.7320	6.5070	**.975**	-20.257	14.793
	Saline	17.2590	6.5070	.048	-.266	34.784
Chitosan	EDTA	-160.9390*	6.5070	.000	-178.464	-143.414
	Phytic acid	2.7320	6.5070	**.975**	-14.793	20.257
	Saline	19.9910*	6.5070	.020	2.466	37.516
Saline	EDTA	-180.9300*	6.5070	.000	-198.455	-163.405
	Phytic acid	-17.2590	6.5070	.048	-34.784	.266
	Chitosan	-19.9910*	6.5070	.020	-37.516	-2.466
*: The mean difference is significant at the 0.05 level.						

Table 2: *Mean concentration of calcium ion removal between groups by Tukey's Post-hoc test.*

ions from root canal between the groups and was found highest in the EDTA, phytic acid, chitosan and normal saline showed the least effect as depicted in table 2.

The pre-treatment and post treatment Vickers microhardness values (mean + standard deviation) for all tested specimens at the cervical, middle and apical levels

Group	Cervical third			Middle third			Apical third		
	Pre treatment Mean ± SD	Post treatment Mean ± SD	%Reduction Mean ± SD	Pre treatment Mean ± SD	Post treatment Mean ± SD	% Reduction Mean ± SD	Pre treatment Mean ± SD	Post treatment Mean ± SD	%Reduction Mean ± SD
EDTA	51.74 ± 1.1	41.41 ± 1.6	10.33 ± 1.7*	48.55 ± 0.8	42.73 ± 0.8	5.82 ± 1.3*	47.86 ± 0.8	46.88 ± 0.6	0.98 ± 0.9*
Phytic acid	52.41 ± 0.7	48.21 ± 1.3	4.21 ± 1.8*	51.14 ± 0.7	48.63 ± 0.8	2.51 ± 0.6*	51.86 ± 0.8	49.58 ± 1.6	2.28 ± 1.8*
Chitosan	51.83 ± 1.03	48.64 ± 0.8	3.18 ± 1.3*	48.31 ± 1.2	44.36 ± 2.1	3.94 ± 1.9*	47.28 ± 1.7	44.4 ± 1.91	2.88 ± 1.5*
Saline	69.52 ± 1.1	68.4 ± 1.1	1.12 ± 2.1	56.26 ± 2.2	55.26 ± 2.2	1.04 ± 0.6*	64.11 ± 1.5	63.75 ± 1.5	0.35 ± 2.5*

Table 3: *Mean Vickers microhardness values of the radicular dentin specimens at different levels with respect to the type of treatment (Paired t-test).*
**p < 0.05 statistically significant.*

were shown in table 3. All the chelators on application reduced the dentin microhardness at all levels. The percentage of reduction was least at the apical third than the cervical third and middle third.

Groups	Mean % Reduction ± SD
EDTA	5.71 ± 0.6*
Phytic acid	2.99 ± 1.1*
Chitosan	3.33 ± 0.9*

Table 4: *Percentage decrease in Vickers microhardness values of radicular dentin after the use of the tested chelating agents.*
**p < 0.05 Statistically significant.*

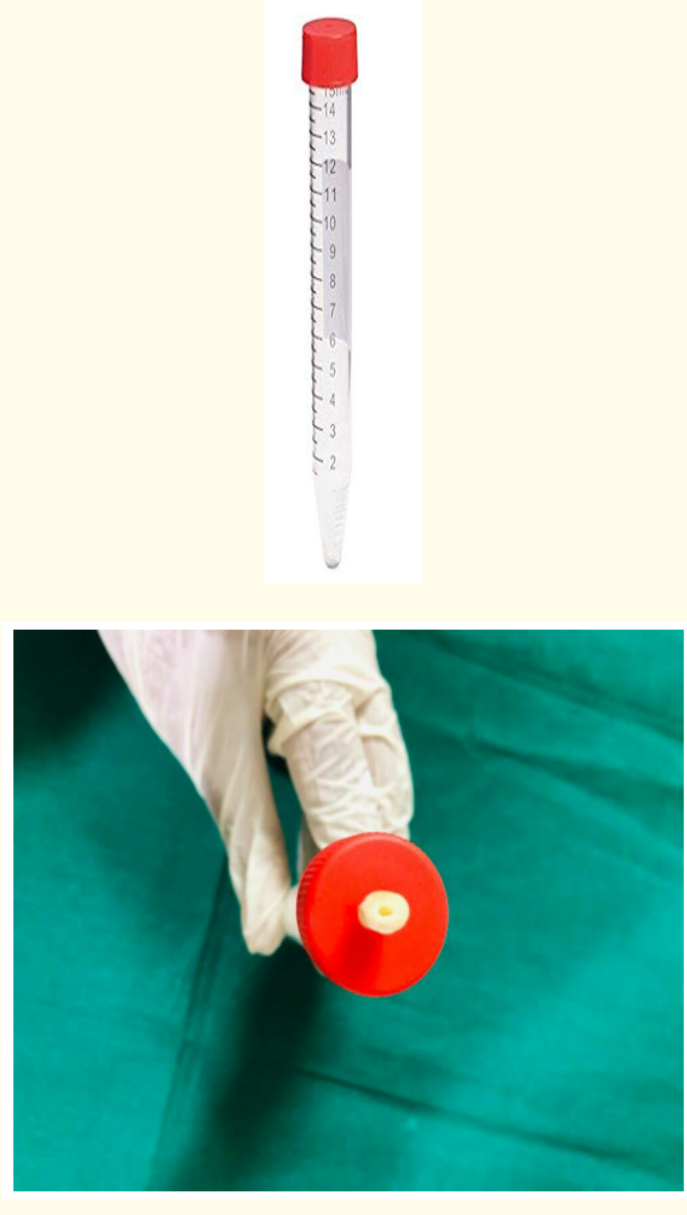


Figure 1: *Tooth placed inside the falcon tubes with the crown portion of tooth exposed.*

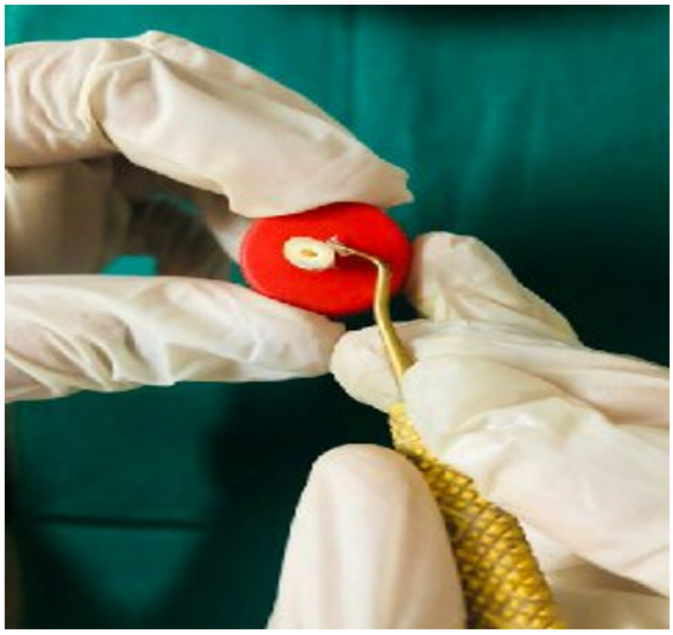


Figure 2: *Gap between the tooth and the lid sealed with Filtek Z250 Composite resin.*

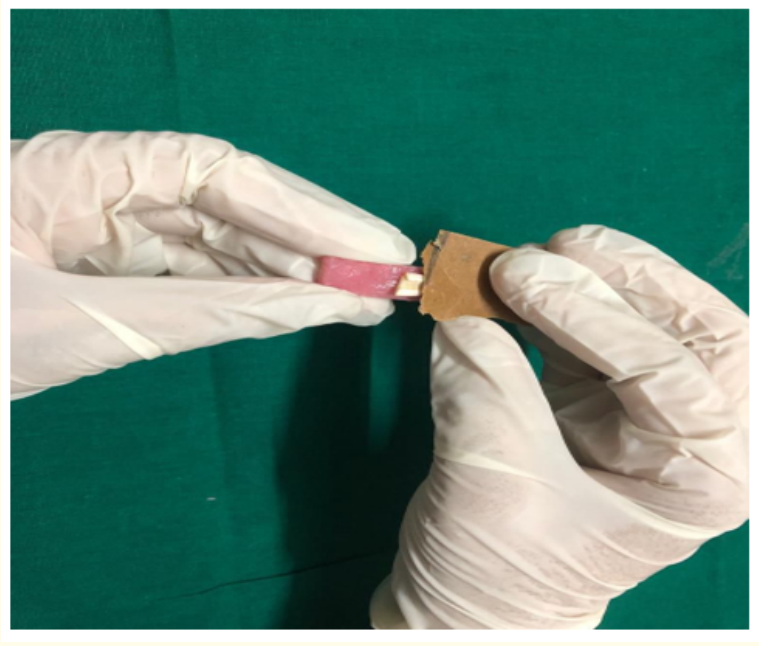
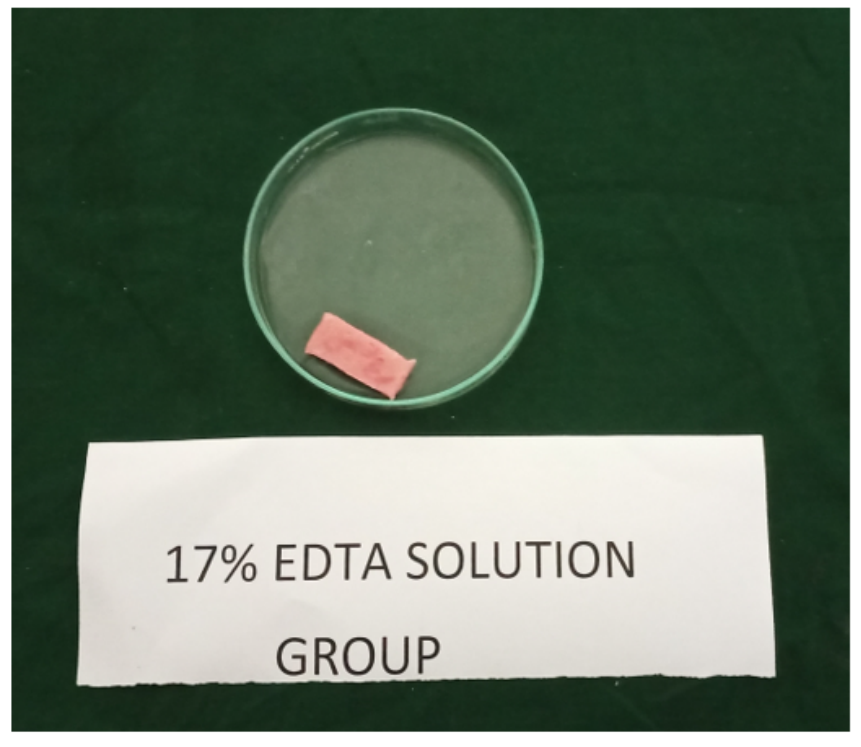
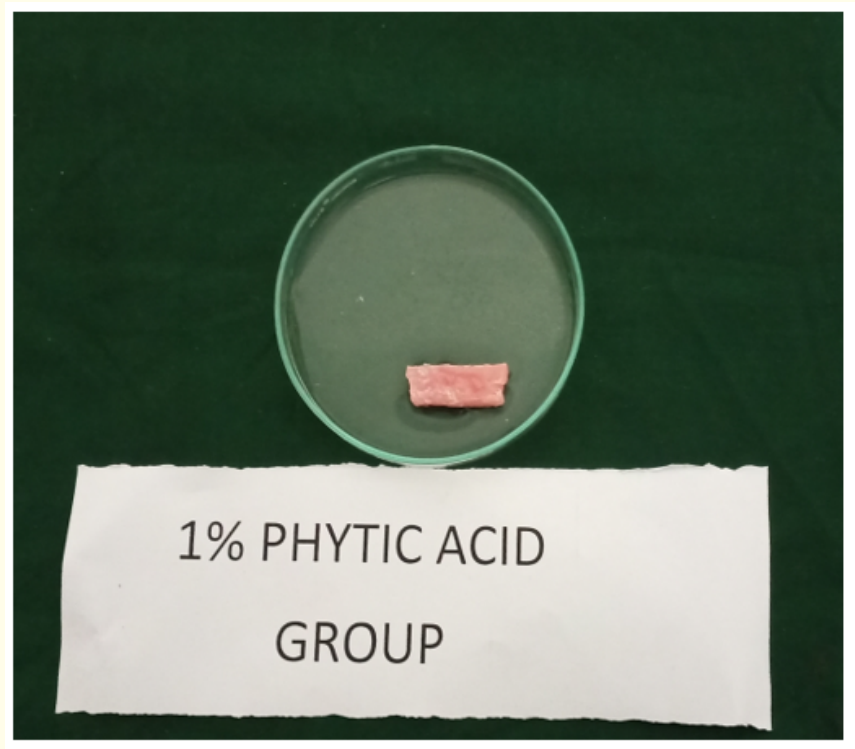


Figure 3: *Dentin surfaces of the mounted specimens were grounded flat with a silicon grit paper.*


17% EDTA SOLUTION
GROUP


1% PHYTIC ACID
GROUP

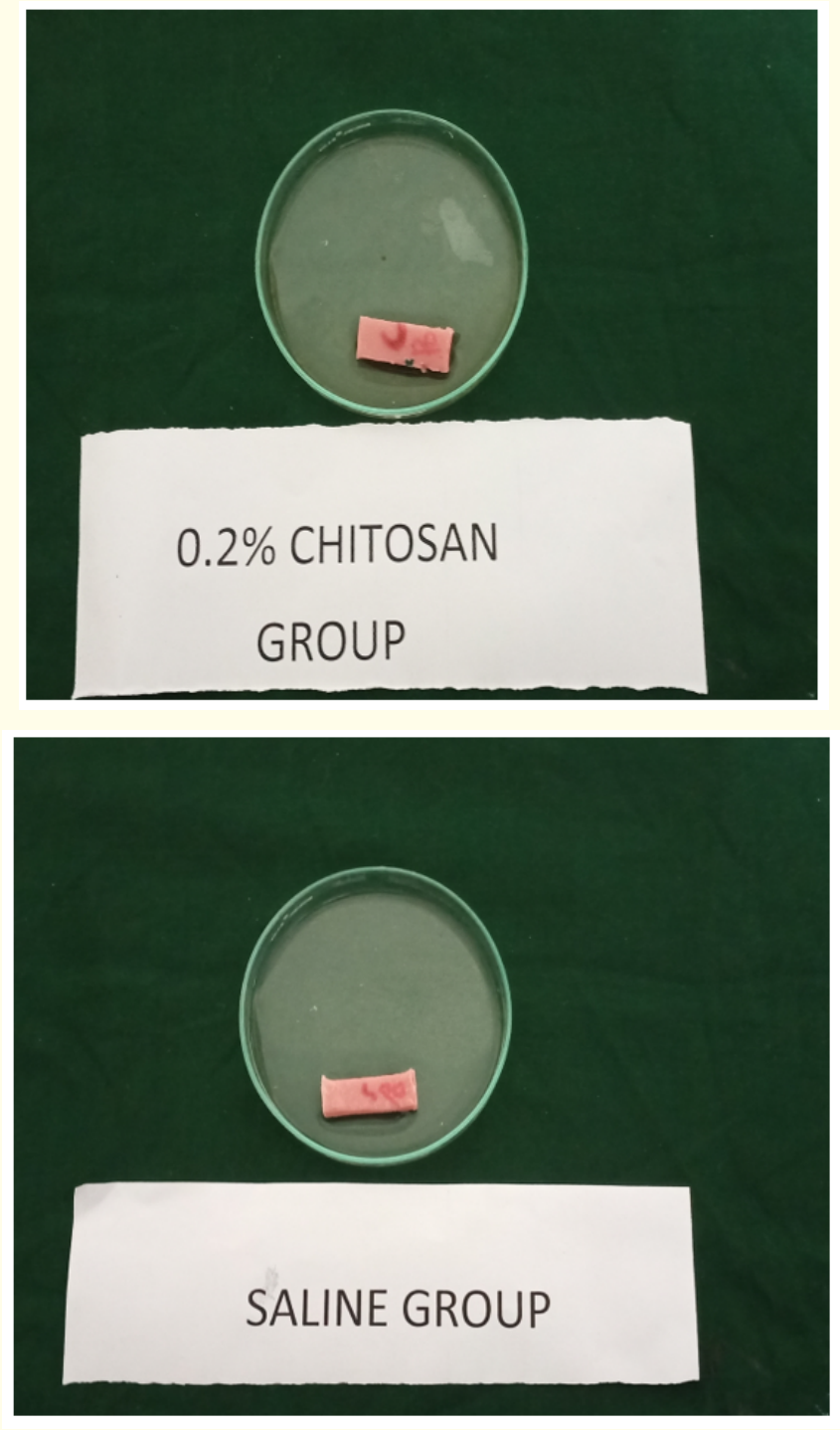


Figure 4: *Samples immersed in the 5 ml of the test irrigants for 5 minutes.*

EDTA caused the greatest percentage reduction in dentin microhardness (5.71 + 0.6) and the pairwise comparison indicated that this reduction was significantly more ($P < 0.05$) than phytic acid (2.99 + 1.1) and chitosan (3.33 + 0.9), which were similar to each other ($P > 0.05$) and were effective in terms of their effect on dentin microhardness (Table 4).

Discussion

The results of the present study indicated that 17% EDTA showed highest amount of chelated ions. 1% Phytic acid and 0.2% chitosan are associated with less calcium loss and less reduction in dentin microhardness.

Chelating agents are used to improve the chemomechanical debridement of root canal treatment by elimination of the smear layer. They cause a relative softening of the dentinal walls by the uptake of multivalent positive ions, thereby facilitating the preparation of root canals. This process usually causes changes in the microstructure of dentin and changes in the Ca/P ratio, which in turn affects the microhardness of dentin [23].

The decalcifying effect of different chelators in the removal of the inorganic component of smear layer and the negotiation of the fine, tortuous and calcified canal to obtain patency mainly depends on the root length, application time, diffusion in the dentin, relationship between the amount of available active substance (chelator) and the canal wall surface area, in addition to the pH of the solution, because the demineralization process continues until all chelating agents have formed complexes with calcium [3].

The recommended pH for EDTA solutions for decalcification is around 7.3. In a gravimetrical analysis done by Seidberg and Schilder, determined that the properties of EDTA were self-limiting, because of pH changes during the demineralization of dentin [3,11]. Usually under normal conditions, most of the chelators have an almost neutral pH. Due to the release of the acid by exchange of calcium from dentin with hydrogen, the efficiency of EDTA decreases with time; on the other hand, the reaction of the acid with hydroxyapatite affects the solubility of dentin. In a study done by Hülsmann and Hahn, the authors demonstrated that EDTA solutions demineralized dentin up to a depth of 50µm per canal wall [14]. The fact that EDTA acts efficiently to reduce the dentinal microhardness is mainly attributed to its chelating property. Among the many theories which try to explain this chemical reaction, one significant among them is the crystalline field theory, which maintains that the force of attraction between the central metal and ligands is purely electrostatic. Thus, the metallic ion exerts an attracting force which will be greater than the repulsion created by the atoms of the EDTA molecule [14,15].

Currently, there are many disagreements regarding the ideal chelator, the application time, and the association with sodium hypochlorite. The time these solutions stay in contact with the canal walls has been reported to vary from 30s to 10 mins [14,16]. In the present study, we have used 5 ml of test irrigants for 5 minutes (1 ml/min), with a considerable time and volume of irrigation. This regimen was used because during irrigation, chelation is not necessarily an equilibrium reaction and is determined by a standard stability constant, as the rate and ligand exchange reactions might considerably affect the chelation process [17]. Although an irrigation time of 1 min was relatively short, it was performed as longer irrigation times with effective chelators such as EDTA, might affect the dentin surface micro structure. In a study done by Calt., *et al*. the researchers demonstrated, that a 1 min EDTA irrigation was effective in removing the smear layer; however, a 10-min application of EDTA caused excessive peritubular and intertubular dentinal erosion [16].

In the present study, 1% phytic acid was associated with less calcium loss. The results are in agreement with a previous investigation done by Nassar., *et al*. and it was observed that 1% phytic acid removed the smear layer more effectively from the coronal dentine surfaces compared with 17% EDTA. Additionally, better results were observed with 1% phytic acid at the middle and apical thirds of the root canal in comparison with 17% EDTA, with phytic acid being biocompatible to MC3T3-E1 cells [18]. In the present research, phytic acid is used in the concentration of 1%, which has a pH of 1.2. This acidity, along with its favourable chelation ability, is attributed to effective smear layer removal and Ca^{++} ion extraction.

The findings of the present study also demonstrated that 0.2% chitosan was associated with less calcium loss, which in turn showed reduction in dentin microhardness. These results are in agreement with previous studies. In a study done by Pimenta., *et al*. investigators found that the application of the 0.2% of chitosan solution for 3-5 min was the most viable combination for use on the root dentin. They also found that 0.2% chitosan was equally effective in removing smear layer as 15% EDTA [1,19]. In a study done by Darrag., *et al*. the authors found that the final irrigation with 0.2% chitosan solution was more efficient in smear layer removal than 17% EDTA [20]. Although the mechanism of action of chitosan is not fully known, it is believed that adsorption, ionic exchange and chelation are responsible for the formation of complexes between the substance and the metallic ions. The type of interaction that occurs depends on the ion involved, the chemical structure of chitosan and the pH of the solution [1,21].

Currently, there are two versions to explain the chelation process of chitosan. The first, known as the bridge model, is grounded in the theory that two or more amino groups of one chitosan chain will bind to the same metallic ion [22]. The other defends the thesis that only one amino group of the structure of the substance is involved in the binding, that being the metallic ion "anchored" to the amino group [23].

Also chitosan is insoluble at neutral and high pH regions due to its molecular structure and pKa (6.2 - 7.0). At low pH in aqueous solutions the chitosan can be protonated. Therefore, acidulated diluted solutions like acetic acid (1% - 3% W/V) and citric acid (3% - 4%) are most commonly used to prepare chitosan solutions [24]. In our study, 1% acetic acid is used to form a solution, as this was proven to be the minimum concentration able to obtain a stable effective chitosan solution. Higher concentrations were found to leave behind acidic residues which compromised its chelating effects and efficacy. Some studies have stated that this acid might enhance the chelating efficacy of chitosan [11]. However, previous studies have reported that the capacity of 5% acetic acid for reducing dentin microhardness, removing the smear layer and chelating calcium ions in the root canal was insignificant in relation to 15% EDTA and 10% citric acid. In this way, it is highly evident that the effect caused by chitosan on dentin was not due to acetic acid [25].

In a study, by El Naby Bastawy Hagar A., *et al*. the researchers concluded that 0.2% chitosan showed less reduction in dentin microhardness and chelated calcium ions from the root dentin compared to 2% chitosan and 17% EDTA [25]. Hence, in the present study, 0.2% chitosan was used which has a pH of 3.7.

The results observed in the present research demonstrated that 0.2% chitosan and 1% phytic acid showed similar chelating effect and lower percentage reduction in microhardness compared to 17% EDTA solution. There is no statistical difference seen between 0.2% chitosan and 1% phytic acid. Also, as a lower concentration is required for removing smear layer without alteration of the intertubular dentin, this could indicate that these chelating solutions should be preferred for dentin decalcification compared to 17% EDTA. The results are also in agreement with the previous study done by Nikhil., *et al*. who reported that 0.2% chitosan and 1% phytic acid showed less reduction in dentin microhardness than 17% EDTA solution [11]. The findings of the study showing the mean percentage reduction in microhardness between EDTA, 0.2% chitosan and 1% phytic acid is depicted in the table (Table 4).

In the present study, for calcium ion quantification, inductively coupled atomic emission spectrophotometry (ICP-AES) was used. It is a more sensitive method than atomic absorption spectroscopy (AAS). ICP generates a much higher temperature than does a flame; 6000 - 8000 K for ICP-AES versus 1700-2700 K for AAS. This higher temperature results in greater atomization and excitation efficiencies of elements. Hence, the detection limits are approximately 1 part per billion for ICP-AES vs. approximately 0.1 parts per million for flame AAS. The application field originally assigned to AAS, using both the flame and graphite furnace atomic absorption spectrometry, has been relinquished to the ICP [9]. In this research investigation, for microhardness testing, Vickers microhardness indenter was used. Vickers hardness test was selected as it is a suitable and practical method to evaluate the change in the surface of the deeper hard tissue structures [11]. This test is widely accepted because of its extremely accurate readings and the fact that in this method, just one type of indentation is

used for all types of surface treatment [11]. Thus, in the present study, to measure the Vickers hardness values for the dentin, indentations were made in the cervical, middle and apical thirds of the radicular dentin and were done at the 0.5-mm level from the root canal walls for standardization. For evaluation of a reduction in hardness, uniformity was maintained by estimating the pre-treatment hardness of every sample and then comparing with the post treatment hardness.

Conclusion

Within the limitations of the current study, it was concluded that the impediments of the commonly employed chelating agent, EDTA when used in conjunction with sodium hypochlorite can be minimized by using 0.2% chitosan solution and 1% phytic acid. Therefore, use of 0.2% chitosan and 1% phytic acid can be thought of as suitable alternatives to EDTA when used as a chelating agent along with sodium hypochlorite.

Prior, to the clinical use of these new substances or products, further studies are needed to investigate in detail its physical, chemical and biological properties in order to verify the benefits and their consequences to human root dentin.

Clinical Relevance of the Research

The present research study quantified the calcium ions chelated by 17% EDTA and its potential effects on the adverse softening of the calcified components of dentin and subsequently the reduction in the dentin microhardness was investigated.

It was noticed that there is an inverse correlation between calcium ions loss and the microhardness of root dentin, as when the loss of calcium ions increases, the reduction in dentin microhardness is observed.

The significant alteration in the dentin hardness after the direct effect of the chelating agents like EDTA in an attempt to remove the smear layer is noted. Hence suitable alternating newer irrigating regimens like 1% phytic acid and 0.2% chitosan in conjunction with NaOCl for removal of the smear layer without negatively impacting the microhardness of dentin is analyzed in order to strike a critical balance between canal cleaning efficiency and tooth structural integrity. This understanding will help the clinician to better decide on an

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