



Impact of Chitosan as Chelating Agent on Microhardness and Mineral Content of Intraradicular Dentin

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ABSTRACT

Aim: This study was designed to evaluate the effects of 0.2% chitosan, 2% chitosan and 17% EDTA chelating agents on the microhardness and mineral content of human root canal dentin. **Materials and Methods:** Sixty extracted single-rooted human teeth were longitudinally sectioned into 120 segments. The specimens were randomly divided into four groups according to the chelating agent used; G1: 0.2% chitosan, G2: 2% chitosan, G3: 17% EDTA and G4: saline (control group). Dentin microhardness was measured in a total of 80 specimens using Vickers diamond indenter with 300 g load and a dwell time of 20 s. A total volume of 5 mL of each solution was used for 3min and then specimens finally flushed with 10 mL distilled water. In a total of 40 specimens, the level of three elements (calcium, phosphorus and magnesium) in each specimen was analyzed using inductively coupled plasma atomic emission spectrometry (ICP-AES). **Results:** All tested chelating agents significantly decreased the microhardness of root canal dentin ($P \leq 0.05$) and reduced the mineral content of dentin. Chitosan 2% and 17% EDTA had the statistically significant highest mean percentage reduction in microhardness compared to the control group ($P \leq 0.05$) and showed the maximum loss of Ca ions from root canal dentin. Chitosan 0.2% showed less reduction in dentin microhardness and chelated Ca ions from root dentin. **Conclusions:** Chitosan 0.2% solution is equally effective to 17% EDTA in removing Ca ions from root canal dentin without much altering its microhardness. Accordingly, the natural product of 0.2% chitosan attracts its use as dentin chelating agent.

KEYWORDS

Chitosan,
Chelating agents,
Microhardness,
ICP-AES,
Intraradicular dentin.

INTRODUCTION

Cleaning and shaping are considered the most important and most demanding aspect of endodontic therapy. During this phase, an amorphous, irregular layer of organic and inorganic materials known as smear layer adheres to the canal wall by the action of the instruments⁽¹⁻²⁾. It has been shown that the smear layer may contain and harbor bacteria,

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preventing the canal from being disinfected⁽³⁾, limit the penetration of intracanal disinfectants⁽⁴⁾ and sealers into dentinal tubules⁽⁵⁾ and interferes with a tight adaptation of root canal sealers to dentin walls⁽⁶⁾.

An ideal irrigation material should be capable of removing the smear layer entirely from the root canal walls. Sodium hypochlorite (NaOCl) is the most widely used irrigant in root canal treatment due to its unique tissue dissolving property, where it dissolves necrotic and vital organic tissue, and its antibacterial activity⁽⁷⁻⁸⁾. However, its capacity to remove the smear layer from the root dentin appears to be limited.

Chelating agents are believed to aid root canal irrigation and removal of the inorganic component of smear layer. The most common chelating solutions are based on ethylenediamine tetra acetic acid (EDTA) which reacts with the calcium ions in dentin and forms soluble calcium chelates⁽⁹⁾. The demineralizing effect of EDTA acts indistinguishably on the smear layer and the root dentin, with consequent exposure of collagen fibrils that contributes to interfacial nanoleakage at the dentin-sealer interface⁽¹⁰⁾.

Alterations in the chemical composition of dentin and change in the Ca/P ratio may alter the original proportion of organic and inorganic components, which in turn change the permeability, solubility characteristics and microhardness of dentin⁽¹¹⁻¹⁴⁾. When using chelating agents, there is no consensus on the ideal amount of reduction in root dentin microhardness that both facilitates mechanical instrumentation and avoids mineral loss and weakening of dentin. It has been reported that when root dentin was exposed to 17% EDTA solution for 3 minutes⁽¹⁴⁾ or 15 minutes⁽¹³⁾, its microhardness significantly decreased.

Cruz-Filho et al⁽¹⁵⁾ evaluated the chelating capacity of different substances on root canal lumen dentin after 5 minutes of application. They found that 15% EDTA and 10% citric acid promoted greater

reduction in microhardness. In a subsequent study, 17% EDTA significantly reduced the microhardness of root dentin when compared to MTAD and NaOCl after 5 minutes of application⁽¹⁶⁾.

Unfortunately, no irrigating solution is capable of acting simultaneously on the organic and inorganic elements of the smear layer. The use of a combination of NaOCl and EDTA has been recommended for efficient smear layer removal⁽¹⁷⁻¹⁸⁾. However, there is concern that this combined irrigation regimen causes inadvertent erosion of the intertubular and peritubular dentin⁽¹⁹⁻²¹⁾ and produced maximum decrease in dentin microhardness⁽²²⁻²³⁾. The search for more biocompatible solutions than EDTA, aiming at minimizing its harmful effect continues. Recently, more attention has been given to crosslinking and reinforcement of collagen matrix which can be achieved by incorporating biopolymers such as chitosan⁽²⁴⁾.

Chitosan is a natural polysaccharide biopolymer produced by the alkaline deacetylation of chitin which is obtained from shells of crustaceans and shrimps. It is economically attractive because chitin is the second most abundant biopolymer in the nature after cellulose⁽²⁵⁻²⁶⁾. It has the properties of biocompatibility, biodegradability, bioadhesion, high chelating ability for various metal ions in acidic conditions and lack of toxicity⁽²⁶⁻²⁷⁾. Owing to these properties, chitosan was applied as a chelating agent in several studies⁽²⁸⁻³¹⁾. It has been reported that chitosan, even at the lowest concentration, has similar smear layer removal capacity to EDTA⁽³⁰⁾ or more efficient than EDTA⁽³¹⁾.

There is a little information available about the effect of chitosan on root dentin microhardness. *Pimenta et al*⁽³²⁾ revealed that 0.2% chitosan reduced root dentin microhardness similarly to 15% EDTA and 10% citric acid solutions. Moreover, *Nikhil et al*⁽³³⁾ reported that 0.2% chitosan caused less reduction in dentin microhardness than EDTA.

A reduction in microhardness facilitates the instrumentation throughout the root canal⁽¹⁴⁾.

However, when it becomes substantial, it may also weaken the root structure. It is also important to determine the mineral content that is removed from teeth when different chelators are used. Therefore, the purpose of this study was to evaluate the effects of 0.2% chitosan, 2% chitosan and 17% EDTA chelating agents on the microhardness and mineral content of human root canal dentin.

MATERIALS AND METHODS

Specimen preparation and grouping

Sixty extracted single-rooted human teeth were used. All teeth were stored in distilled water until use. The crowns were removed at the cemento-enamel junction (CEJ) using a low speed diamond disc (Isomet, Buehler Ltd., Lake Bluff, NY) under water cooling. The roots were sectioned longitudinally into two segments using a diamond disc under water cooling and chisel (Dentsply, Switzerland) to obtain one-hundred twenty specimens.

The specimens were randomly divided into four groups according to the chelating agent used (30 specimens obtained from 15 teeth):

Group 1: Irrigated with 0.2% chitosan (Sigma Co., Egypt).

Group 2: Irrigated with 2% chitosan.

Group 3: Irrigated with 17% EDTA.

Group 4: Irrigated with saline (control group).

Chitosan 0.2% and 2% solutions were prepared with 0.2 g and 2 g of chitosan respectively in 100 mL of 1% acetic acid. The mixture was agitated using a magnetic agitator for 2 h. The pH of each solution was determined by using Thermo Scientific Orion 2-star benchtop pH meter with refillable glass electrode (Thermo Fisher Scientific Inc, Beverly, MA, USA). The accuracy of the pH meter was ± 0.002 . Chitosan 0.2% (pH 3.7), chitosan 2% (pH 3) and 17% EDTA (pH 7.4).

Part I: Evaluation of microhardness

Eighty specimens were horizontally embedded in auto polymerizing acrylic resin exposing the root-dentin surfaces and ground polishing with felt discs embedded in aluminum oxide paste (Diamond, FGM, Joinville, SC, Brazil). Within each group, the initial microhardness value (M_i) of one-half of each tooth (untreated specimen) was measured with microhardness tester (INNOVATEST NEXUS 4000 SERIES hardness tester, Maastricht, Netherlands) using Vickers diamond indenter and a 20X objective lens, with 300 g load and a dwell time of 20 s^(13,21,22,23). Three separate indentations were made at 0.5 mm level to root canal wall in the mid-region of the root^(13,22).

A total volume of 5 mL of each solution was used for 3min in each treated specimen. The samples were finally flushed with 10 mL distilled water after the application of chelating agent and were dried with paper points (Meta Dental Co.,Ltd. Korea). Posttreatment microhardness value (M_p) was measured for the treated specimen after application of the chelating solution in the same way as initial microhardness. Mean Vickers hardness number was calculated for each specimen. The initial and Posttreatment microhardness values were measured for the two-halves of each tooth. Accordingly, each tooth served as its own control.

The change in microhardness was calculated as the difference between initial and posttreatment values. Also the percentage change in microhardness values was calculated as follows:

$$\frac{M_i - M_p}{M_i} \times 100$$

Where M_i initial microhardness and M_p posttreatment microhardness values.

Part II: Mineral content of root canal dentin

Forty specimens were used in this part. Within each group, before application of the chelating

agents, the mineral content of root canal dentin of one-half of each tooth (untreated specimen) was measured using inductively coupled plasma atomic emission spectrometry (ICP-AES) (Vista AX; Varian Inc., Melbourne, Australia), where each specimen was digested with nitric acid (HNO_3) (69-70%) followed by 10 mL HClO_4 , and then diluted to specific volume. The levels of three elements: calcium (Ca), phosphorus (P), and magnesium (Mg) in each specimen were calculated with AOAC Official Methods of Analysis, for Ca ions AOAC (935.13), P ions AOAC (965.17) and Mg ions AOAC (937.02)⁽³⁴⁾.

Posttreatment mineral content value was measured for the other half of each tooth (treated specimen) after application of the chelating solution as mentioned previously. Changes in the levels of the mineral content were recorded and measured as difference between pre treatment mineral content values and posttreatment values after application of the chelating solution. Also the percentage change in mineral content was calculated.

Statistical analysis

Statistical analysis was performed with IBM SPSS 20 (SPSS, Inc., Chicago, IL, USA). Changes

and percentage change in the microhardness and mineral content were presented as mean values and standard deviations. Repeated measures ANOVA was used for comparison of mean values among the tested groups, where the significance level was set at $P \leq 0.05$. Tukey's post-hoc test was used for pairwise comparison between means when ANOVA was significant. Kruskal-Wallis test was used to compare between changes and percentage changes among the four groups.

RESULTS

(a) Microhardness: (Table 1) and (Fig. 1)

All tested chelating agents (0.2% chitosan, 2% chitosan and 17% EDTA) significantly decreased the microhardness of root canal dentin compared to the control group ($P \leq 0.05$).

Chitosan 2% and 17% EDTA had the statistically significant highest mean percentage reduction in microhardness compared to the control group ($P \leq 0.05$), with no statistical significant difference between them. Chitosan 0.2% was associated with less reduction in dentin microhardness, with none statistically significant difference from the control group.

Table 1. Descriptive analysis of the microhardness and its change among the tested groups.

	Chitosan 0.2%		Chitosan 2%		17% EDTA		Saline		P-value (Between groups)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Initial microhardness	48.2	8.8	46.9	4.6	46.2	8.5	48.0	5.2	0.967
Post treatment microhardness	41.5 ^{AB}	6.3	36.1 ^B	3.2	37.2 ^B	4.5	44.9 ^A	3.7	0.026*
P-value (within group)	0.027*		0.001*		0.023*		0.411		
Change in microhardness	-6.7	5.1	-10.8	6.0	-9.0	7.4	-3.0	1.6	0.237
Percentage change in microhardness	-13.3 ^{AB}	9.2	-22.3 ^A	11.3	-18.0 ^A	13.2	-6.1 ^B	3.0	0.048*

* Significant at $P \leq 0.05$, different superscripts are statistically significantly different

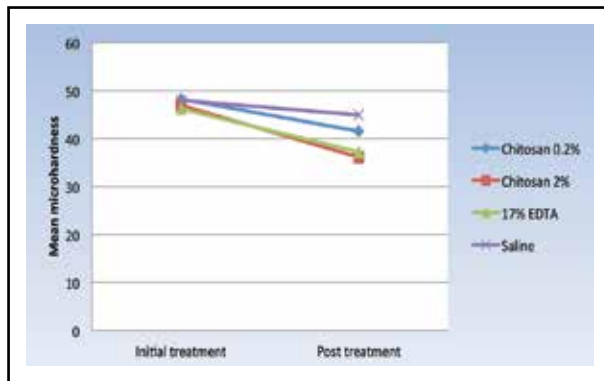


Fig. (1) Line chart representing the mean initial and posttreatment microhardness values among chitosan 0.2%, chitosan 2%, 17% EDTA and saline.

(b) Mineral content: (Table 2) and (Fig. 2)

The maximum calcium ions loss from dentin was observed in specimens treated with 2% chitosan followed by 17% EDTA and 0.2% chitosan with none statistically significant difference from the control group ($P > 0.05$).

The level of P ions decreased after treatment with 17% EDTA followed by 2% chitosan. These changes were statistically non significant ($P > 0.05$). The use of 17% EDTA and 0.2% chitosan increased the Mg ions level compared to 2% chitosan and the control group ($P > 0.05$).

Table 2. Descriptive analysis of the mineral content (mg/kg) and its change among the tested groups.

Mineral		Chitosan 0.2%		Chitosan 2%		17% EDTA		Saline		P-value (Between groups)
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Ca	Pre-treatment	246450.0	2474.9	245350.0	3464.8	247450.0	1626.3	236550.0	3606.2	0.169
	Post treatment	241250.0	2474.9	234000.0	2841.7	241850.0	4030.5	232150.0	2474.9	0.185
	P-value (within group)	0.269		0.200		0.241		0.180		
	Change	-5200.0	4949.7	-8900.0	6123.8	-5600.0	5656.9	-4400.0	6081.1	0.639
	Percentage change	-2.1	2.0	-3.7	1.5	-2.3	2.3	-1.8	2.5	0.682
P	Pre-treatment	119400.0	1131.4	118100.0	1258.1	123600.0	4101.2	114900.0	141.4	0.128
	Posttreatment	117650.0	1202.1	115000.0	1367.2	116800.0	989.9	113050.0	495.0	0.169
	P-value (within group)	0.514		0.423		0.064		0.180		
	Change	-1750.0	2333.5	-3100.0	1984.2	-6800.0	3111.3	-1850.0	353.6	0.254
	Percentage change	-1.5	1.9	-2.6	1.1	-5.5	2.3	-1.6	0.3	0.254
Mg	Pre-treatment	7313.0	527.5	7655.5	638.5	7482.5	696.5	7556.0	411.5	0.761
	Posttreatment	7405.5	10.6	7184.5	113.8	7873.5	194.5	7621.0	1183.7	0.446
	P-value (within group)	0.849		0.360		0.439		0.655		
	Change	92.5	55.2	-471.0	524.7	391.0	502.0	65.0	72.2	0.446
	Percentage change	1.5	7.5	-5.9	6.4	5.6	7.2	0.6	1.2	0.446

*: Significant at $P \leq 0.05$

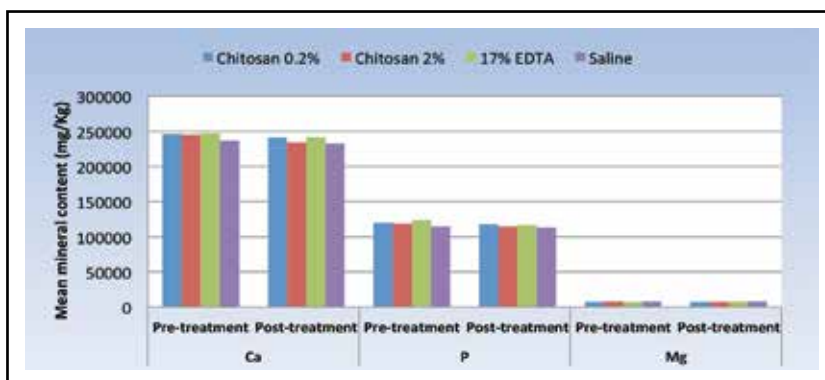


Fig. (2) Bar chart representing the mean change and percentage change in mineral content among chitosan 0.2%, chitosan 2%, 17% EDTA and saline.

DISCUSSION

Chelating agents are used to improve the chemomechanical debridement of root canal treatment by removing the smear layer. They create a relative softening of the dentinal walls by the uptake of multivalent positive ions, which in turn facilitate the preparation of root canals⁽⁹⁾. However, this process can cause changes in the microstructure of dentin and changes in the Ca/P ratio, which in turn change the microhardness^(12,14). Therefore, this study was conducted to evaluate the effects of 0.2% chitosan, 2% chitosan and 17% EDTA chelating agents on the microhardness and mineral content of human root canal dentin.

Before irrigating the root canal lumen with the chelating agents, dentin surface was polished as the indentations of microhardness test are only visible on polished dentin surfaces⁽¹⁵⁾. The use of chitosan at 0.2% concentration in the present study is justified by preliminary tests in which the comparison between chitosan prepared with different concentrations and action times on dentin showed that application of the 0.2% concentration for 3 min was the most efficient for smear layer removal⁽²⁹⁾. Also, the selection of 2% chitosan based on previous study in which 2% chitosan acetate was effective in removal of smear layer⁽²⁸⁾.

Microhardness of dentin may vary considerably between teeth, so in the present study the

microhardness measurement was performed for each tooth at baseline (untreated halve) and posttreatment (treated halve) with chelating agents to minimize the effect of the structural variations of different teeth and to establish a reasonable evaluation for the effect of the chelating agents on the dentin surface^(21,23). Previous investigations have shown the suitability and practicality of Vickers microhardness test for evaluating surface changes of dentin treated with chemical agents^(13,22,35).

It has been reported that the microhardness of dentin declined when indentations are made closer to the pulp as dentin hardness is related to location⁽³⁶⁾. Moreover, it has been showed that tubule density decreased from cervical to apical dentin, where there is an inverse correlation between dentin microhardness and tubular density. This histological pattern probably contributes to the hardness reduction at the cervical region of the root⁽³⁷⁾. Accordingly, in the present study, the measurements were obtained from three indentations located at 0.5 mm level to root canal wall in the mid-region of the root, where the dentin surface was more uniform^(13,22).

In the present study, the root canals were not prepared; thus no smear was present on the dentin surface. The rationale behind excluding this step was to enable measurement of Ca ions loss that occurred solely on intact root dentin, whereas

avoiding any possible changes in the readings that could have been caused by the Ca ions incorporated into the loosely bound smear layer^(12,13).

The results of the present study indicated that irrigation of root canals either with 2% chitosan or 17% EDTA solutions significantly reduced the microhardness of root canal dentin compared to the control group ($P \leq 0.05$), while 0.2% chitosan was associated with less reduction in dentin microhardness compared to 2% chitosan or 17% EDTA. However, the results were not statistically significant different.

The mechanism of action of chitosan is not fully known. It is believed that chitosan polymer being hydrophilic favors intimate contact with root canal dentin and gets adsorbed to root canal walls. Its powerful ability to chelate metal ion originate from the high charged density of the amino group on its polymer chain⁽²⁵⁾.

It has been shown that the higher the concentration of a solution, the stronger the chelating effect is⁽³⁸⁾. The concentration of 2% chitosan is ten times that of 0.2 concentration, which probably intensified the demineralizing action of this solution. Moreover, the pH of the solution is an important factor to demineralization. This might be due to a balance between the decrease in pH and the increase in viscosity of the solution caused by the increase in the constituent concentration⁽³⁹⁾. In the present study, the chitosan 2% has pH=3, while chitosan 0.2% has pH=3.7. This might explain the high percentage reduction in microhardness and chelated calcium ions from root dentin in specimens treated with 2% chitosan.

The effect of EDTA on reducing dentin microhardness has been reported by many authors^(13,15,22,23). EDTA efficiently reduces dentinal microhardness due to its chelating property. This might be attributed to that, EDTA form a stable complex with the calcium ions in dentin. Accordingly, carboxyl groups of the EDTA molecule are ionized, releasing hydrogen atoms that

compete with the calcium ions. When all available ions have been bound, equilibrium is formed and no further dissolution takes place⁽⁹⁾. Moreover, the organic matrix of dentin may act as a limiting factor in the dissolution of the inorganic component, thus the decalcifying action of EDTA stops⁽¹²⁾.

The results are in accordance with *Nikhil et al*⁽³³⁾ who reported that 0.2% chitosan caused less reduction in dentin microhardness than 17% EDTA. On the other hand, the results are in disagreement with those of *Pimenta et al*⁽³²⁾ who found that 0.2% chitosan equally effective to 15% EDTA and 10% citric acid in microhardness reduction. This divergence in results could be related to factors that affect the efficiency of chelating agents, such as usage time and concentration of the solution⁽¹⁹⁾.

ICP-AES was one of the most attractive detection systems for determination of trace elements in dentin⁽¹³⁾. They showed that polishing was not necessary and multiple elements can be measured at the same time. Trace metal concentrations in teeth are most often determined by bulk technique, in which whole teeth or some portion of enamel or dentin is digested in a suitable acid⁽⁴⁰⁾.

Reduction in microhardness of superficial layer of dentin is because of chelating properties and the release of Ca ions from root dentin. The results obtained from the quantification of chelated calcium ions showed that the highest amount of chelated Ca ions was observed with 2% chitosan, with no significant difference when compared with 17% EDTA and 0.2% chitosan, which chelated the least amount of Ca ions. The results of the present study was similar to those obtained in a previous study in which 0.2% chitosan had less effect on calcium ions extraction from root canal than EDTA with no statistical significant difference between them⁽³⁰⁾.

The results of the present study are supported by previous studies that compared the effect of EDTA with other irrigants on mineral content^(13,41-43). They showed that EDTA presents more potent effects on Ca ions extraction from dentin than other irrigants.

On the contrary, it has been reported that EDTA showed less decalcifying capability significantly than other irrigants⁽⁴⁴⁾. The discrepancy in the results may be attributed to difference in the methodology, where the Ca ions concentration was detected in the solution rather than in the specimen.

In addition to Ca and P ions, a small amount of Mg ions that is always detectable in the mineralized tissues has been considered to influence the mineralization process, especially crystal growth⁽⁴⁵⁾. In the present study, the use of 17% EDTA and 0.2% chitosan increased the Mg ions level in the root dentin. This could be due to that, magnesium replaces calcium in dentin⁽¹²⁾. The results are in agreement with previous study that reported insignificant increase in the Mg ions level of root dentin after treatment with EDTA compared with the other groups⁽⁴³⁾. On the other hand, earlier studies showed that the use of EDTA did not alter the Mg ions level^(12,13).

An inverse correlation between calcium ions loss and microhardness of root dentin was observed in all experimental and control groups. As the loss of calcium ions from root dentin increased, a reduction in microhardness of root dentin was reported. Chitosan 0.2% solution associated with less reduction in dentin microhardness and mineral content than chitosan 2%, accordingly, the less concentrated solution should be preferred. In addition, chitin polysaccharide, the precursor to chitosan, is the most abundant substance in nature after cellulose⁽²⁷⁾. The production cost of chitosan is considered to be low; making its use ecologically attractive as dentin chelating agent.

CONCLUSIONS

Within the limitations of this study, the following conclusions can be drawn:

- Irrigation of root canal with 2% chitosan, 17% EDTA and 0.2% chitosan lead to structural changes in root dentin as evidenced by loss of calcium ions and reduction in intraradicular dentin microhardness.

- Chitosan 0.2% solution is equally effective to 17% EDTA in removing calcium ions from root canal dentin without much altering its microhardness.
- Chitosan 2% solution showed more reduction in microhardness and chelated calcium ions from root dentin compared to other chelating agents with none statistically significant difference.
- The natural product of 0.2% chitosan which cause lower percentage reduction in dentin microhardness and mineral content attracts its use as dentin chelating agent.

Further studies are recommended to determine the extent to which these structural changes may affect the surface roughness of root dentin and the adhesion of sealers to the dentin surfaces treated with 0.2% chitosan solution.

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