Evaluation of the chelating effect of chitosan solubilized in different acids

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Abstract

Aim: This study aimed to compare, through dentin microhardness and colorimetric analysis, the chelating effect of 0.2% chitosan solubilized in different acids.

Materials and Methods: The second and third cuts of the cervical region of maxillary central incisors were divided into four quadrants, resulting in eight specimens, which were treated with 50 µL of solution for 5 min according to their group (n = 10): GI – 0.2% chitosan solubilized in 1% acetic acid; GII – 0.2% chitosan solubilized in 3.3% citric acid; GIII – 0.2% chitosan solubilized in 0.00145% hydrochloric acid; and GIV – 0.2% chitosan solubilized in 0.00112% nitric acid. A control was made from the chelating properties of the following acids: GV – 3.3% citric acid, GVI – 0.00145% hydrochloric acid, GVII – 0.00112% nitric acid, and GVIII – control (distilled water). Afterward, they were subjected to the Knoop microhardness tester with a load of 10 g for 15 s, resulting in three indentations of the root canal toward the cement. The measurements obtained were subjected to the one-way ANOVA test followed by Tukey’s test (α = 0.05). Subsequently dispensing the chitosan solutions, the same were subjected to colorimetric analysis.

Results: Chitosan solubilized in acetic acid, followed by chitosan in citric acid, provided a greater reducing effect compared to the other groups. Similar results were observed in the colorimetric analysis.

Conclusion: It was concluded that the chelating ability of the chitosan solution solubilized in acetic acid is higher than solubilization in citric, hydrochloric, and nitric acids.

Keywords: Chelating agents; chitosan; microhardness; solubility

INTRODUCTION

Chitosan is a natural substance recognized for its properties of biocompatibility, biodegradability, bioadhesion, and nontoxicity to the human cell. It is obtained by the deacetylation of chitin, a substance extracted from crustacean shells such as crab and shrimp.

Its application in the dental field has been observed in different specialties as a modulator of inflammation, assistant in the periodontal regeneration process in intraosseous defects, an aid in intracanal medication, and as an antimicrobial agent associated with bonding agents and composite resin.

At acidic pH, chitosan presents remarkable chelating ability for several metal ions. The studies specifically directed to the chelation of calcium ions showed that chitosan removes the smear layer from the walls of the root canal similar to ethylenediaminetetraacetic acid (EDTA) and citric acid, but without promoting erosion of the root dentin. Its demineralizing effect on the dentin tissue results in reduced microhardness similar to the EDTA standard chelator solution.

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Research about chitosan solution is directed at its ability to clean the root canal and reducing dentin microhardness, using the solution prepared from chitosan, with a degree of deacetylation >90%, solubilized in acetic acid.\textsuperscript{10-12} Chitosan is insoluble in aqueous medium, organic solvents, and bases; however, it is soluble in most organic acid solutions with a pH below 6.0 and in diluted inorganic acids such as nitric, hydrochloric, perchloric, and phosphoric.\textsuperscript{[13]} There is no information in the literature whether the chelating property of chitosan is enhanced or decreased when solubilized in other acids. Therefore, the objective of this study was to compare, by means of dentin microhardness and a colorimetric analysis, the chelating effect of chitosan solubilized in citric, hydrochloric, and nitric acids to chitosan solubilized in acetic acid. The null hypothesis of this study is that none of the evaluated chitosan solutions have a chelating effect, independently of the acids used for solubilization.

**MATERIALS AND METHODS**

After submission and approval of this project by the local Ethics and Research Committee (23800613.4.0000.5419), ten human maxillary central incisors were selected. The specimens were examined using a magnifying glass ($\times10$), and those with the presence of fractures, dental caries, abrasion or attrition, and incomplete root formation were replaced. Subsequently, a radiographic examination was performed to rule out the possibility of calcifications and internal resorption.

**Preparation of solutions**

The chitosan solutions were dispensed with an analytical reagent grade and water purified by reverse osmosis with ultraviolet (UV) light (Quimis, Diadema – SP - Brazil), with electric conductivity of $<$ 1 $\mu$S/mm.\textsuperscript{[9]} Initially, the hydrogen ion concentration of the acids used to solubilize the chitosan was standardized since its solubilization capacity is directly related to the concentration of free acid hydrogen. The preliminary calculations of the equivalent hydrogen ion concentration for all of the acids studied showed the following percentages: citric acid (3.3%), hydrochloric acid (0.00145%), nitric acid (0.00112%), and acetic acid (1.0%).

In a 200 mL beaker, 0.2 g of chitosan (ACROS Organics, Belgium) was weighed, degree of deacetylation $>$90%, and added to 100 mL of the solubilizing acid solution. The mixture was stirred by a magnetic stirrer until the complete dissolution of the chitosan was observed when crystalline proved to be homogeneous. The pH was measured by a pH meter.

**Preparation of the specimen**

The dental crown was sectioned transversely, using a carborundum disk, attached to the handpiece at low speed. The root portion in acrylic resin was included, and the set formed was taken to the cutting machine (Struers A/S, Copenhagen, Denmark). Three dentin slices with 1 mm of thickness each were obtained from the cervical third. The first slice was discarded, and the cervical surface of the second and third slices was polished with 400-, 500-, and 600-grit wet sandpaper. Afterward, the specimens were polished in a polishing machine with a felt disk and aluminum paste. Each slice was divided into four quadrants, using a scalpel blade and the visual aid of a magnifying glass ($\times30$); thus obtaining eight specimens per tooth, one for each solution test ($n = 10$).

**Evaluated solutions**

The 0.2% chitosan solutions were distributed according to groups: GI-solubilized in 1% acetic acid (pH 3.4); GII-solubilized in 3.3% citric acid (pH 2.0); GIII-solubilized in 0.00145% hydrochloric acid (pH 5.6); and GIV-solubilized in 0.00112% nitric acid (pH 5.7). Then, the isolated acid groups were formed: GV – 3.3% citric acid (pH 2.0); GVI – 0.00145% hydrochloric acid (pH 3.6); G VII – 0.00112% nitric acid (pH 3.5); and GVIII – distilled water (control).

**Evaluation of dentin microhardness**

Each tooth specimen received 50 $\mu$L of one of the test solutions, by means of an automatic pipette. After 5 min, the specimen was rinsed in distilled water, dried with gauze, and subjected to the Knoop microhardness tester (Shimadzu HMV-2000, Shimadzu Corporation, Kyoto, Japan). A load of 10 g was applied for 15s.\textsuperscript{[13]} In total, three indentations were obtained-conducted in the root canal toward the cement-with standard lengths of 200 $\mu$m between them. The results obtained were subjected to statistical analysis.

**Colorimetric analysis**

Immediately after the preparation of solutions, the colorimetric analysis was performed with two main objectives: (a) to verify that the solution formed presented chelating ability; (b) to quantify the volume of chitosan solution sufficient to chelate all calcium ions from the calcium carbonate (CaCO$_3$) solution.

Initially, 300 $\mu$L of a standard solution of 0.3% CaCO$_3$ was prepared, which was added to the indicator of calcium ions (Eriochrome Black T), resulting in a violet-colored complex. Added to the complex were 10 $\mu$L fractions of the test solution, until the violet color assumed the blue color, indicating total chelation of calcium ions. Failure to change color resulted in the absence of the chelating effect. The variation in color was detected by the UV/Vis spectrophotometer and expressed as a graph (wavelength versus absorbance).
**Statistical analysis**

The results obtained from the evaluation of dentin microhardness were subjected to the one-way ANOVA test followed by the Tukey complementary test ($\alpha = 0.05$).

The colorimetric assay is a qualitative and independent test to the microhardness analysis and served only as complementary test. The coloration of the chitosan/CaCO3 complex, expressed by means of a graph, showed whether or not the solution had a chelating effect.

**RESULTS**

Tukey’s test [Table 1] showed that the ability to reduce dentin microhardness of the solutions followed the subsequent descending order: chitosan with acetic acid (41.0 ± 3.7) and with citric acid (51.3 ± 4.3), and chitosan solubilized in hydrochloric acid (58.4 ± 5.3) and nitric acid (59.8 ± 3.6), with no difference between the last two ($P = 0.998$). The acids used alone slightly reduced the microhardness; however, statistically different from the control ($P < 0.05$).

The graph of the colorimetric analysis [Figure 1] demonstrated a peak at 234 nm, indicating the total chelation of calcium ions to the chitosan solutions solubilized in acetic acid and citric acid. The absence of the peak for the solutions solubilized in nitric and hydrochloric acid indicates remnants of calcium ions in the standard solution.

**DISCUSSION**

All the solutions evaluated by the microhardness test and colorimetry assay, reduced dentin microhardness, and presented total or partial chelation of calcium ion, thus rejecting the null hypothesis.

The efficiency of the chelating agent depends on not only its concentration and application time but also the volume of solution available. Thus, the volume of solutions was standardized to be evaluated in 50 $\mu$L. The concentration of the solution was based on previous work, which showed that 0.2% chitosan completely removes the smear layer from the dentin walls, promotes unobstructed tubules, without causing erosion of peritubular dentin.

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The application time of the solutions on the dentin was 5 min. At this time, 0.2% chitosan reduces dentin microhardness similar to 15% EDTA solution. The previous studies provided the basis for the hypothesis that the chitosan solution could be a very feasible substitute for EDTA since this acid attacks the periapical tissues, besides being considered a pollutant to the environment.

The reduction of microhardness by one of the chitosan solutions evaluated here could raise doubts about the solution truly responsible for the reducing effect, chitosan, or solubilizing acid. Therefore, the effect of each acid solution on dentin microhardness was evaluated. The results showed that citric, hydrochloric, and nitric acids used alone are virtually devoid of any reducing effect. The 1% acetic acid was not evaluated in this study since the previous studies have shown through atomic absorption spectrometry flame, that the demineralizing effect of 5% acetic acid is very low. Reports showed that the 1% acetic acid is neither able to remove the smear layer nor to promote demineralization of the dentin walls, being statistically similar to the control group and different than the 0.2% chitosan solubilized in acetic acid.

The colorimetric assay by spectrophotometry has been used to obtain and determine qualitative values, being considered an accurate and flexible method, which provides objective analyses of the samples.

By means of colorimetric analysis, it was possible to observe that the chitosan solubilized in hydrochloric and nitric acids showed no peak in the wavelength referring to the total chelation of calcium ions, demonstrating deficiency in chelating ability. After this observation, it was expected that the chelating effect of these solutions on dentin microhardness would be ineffective, as proven in the Knoop hardness test.
Citric acid is a weak organic acid with the ability to react quickly with calcium ions, in addition to presenting relatively low cytotoxicity.[23] Studies have shown that 10% citric acid can reduce dentin microhardness similar to 15% EDTA[12,24] and 0.2% chitosan.[12] The chelating action of citric acid becomes greater as its concentration increases.[25] Although the concentration of citric acid (3.3%) used in this study to solubilize the chitosan be well below that employed by previous authors, more promising results could be expected. The colorimetric analysis showed that chitosan solubilized in acetic acid, as well as in citric acid, showed a peak of 234 nm. The qualitative analysis made it possible to establish that both solutions completely chelated the calcium ions of the CaCO₃ standard solution. However, when applied on root dentin, the effect of reducing microhardness promoted by chitosan solubilized in citric acid was lower than that solubilized in acetic acid. This observation helped to confirm that even the solubilizing acid presenting affinity to calcium ions and demineralization ability does not enhance the chelating effect of the final chitosan solution.

Adsorption processes, ion exchange, and chelation are the probable mechanisms responsible for the formation of complexes between chitosan and metal ions. The type of interaction that occurs depends on the ion involved, the chemical structure of chitosan, and the pH of the solution.[26,27] By colorimetric analysis, it was evident that citric acid as a solubilizer did not influence the complex formation mechanisms of chitosan with free calcium ions. However, it decreased the chelating ability of chitosan in the presence of calcium overlapped in the hydroxyapatite matrix. A previous work[28] demonstrated that adding citric acid to the suspension of chitosan/hydroxyapatite promotes structural change of the biopolymer with the formation of an ionic complex, but without altering the hydroxyapatite. Thus, the reduction of chelating ability of the solution formed between the chitosan and citric acid, in relation to chitosan solubilized in acetic acid, is probably due to the conformational differences between the molecules of the solutions.[29]

The findings of the present study do not endorse the clinical use of the chitosan solution. Biological studies must be performed previously. However, based on the current literature and the results found in the present study, it can be inferred that the 0.2% solution chitosan solubilized in acetic acid presented promising results. The solution actually has a chelating effect, the capacity to remove the smear layer and according to some authors, may be a viable alternative to the use of EDTA.

CONCLUSION

The chitosan solution has been shown capable of reduce dentin microhardness as well as cleaning of root canals. Among the chitosan solutions investigated in this study, the solution solubilized in acetic acid had a more efficient chelating capacity than the same solution solubilized in the other acids.

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Conflicts of interest

There are no conflicts of interest.

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