



Cytotoxicity of Chelating Agents Used In Endodontics and Their Influence on MMPs of Cell Membranes

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This study evaluated the cytotoxic effect and the ability to inhibit matrix metalloproteinases (MMP-2 and MMP-9) of 0.2% chitosan (CH) and 1% acetic acid (AA) compared with 17% ethylenediaminetetraacetic acid (EDTA). Cell viability assay was performed according to ISO 10993-5 with mouse fibroblasts (L929). The culture was exposed to 0.2% CH, 1% AA, and 17% EDTA. The chelating agents were evaluated immediately after contact with the cells and after 6 h, 12 h, and 24 h of incubation. Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Inhibition of the gelatinolytic activity of MMP-2 and MMP-9 was evaluated by gelatin zymography. Different concentrations of CH were evaluated: 50 mM, 5 mM, 0.5 mM, and 0.05 mM. EDTA (0.5 mM) was used as a positive control. The results demonstrated that CH and AA had an initial cytotoxic effect, which decreased after 6 h, 12 h, and 24 h, being statistically similar to EDTA ($P > 0.05$). Additionally, CH at concentrations of 50 mM, 5 mM, and 0.5 mM had an inhibitory effect on MMP-2 and MMP-9, similar to that of the control with EDTA. The chelating agents had no cytotoxic effects after 24 h. MMP-2 and MMP-9 were inhibited by the experimental solutions.

Key Words: chelating agents, chitosan, matrix metalloproteinases, root canal therapy.

Introduction

Chelating agents are claimed to remove the inorganic component of the smear layer from root canal dentin, being commonly used for final irrigation during endodontic treatment (1). The most common chelating agent is ethylenediaminetetraacetic acid (EDTA), which, at neutral pH, reacts with the calcium ions in dentin and forms soluble calcium chelates (1,2). However, if extruded into the periapical tissues, it can cause apoptosis, necrosis (3), inflammation (4), and cytotoxic effects (5). Acetic acid (AA) can remove dentin calcium ions, favoring smear layer removal, but it can also reach the inorganic structure of dentin, causing subsequent erosion and changes in dentin microhardness (6). In order to obtain a chelating effect without harming the dentin content or periapical tissues, other solutions have been proposed and tested, such as chitosan (CH) (7-11).

The chelating effect of CH is chemically explained by its nitrogen content (6.89%): nitrogen atoms of amino groups hold free electrons that react with metal cations, leading to the binding of metal cations by chelation (12). In dentistry, CH has shown antibiofilm (8) and chelating effects (7-10). Its ability to remove the smear layer, similar to that of 17% EDTA and of calcium hydroxide intracanal medicament, has been confirmed by previous studies (6,7,9-13).

Periapical extrusion of chelating solutions can promote cytotoxic effects (14). Injuries caused to the tissues are

enhanced by the effect of the irrigant on the maintenance of cell viability (15). Impairments in cell metabolism and function may be expressed as vascular alterations with activation of inflammatory cells, decrease in proliferative or colony-forming capacity, production of chemical mediators, alteration in macrophage function, necrotic cell death, apoptosis, and degradation of dentin collagen (3,4,14-17). All of these reactions can affect metabolism and cellular repair. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the process of extracellular matrix degradation that occurs both physiologically and pathologically (18). MMP-2 and MMP-9 are present in root dentin and can be inhibited or activated by metal-based restorative materials, dental adhesives, cements, toothpastes, and mouthwashes (19).

Considering the demand for alternatives that are less damaging to the periapical tissues than those currently used (i.e., EDTA), the purpose of this study was to evaluate the cytotoxic effect and the ability to inhibit MMP-2 and MMP-9 of CH and AA compared with EDTA. The null hypothesis tested was that the chelating agents would have non-cytotoxic and inhibitory effects on MMP-2 and MMP-9.

Material and Methods

Chelating Agents

The test solutions were 0.2% CH (Chitosan Medical Grade, Sheijiang Chemicals, China), 1% AA (Merck & Co

Inc., St. Louis, MO, USA), and 17% EDTA (Merck & Co Inc.). The 0.2% CH solution was prepared by diluting 0.2 g of CH in 100 mL of 1% AA under magnetic stirring for 2 h (10).

Cell Culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). Mouse fibroblasts of the L929 immortalized cell line were maintained as a stock culture in DMEM and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air until achieving subconfluence. This study was approved by the institutional ethics committee (protocol #92.078.368).

Cytotoxicity Assay (MTT Assay)

The cell viability assay was performed according to ISO 10993-5 (2009). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Company, St. Louis, MO, USA) was used to assess cell metabolic function by mitochondrial dehydrogenase activity. L929 mouse fibroblasts (2×10^4 cells/well) were maintained in 96-well plates in DMEM for 24 h. Five samples were prepared for each test group. The solutions were filtered with sterilized syringe filters (pore size, 0.22 µm; Kasvi, São José dos Pinhais, PR, Brazil). After incubation, 200 µL of the medium was removed and 200 µL of each test solution was added. During the cytotoxicity assay, one sample containing only fibroblasts in DMEM was used as a negative control. The cytotoxicity of 0.2% CH, 1% AA, and 17% EDTA was evaluated as diluted in DMEM. Each well was incubated at 37°C for 6 h, 12 h, and 24 h under static conditions. A pilot study demonstrated that 2×10^4 cell/well was more appropriate to obtain a cell concentration suitable for analysis in time periods of less than 24 h (20,21).

The test solutions were added and remained in contact with the fibroblasts for 3 min for CH (7,22) and 5 min for EDTA and AA (6). Immediately after this time (0 h) and after 6 h, 12 h, and 24 h of contact with the cells, the solutions from each well were removed and replaced with 200 µL of MTT solution (5 mg/mL). After 4 h of incubation at 37°C in the dark, the MTT solution was removed and 200 µL of dimethyl sulfoxide (DMSO) was added to each well, and the plates were placed on a shaker for 5 min. Absorbance was determined using a spectrophotometer at a wavelength of 540 nm.

The cell viability (absorbance) data were analyzed using SigmaPlot 12 (Systat Inc., San Jose, CA, USA). Two-way analysis of variance followed by Tukey post hoc test was used for statistical analysis. The level of significance was set at $p < 0.05$.

Zymography

Proteolytic activity was analyzed on 10% polyacrylamide gels containing 0.05% gelatin. The conditioned medium was mixed with an equal volume of non-reducing sample buffer (2% sodium dodecyl sulfate [SDS]; 125 mM Tris-HCl [pH 6.8], 10% glycerol, and 0.001% bromophenol blue) and then electrophoresed.

After electrophoresis at 80V for 6 h, the gels were washed twice in 2% Triton X-100 for 60 min at room temperature. In order to examine the effect of different concentrations of CH and AA on enzyme activity, a conditioned medium containing MMPs was loaded onto preparative gelatin-containing polyacrylamide gels. Gels were cut into strips of approximately 1 cm, and each strip was incubated at 37°C for 24 h in Tris-CaCl₂ buffer containing 50 mM, 5 mM, 0.5 mM, and 0.05 mM of CH. Because CH was solubilized in AA, an AA control containing the same concentration used to dilute CH was used for all CH concentrations. AA and EDTA (0.5 mM) were used as positive controls, since they are known to be MMP inhibitors (23), while 0.5 mM N-ethylmaleimide (NEM) was used as a non-inhibitory control. The nature of lytic bands observed in the conditioned media was confirmed by performing parallel proteinase inhibition assays. Gelatin-containing gels were incubated in Tris-CaCl₂ buffer at 37°C for 24 h, with the addition of 0.5 mM EDTA (Reagen, São Paulo, SP, Brazil) to inhibit the lytic activities of MMP-2 and MMP-9.

After the addition of chelators to the solution, the pH was adjusted to 7.4 and the solution was incubated at 37°C for 24 h in 50 mM Tris-HCl buffer containing 5 mM CaCl₂ (Tris-CaCl₂). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250. Inhibition of enzyme activity was plotted against the solution concentration. Each assay was performed in triplicate and repeated at least twice.

Results

CH had an initially high cytotoxic effect (0 h), with a cell viability of 13.7% which was similar to that of AA (11.4%) and EDTA (8.8%) (Table 1). The cytotoxic effect of CH and AA decreased with time, consistent with the decrease observed in EDTA. At all time points, the viability of CH-treated cells was statistically similar to that of AA-treated cells ($p > 0.05$). At the end of 24 h, CH, AA, and EDTA groups were statistically similar ($p > 0.05$) (Table 1).

The results of zymography (Fig. 1) showed that CH was able to inhibit the gelatinolytic activity of MMP-2 and MMP-9 at concentrations of 50 mM, 5 mM, and 0.5 mM, with inhibitory effects similar to those of the respective AA controls. AA and EDTA control groups showed bands of 92 kDa (Pro MMP-9), 77 kDa (Act MMP-9), 72 kDa (Pro MMP-2), and 66 kDa (Act MMP-2) (Fig. 1).

Discussion

Based on the results of the present study, the null hypothesis was partially accepted, since both CH and AA had a low cytotoxic effect on fibroblasts after 6 h, 12 h, and 24 h; EDTA, after 12 h and 24 h. In addition, all chelating agents were able to inhibit MMP-2 and MMP-9. However, the effect of CH did not differ from that of AA, which is one of the solutions used to solubilize CH (10). Previous studies have shown that 1% AA is ineffective in removing the smear layer, without promoting the demineralization of the dentin walls and differing statistically from the demineralization effect of 0.2% CH (6,7,10). In this study, a group with 1% AA was used to demonstrate the effect of AA on cell viability and to compare it with the effect of 0.2% CH solubilized in AA. Although AA presented a higher percentage of cell viability than CH at 6 h, 12 h, and 24 h, there was no statistical difference between them.

Regarding chelating action, 0.2% CH and 17% EDTA solutions are statistically similar in their capacity for chelating ions, removing the smear layer, and reducing dentin microhardness, but these effects are statistically different from the effect of 1% AA (6,7,10), reason why 0.2% CH appears as a promising chelator in endodontic

treatment. The chelating agents were tested at clinically used concentrations: 1% AA, 0.2% CH, and 17% EDTA. Cell viability assay evaluates cytotoxic effects by measuring the behavior of cell cultures against a given substance and may be indicative of effects caused in vivo, although its results cannot be directly extrapolated to the in vivo condition (5). Different time points were used to mimic the effect of the chelating agents immediately after contact with the cells and the effect of residual components over time.

CH had no cytotoxic effect on fibroblasts at 24 h. The cell viability of CH increased from 13.7% at baseline (0 h) to 100% after 24 h, with a value similar to those of 17% EDTA (99.5%) and 1% AA (103.4%). AA had the highest percentage of cell viability compared with the other test solutions during the experiment, with the initial cytotoxic effect also being reversed at 24 h. An increase in the number of viable cells depends on a number of factors, such as nutritional reserve, ability to disperse the substances within the medium, adaptation of the cells to the medium, and the difference in the cytotoxic effect of each substance. Furthermore, phagocytic cells and lymph and blood vessels help to dilute and carry away the drug in the body (15). In the present study, the self-renewal capacity of the cells

provided support for a non-cytotoxic effect of all test solutions after the 24-h interval.

The 17% EDTA solution had a cell viability lower than 30% at baseline (0 h), indicating an initial cytotoxic effect, which was reversed at 24 h, in agreement with a previous study (24). The result obtained here at 24 h with

Table 1. Cell viability and standard deviation (%) of the different test groups

Groups	0 h	6 h	12 h	24 h
EDTA	8.8 ±8.5A,a	31.6±11.1A,b	79.7±15.6B,c	99.5±4.7A,d*
Chitosan	13.7±4.3A,a	24.8±8.9A,a	97.3±15.7AB,b	100.5±6.0A,b
Acetic acid	11.4±4.0A,a	43.7±12.0A,b	98.3±7.6A,c	103.4±6.7A,c

*Different lowercase letters in the same row and different uppercase letters in the same column indicate statistically significant differences (p<0.05).

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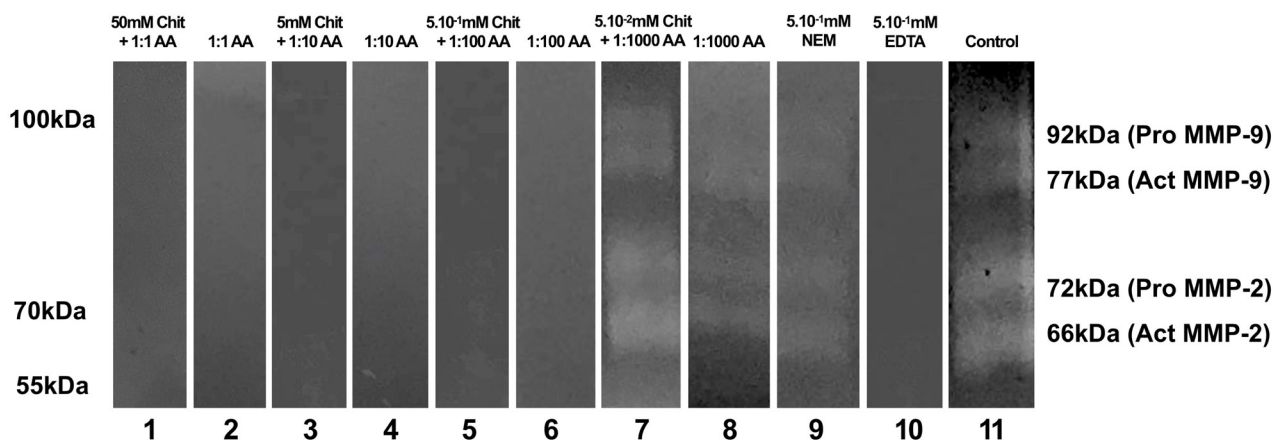


Figure 1: Results of MMP-2 and MMP-9 inhibition by chelating agents as determined by gelatin zymography. Gelatinolytic activities of MMP-2 and MMP-9 were detected by electrophoresis in gelatin containing 10% polyacrylamide gel. Ethylenediaminetetraacetic acid (EDTA; 0.5 mM), a known MMP inhibitor, was used as a positive control, while N-ethylmaleimide (NEM; 0.5 mM) was used as a non-inhibitory control. Chitosan (CH) solution was prepared by diluting CH in acetic acid (AA), and an AA control containing the same concentration used to dilute CH was therefore used for all CH concentrations. The control shows bands of 66 and 72 kDa, related to Act MMP-2 and Pro MMP-2, respectively, and of 77 and 92 kDa for Act MMP-9 and Pro MMP-9, respectively. Lysis zones indicate the activity of MMPs in the digestion of gelatin. The absence of lysis zones indicates MMP inhibition by the chelators.

17% EDTA, diluted to 0.1%, was also consistent with the cell viability of 87% reported in that same study at 24 h (24). However, with EDTA diluted to 0.5%, the results were conflicting (16). In addition, our result was contrary to that of other investigators who concluded that the cytotoxic effect of 15% and 17% EDTA is time- and dose-dependent (3,5,14,15,17,25). These conflicting results may be explained by the use of a variety of methods other than the MTT assay used in the present study to evaluate the cytotoxic potential of chelating agents (25). Another methodological difference is the use of macrophages and non-fibroblast cells to evaluate cytotoxic effects (14). In the present study, the ISO 10993-5 standard was followed, which recommends the use of fibroblasts for cytotoxicity testing.

The results obtained by zymography defined the clinically used chelating agents as inhibitors of MMP-2 and MMP-9. Gelatinases A (MMP-2) and B (MMP-9) are involved in the processes of cell migration and re-epithelialization. According to a study in a rat model, MMP-2 stimulates epithelial cell migration, while MMP-9 stimulates healing by promoting the migration of several cell types (18). MMP-2 and MMP-9 identified in root dentin are part of a family of more than 20 MMPs (18). They are categorized according to their structure and substrate specificity into collagenases, gelatinases, membrane type (MT-MMPs), stromelysins, and matrilysins (18,19). Like the other MMPs, MMP-2 and MMP-9 play a role in cell invasion, cartilage degradation, tissue remodeling, wound healing, and embryogenesis (18) and are also present in pathological processes, such as rheumatoid arthritis, cancer, obstructive pulmonary disease, and periodontal inflammation, among others (18,19).

CH, with different molecular weights, has been shown to inhibit MMP-2 in gingival fibroblasts when evaluated by zymography (26). This suggests that the inhibitory capacity of CH is related to its proven ability to inhibit zinc, supporting its commercial use as a chelator (26). This is consistent with the present study in which CH at concentrations of 50 mM, 5 mM, and 5.10⁻¹ mM had an inhibitory effect on MMP-2 activity in L929 mouse fibroblasts assessed by zymography. EDTA was used as a parameter for comparison in zymography, providing the minimum inhibitory concentration values for a chelating agent (23). Also, our results showed that CH had an inhibitory effect similar to that of AA, which was used as a solubilizing agent to prepare the CH solution. One can speculate that the inhibitory effect of CH on MMP-2 and MMP-9 may be mediated by the AA present in the CH solution, a hypothesis that needs to be further investigated.

Most MMPs are secreted as latent precursors (zymogens) or inactive enzymes that remain anchored to the cell

surface, limiting their catalytic activity to membrane proteins or proteins within the secretory pathways or extracellular space (18). The structural catalytic domain of MMPs contains three histidines that bind to Zn²⁺ sites and one that binds to a water molecule, with the cysteine-thiol-zinc linkage maintaining the MMPs in a latent form (18). Some MMPs, such as MMP-2, MMP-19, MMP-28, and some MT-MMPs, play an important role in homeostasis and are expressed in normal tissues. However, most MMPs remain latent, being expressed only when they are activated for repair, for remodeling or in diseased or inflamed tissues (18).

The ability of metal salts, such as ZnSO₄, CuSO₄, HgSO₄, and SnCl₂, to inhibit MMP-2 and MMP-9 justifies the classification of these enzymes as zinc-dependent (2,18,19). Although not fully understood, the relationship between the accumulation of metals in connective tissue and their interference with the formation or resorption of extracellular matrix components has already been proven (18,19). The ability of EDTA, CH, and AA to remove calcium, potassium, magnesium, sodium, sulfur, and zinc phosphate from root dentin may be governed by their metal-related mechanisms of action, i.e., through adsorption, ion exchange, and chelation (7,10,26,27). These interactions vary according to the binding ion, the chemical structure of CH, and the pH of the solution (7,8,27). Two models may explain the chelation process of CH. One of them, known as the bridge model, is based on the theory that two or more amino groups of a CH chain bind to the same metal ion (7,8,27). The other model is based on the theory that only one amino group of CH participates in the binding, and the metal ion is anchored to the amino group (7,8,27). The adsorption capacity of CH is influenced by the amount of free amino groups and by the hydrophilicity of the adsorbed molecules (27).

It is necessary to consider that the present study is an *in vitro* evaluation. Despite following the same steps used in clinical practice, the results cannot be extrapolated *in vivo*, but they may serve as reference data for future evaluations. It is important to note that the results of this study are limited to the cells and enzymes evaluated here, and further studies involving other cell types and biological properties of CH are still required. Therefore, the perspective that a new chelating agent has potential for use in endodontic treatment demands elucidation of its mechanisms of action and effects when in contact with the periapical tissues, aiming to ensure safety in its application.

In conclusion, CH had a cytotoxic effect similar to that of EDTA immediately after contact with fibroblast cells, which decreased after 6 h, 12 h, and 24 h of incubation. In addition, CH had an inhibitory effect on MMP-2 and MMP-9 similar to that of EDTA.

Resumo

Este estudo avaliou o efeito citotóxico e a capacidade de inibição das metaloproteínas da matriz extracelular (MMP-2 e MMP-9) pela quitosana 0,2%(CH) e o ácido acético 1% (AA) em comparação com o ácido etilendiaminotetracético 17% (EDTA). O ensaio de viabilidade celular foi realizado de acordo com a ISO 10993-5 com fibroblastos de camundongo (L929). A cultura foi exposta a CH 0,2%, AA 1% e EDTA 17%. Os agentes quelantes foram avaliados imediatamente após o contato com as células e após 6 h, 12 h e 24 h de incubação. A viabilidade celular foi analisada utilizando o ensaio de brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio (MTT). A inibição da atividade gelatinolítica de MMP-2 e MMP-9 foi avaliada por zimografia de gelatina. Diferentes concentrações de CH foram avaliadas: 50 mM, 5 mM, 0,5 mM e 0,05 mM. EDTA (0,5 mM) foi usado como controle positivo. Os resultados demonstraram que CH e AA apresentaram um efeito citotóxico inicial, que diminuiu após 6 h, 12 h e 24 h, sendo estatisticamente similar ao EDTA ($P > 0,05$). Adicionalmente, CH a concentrações de 50 mM, 5 mM e 0,5 mM tiveram um efeito inibidor sobre MMP-2 e MMP-9, semelhante ao controle com EDTA. Os agentes quelantes apresentaram efeitos não citotóxicos após 24 h. MMP-2 e MMP-9 foram inibidas pelas soluções experimentais.

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