Sodium Hypochlorite and Chlorhexidine Downregulate MMP Expression on Radicular Dentin

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Highlights of the Study

- Radicular dentin matrix metalloproteinases have potential implications on regenerative therapies.
- Endodontic irrigant solutions affect the expression of matrix metalloproteinases on radicular dentin.
- Sodium hypochlorite and chlorhexidine downregulate the expression of matrix metalloproteinases.
- Conditioning with ethylenediaminetetraacetic acid could improve the environment for dentin remineralization.

Keywords

Matrix metalloproteinases · Root canal irrigants · Endodontics

Abstract

Objective: Matrix metalloproteinases (MMPs) are present in radicular dentin and can convert structural matrix proteins into signaling molecules; thus, these enzymes play an essential role in dentin biomineralization and tissue regeneration therapies. Their expression on radicular dentin may be affected by the irrigation solutions used during root canal treatments. This study aimed to evaluate the effects of the most common irrigants on radicular dentin MMP expression. **Materials and Methods:** The experimental solutions were distilled water (control), 5% sodium hypochlorite (NaOCl), 18% ethylenediaminetetraacetic acid (EDTA), and 2% chlorhexidine (CHX). Samples were prepared from extracted human teeth. For zymography analysis, root sections were powder-

ized, and dentin proteins were extracted to observe gelatinolytic activity. Root dentin slices were treated with the experimental solutions for immunohistochemical analysis using anti-MMP-2 and anti-MMP-9 antibodies. ANOVA and the Tukey test were performed. **Results:** Zymograms revealed the presence of MMP-2, MMP-8, and MMP-20 in the control group and the EDTA-treated group. Immunohistochemistry confirmed the presence of MMP-2 and MMP-9 mainly associated with the dentinal tubule lumens and occasionally with intertubular dentin. NaOCl- and CHX-treated groups showed lower expression of MMPs than the control group. Immunostaining for both proteinases in the EDTA-treated group showed higher expression compared to the other experimental groups. **Conclusion:** Our results showed that most common irrigants affect MMP expression on radicular dentin. Treatment with NaOCl and chlorhexidine resulted in lower expression of MMPs, while EDTA increased their expression in root canal dentin.

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Introduction

Dentin is a mineralized tissue composed of extracellular collagen matrix (ECM) with immersed apatite crystals. Type I collagen is the major constituent (~90%) of the ECM, conferring dentin with most of its functional properties. On the other hand, noncollagenous proteins (NCPs) (~10%) play fundamental roles during dentinogenesis and pathophysiological processes [1–3]. NCPs are synthesized and secreted by odontoblasts which remain embedded in ECM [1, 4]. Thus, the dentin matrix is a reservoir of NCPs including enzymes that act as signaling molecules in biomineralization of the dentin and bone [2, 5, 6].

Mineralization of hard tissues is a complex biological process that requires active extracellular enzymatic control, which is mainly performed by different proteinase members of the matrix metalloproteinase (MMP) family [2, 5, 7]. MMPs cleave specific substrates involved in cell signaling, tissue development/remodeling, and tissue breakdown in pathological processes [2–4, 7]. Among MMPs, MMP-2, MMP-8, MMP-9, and MMP-20 in their active or latent forms have been isolated from the mature human mineralized and demineralized dentin matrix [2, 4, 8–10]. Endogenous MMP activity within dentin is mostly related to its ability to degrade all ECM components influencing dental tissue formation and mineralization and showed a regulatory effect on the differentiation of mesenchymal stem cells [3, 11–14]. Thus, new research insights are required in order to elucidate the possible implications of MMPs on endodontic regenerative therapies.

It is widely acknowledged that cleavage by MMPs converts structural matrix proteins such as NCPs into active signaling peptides with specific biological activities [15]. For example, Retana-Lobo et al. [2, 15] reported that MMP-2 becomes active through a radicular dentin demineralization procedure, cleaves the dentin matrix protein 1 (DMP1), and releases a highly phosphorylated DMP1 C-terminal fragment suitable for guiding an attraction and binding site for calcium ions during the biomineralization process; thus, MMPs play a fundamental role in the remineralization procedures.

During endodontic treatment, the root canal is irrigated with antimicrobial solutions in order to remove necrotic tissue and eliminate microorganisms from the root canal system [16–18]. Commonly used irrigants include NaOCl, EDTA, and CHX, either as single solutions or combinations in irrigating protocols [17]. The antibacterial properties of NaOCl and CHX and the ability of EDTA to remove the smear layer have been widely dem-

onstrated. Further, previous studies have analyzed the effects of these chemicals on the mineralized-dentin matrix and have reported changes in the dentin structure as a result of erosion, changes in the elastic modulus, fracture toughness, and bond strength [16, 18–21]. However, the effect of these irrigating solutions on the signaling molecules embedded on the ECM remains unclear.

NaOCl (0.5–6.15%) is the most widely used irrigation solution because of its effective antimicrobial properties. NaOCl is highly alkaline and can dissolve pulp tissue remnants from the canal space because of its proteolytic ability [16, 17, 22] and enhance debridement and lubrication; nevertheless, due to the effect on the organic components of dentin, studies report alterations in the chemical and structural composition of the dentin canal wall [6]. CHX is used as an endodontic irrigant due to its antimicrobial and substantive properties and is also widely reported to be an MMP inhibitor; therefore, it has been recommended to reduce collagen degradation and improve the bonding strength durability [2, 17]. EDTA is an irrigant used to remove the inorganic component of the smear layer. It acts as a specific chelating agent for calcium ions leading to dentin demineralization [6, 16, 20, 22]. Furthermore, EDTA dentin conditioning could release proteins from the dentin matrix, promoting regenerative tissue repair mechanisms [6, 22]. The effect of irrigant solutions on radicular dentin composition, specifically in the expression of MMPs, remains unclear. Strategies for tissue regeneration should be oriented to preserve root canal dentin as a bioactive matrix while ensuring that irrigant solutions do not harm the suitable environment conferred by NCPs specifically MMPs, for cell adhesion, proliferation, differentiation, and mineralization. This study was aimed at elucidating the potential effects of irrigants on the MMPs embedded in mineralized dentin tissue.

We evaluated the effects of the most common irrigants on radicular dentin MMP expression. The tested null hypothesis was that there would be no differences in MMP expression after irrigation with the most common solutions.

Materials and Methods

The research protocol was approved by the Ethics Committee of the Universidad de Costa Rica (VR-467-2018).

Sample Preparation

Forty sound single-rooted extracted human teeth were used. Teeth were extracted for orthodontic reasons not related to this study (patients mean age: 28.3). The criteria for inclusion were sound teeth, single canal, straight root, and fully developed apices.

The exclusion criteria were teeth with caries, resorptive defects, cracks, complex anatomy, and previous endodontic treatment. Organic debris and cementum were removed, and teeth were stored dry at -80°C for no >1 month. The crown portions were resected, and the pulpal soft tissue was removed.

For zymography, dentin from randomly selected 20 roots was reduced to fine powder by cryogenic grinding with MM 400 (Retsch, Haan, Germany), and the mineralized dentin powder was pooled and kept frozen until use. Four 1-g lots were treated: control group – sound radicular dentin powder treated with distilled water; NaOCl group – treated with 5% NaOCl for 30 min; EDTA group – treated with 18% EDTA for 10 min; CHX group – treated with 2% CHX for 30 min. The solutions were washed with distilled water and centrifuged twice. Extraction of dentin proteins was performed in 50 mM Tris-HCl buffer, pH 7.4, according to the Mazzoni et al. [12] protocol, and aliquots obtained were used in the zymographic analysis.

For immunohistochemical analysis, canals from 20 single-rooted teeth were prepared using the Primary file-WaveOne Gold System (Dentsply, York, PA, USA). A standardized method of irrigation was performed using a 27-gauge Endo-Eze irrigation needle (Ultradent-Products Inc., South Jordan, UT, USA), placing the needle as deep as possible into the canal without binding and ejecting the irrigation solution gently with the experimental solutions ($n = 5$): control group – 10 mL distilled water, NaOCl group – 10 mL 5% NaOCl, EDTA group – 10 mL 18% EDTA, and CHX group – 10 mL 2% CHX. Samples were washed with distilled water and formalin fixed for 48 h. Specimens were demineralized and sectioned longitudinally to observe the canal lumen. Using conventional histochemical techniques, samples were dehydrated and embedded in paraffin to obtain tissue sections for the immunolabeling.

Zymography Analysis

Aliquots were subjected to electrophoresis under nonreducing conditions in a 5–20% gradient sodium dodecyl sulfate-polyacrylamide gel containing 1 mg/mL gelatin. Gels were washed and incubated overnight in activation solution (50 mmol/L Tris-HCl and 5 mmol/L CaCl_2 , pH: 7.4). The gel was stained in 0.2% Coomassie Brilliant Blue R-250, destained in 10% acetic acid-10% methanol in H_2O , and photographed using the ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for further analysis.

Immunohistochemical Analyses

Samples were sectioned at 2 μm thickness, mounted on glass slides, and deparaffinized. The samples in each group were subdivided according to the following primary antibodies and respective dilution ratios: rabbit polyclonal anti-MMP-2 (1:600; Novus Biologicals, Centennial, CO, USA) and mouse monoclonal anti-MMP-9 (1:200; Novus Biologicals, Centennial, CO, USA). Proteinase K (Dako Cytomation, Carpinteria, CA, USA) incubation was performed for antigen retrieval, in accordance with the manufacturer's instructions. Nonspecific binding was blocked by incubating sections for 1 h with blocking solution: 1X TBST/5% normal goat serum (Cell Signaling, Danvers, MA, USA). After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the conjugated secondary antibodies SignalStain Boost detection reagent (anti-rabbit/anti-mouse) (Cell Signaling, Danvers, MA, USA) in a humidified chamber for 30 min at room temperature. Samples were washed, and visualization was

Fig. 1. Detection of MMP activity. Zymographic analysis of radicular dentin powder treated with different root canal irrigants, showing the presence of gelatinolytic bands. Gelatinolytic activity detected at 54 kDa corresponding to the molecular weight of MMP-20 (labeled with black arrows) and at 58 kDa and at 66 kDa corresponding to the molecular weight of MMP-2 (labeled with white arrows). Molecular masses expressed in kDa are reported in the standard lane (Std). MMP, matrix metalloproteinase.

completed using the SignalStain DAB Substrate kit (Cell Signaling, Danvers, MA, USA) and counterstained lightly with Mayer hematoxylin solution. Negative controls were performed in parallel by incubating sections with phosphate-buffered saline instead of primary antibody.

Images of the immunohistochemical stained tissue sections were acquired using a light microscope (Eclipse Ti-5; Nikon, Minato, Japan). Images were acquired from the adjacent dentin to the canal lumen. Five images per sample were captured at $\times 40$ magnification. The threshold optical density was obtained using the NIH Image J 1.36b imaging software (National Institutes of Health, Bethesda, MD, USA). The total pixel intensity was determined from the whole image, and data were expressed as optical density. Data obtained were statistically analyzed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). ANOVA and the Tukey test were performed. $p < 0.05$ was considered to be statistically significant.

Results

Detection of MMP Activity

Zymography analyses were performed to observe MMP activity after irrigation with different experimental solutions. Zymograms revealed gelatinolytic activity, with bands observed at 66 kDa related to the molecular weight of the active form of MMP-2, in the control and EDTA lanes; bands at 58 kDa related to the molecular weight of MMP-8 in the control and EDTA lanes and in lower intensity in the CHX lane; and bands at 54 kDa related with the molecular weight of MMP-20 in all the lanes, with a higher intensity in the control and EDTA lanes (shown in Fig. 1).

Fig. 2. Immunohistochemical localization of MMP-2 and MMP-9. Images acquired from dentin adjacent to the canal lumen. **a** Representative images of MMP-2 immunohistochemical analysis ($\times 40$). Scale bar, 100 μ m. **b** Representative images of MMP-9 immunohistochemical analysis ($\times 40$). Scale bar, 100 μ m. Immuno-

staining was observed mainly in the intratubular dentin. At the right of **a**, **b**, immunostaining is expressed as OD. Each bar represents the mean $\pm$ standard error of the mean. * $p < 0.05$ versus the control group. # $p < 0.05$ versus the EDTA group. OD, optical density; MMP, matrix metalloproteinase.

Immunolabeling of MMP-2 and MMP-9

To evaluate the presence and localization of MMP-2 and MMP-9 in the dentin after the irrigation with the experimental solutions, immunohistochemistry analyses were performed. Immunoreactivity analysis confirmed

the presence of MMPs 2 and 9 mainly associated with the dentinal tubule lumens (intratubular dentin) and occasionally with intertubular dentin.

MMP-2- and MMP-9-positive immunostaining presents a similar distribution pattern. MMP-2 immuno-

staining for EDTA showed significantly higher levels than the other experimental groups (NaOCl and CHX) ($p < 0.05$). For MMP-9 immunostaining, EDTA showed significantly higher levels compared with the other groups ($p < 0.05$). NaOCl and CHX showed a minor presence of MMP expression compared to control ($p < 0.05$) (shown in Fig. 2).

Discussion

Although several studies have demonstrated the presence of endogenous proteolytic enzymes in dentin, to our knowledge, this is the first description of MMP expression in the root canal dentin after the use of irrigant solutions. The presence of different expression patterns of MMPs according to the irrigating solution used led to rejection of the null hypothesis.

We observed important changes in the ultrastructural appearance of the dentin matrix, specifically when sodium hypochlorite was used. Previous studies report the effect of NaOCl on dentin surfaces causing erosion, dentin dehydration, and a consequent drop in dentin flexural strength and microhardness, indicating ultrastructural damage [20, 21, 23]. Additionally, NaOCl treatment results in collagen degradation and is associated with the capability to dissolve organic components through the reactions of amino acid degradation, deproteinizing the organic phase in a time- and concentration-dependent manner [16, 19, 23]. Therefore, from a clinical perspective, the use of NaOCl causes irreversible damage to the structural and biological integrity of the canal wall, suggesting the urgent need of alternative and less deleterious irrigants [23].

Zymograms revealed the presence of gelatinolytic activity bands corresponding to the molecular weights of MMP-2 (gelatinase-a), MMP-8 (collagenase-2), and MMP-20 (enamelysin). However, we did not observe the presence of MMP-9 (gelatinase-b) in any of the experimental groups. In agreement with our results, MMP-2 and MMP-8 have been suggested as major MMPs in this substrate [4, 9, 10]. Even though the presence of MMP-9 in dentin is well known, this enzyme is mainly detected after extensive demineralization with EDTA, due to its specific distribution on dentinal tubules walls and on the mineral-organic matrix interface [10]. Furthermore, our extraction protocol did not involve several demineralization cycles with EDTA, as we aimed to observe its effect as an experimental solution; this could explain the absence of MMP-9 in our zymograms in contrast to the pos-

itive immunolabeling that we observed in the immunohistochemical images. On the other hand, the presence of MMP-20 observed in our study is in concordance with the findings reported by Sulkala et al. [11], who found it in mineralized human dentin. They demonstrated that at least some of the MMP-20 previously synthesized by odontoblasts is incorporated into the mineralized dentin matrix.

In this study, we performed gelatinase (MMP-2 and MMP-9) immunolabeling, with both MMPs widely identified as part of the NCPs embedded in the extracellular dentin matrix [3, 4, 10, 12, 24, 25]. These enzymes have been related with degradation of the ECM in physiological or pathological processes of tissue development/remodeling [3] and extensively studied in relation with adhesive procedures in coronal dentin [12]. MMP-2 and MMP-9 in radicular dentin have been recognized as key factors for cleavage signaling molecules (i.e., DMP1) necessary for dentin remineralization mechanisms [2, 15]. These proteins are critical promoters for regenerative endodontic therapies, and conventional root canal treatment decontamination with antimicrobial solutions may counteract the intrinsic action of these bioactive molecules and, therefore, affect the probability of success of these clinical procedures [22].

Immunohistochemistry showed specific changes in the expression of MMPs after contact with experimental solutions. Specimens treated with NaOCl and CHX showed a significant loss of immunoreactivity in the canal walls area for anti-MMP-2 and anti-MMP-9 antibodies. NaOCl showed obvious effects by reducing the expression of both enzymes in all the tests, and its detrimental proteolytic action affected MMP integrity and type I collagen, leading to an important alteration of the ECM-embedded proteins and macromolecules [18]. Based on these observations and considering that most of the endodontic therapies include the use of NaOCl as an irrigant solution, the dentin layer next to the canal lumen could be deproteinized which may subsequently affect the potential role of the signaling molecules. NaOCl was used in a 5% concentration based on previous reports showing larger dentin deproteinization related with higher concentrations of the irrigant [26, 27]. NaOCl at 5% allowed us to observe the maximum effect of the irrigant on MMPs and dentin matrix; however, lower concentrations should be tested attempting for more conservative clinical strategies.

On the other hand, CHX-treated specimens showed lower levels of both enzymes. Our findings are supported by the literature that demonstrate that CHX had a broad-

spectrum MMP-inhibitory effect [28–30], which can be explained by the CHX calcium-chelating mechanism that affects the requirement of MMPs for metal ions for their catalytic activity [29, 30]. Therefore, these solutions may not be suitable in clinical protocols oriented to achieve tissue regeneration [2].

The present study demonstrates that irrigation with EDTA solution significantly increased the amount of immunolabeled MMP-2 and MMP-9, compared to the other experimental solutions. EDTA is a potent chelating agent, and its ability to bind and remove calcium ions from the hydroxyapatite confers dentin demineralization effect, leading to the exposure of collagen fibers and proteins/enzymes present in the dentin extracellular matrix [6, 22]. On the basis of these features, EDTA, through its demineralizing effect, could increase the expression of the enzymes conditioning the root canal dentin, improving the environment for dentin remineralization and tissue regeneration procedures.

Our findings demonstrate that common irrigants used in root canal treatment have a substantial effect on the expression of MMPs. Therefore, the selection of the appropriate disinfectant solution should be considered carefully and according to the purpose of the clinical therapy, in order to optimize the exposure of endogenous NCPs that could provide signaling mechanisms for regenerative therapies [6, 22]. It is very important to avoid degradation of the structural and biological components to take advantage of dentin as a bioactive matrix.

Conclusion

Our results indicate that the use of most common irrigants affects the expression of MMPs on radicular dentin. NaOCl and CHX cause lower expression of MMPs, while EDTA increases their expression in the root canal dentin. As the present study is a preliminary evaluation,

further studies are needed in order to test combinations of irrigant solutions at different concentrations, according to the most common clinical protocols. Development of biocompatible disinfecting solutions is essential to preserve the signaling molecules and maintain a suitable environment when dentin remineralization and/or tissue regeneration are to be achieved.

Statement of Ethics

The research protocol was approved by the Ethics Committee of the Universidad de Costa Rica (VR-467-2018).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

C.R.-L. contributed to methodology, validation, formal analysis, investigation, resources, data curation, original draft preparation, and reviewing and editing of the manuscript. J.M.G.-T. contributed to validation, formal analysis, writing, reviewing, and editing. M.T.-F. contributed to validation, formal analysis, writing, reviewing and editing, and final approval of the version to be published. B.D.M.S. contributed to validation, formal analysis, writing, reviewing and editing, and final approval of the version to be published. J.R.-C. contributed to conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, original draft preparation, reviewing and editing, visualization, supervision, project administration, funding acquisition, and final approval of the version to be published.

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