Subclinical concentrations of chlorhexidine inhibit gelatinase activity of carious dentine *in vitro*

A Mustakis Trufello,* B Urzúa Orellana,* C Lozano Moraga,* C Lefimil Puente,* I Morales-Bozo*

Department of Community and Basic Sciences, Faculty of Dentistry, University of Chile, Santiago, Chile.

ABSTRACT

Background: There is evidence that dentine matrix gelatinases are involved in the destruction of carious dentine after demineralization by bacterial acids. It has also been observed that chlorhexidine, in very low concentrations, inhibits the activity of these enzymes in mammalian cells. The goal of this study was to determine if the gelatinase activity of carious dentine may be inhibited by chlorhexidine in clinical use concentrations.
Methods: Gelatinolytic activity was evaluated through zymography and identified by Western blot. The inhibitory effects of chlorhexidine at concentrations of 0.01%, 0.04%, 0.08% and 1% on the enzymatic activity of softened carious dentine samples were determined.
Results: In carious dentine, five bands of gelatinolytic activity were detected, with molecular sizes of 86, 75, 38, 33 and 32 kDa. The two bands of the greatest molecular size corresponded to latent and active metalloproteinase-9, respectively. Concentrations of chlorhexidine that were greater than or equal to 0.04% were sufficient to inhibit gelatinolytic activity in the observed bands of carious dentine.
Conclusions: These results support the use of chlorhexidine in clinical use concentrations for the treatment and control of dentine caries. Our study demonstrates for the first time the inhibitory effect of chlorhexidine on gelatinases from carious human dentine.

Keywords: Caries, chlorhexidine, dentine, gelatinases, metalloproteases.

Abbreviations and acronyms: CHX = chlorhexidine; MMPs = metalloproteinases; PBS = phosphate buffered saline.

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INTRODUCTION

The process of dental caries has been defined as an infectious and transmissible disease that starts with the demineralization of the enamel and dentine by acids produced by oral bacteria. This demineralization occurs when the pH falls below 5.5 during the first few minutes after the ingestion of sugar and lasts until subsequent neutralization by salivary buffers. After demineralization, this process continues with the destruction of the organic matrix of the dentine.[1,2]

Dentine is a calcified tissue wherein the organic matrix represents 30% of its total volume, which consists of collagen (90%) and non-collagen proteins (10%). During the carious process, the acidic dissolution of the mineral component of dentine exposes the organic matrix to hydrolysis via the action of enzymes derived from both bacteria and the host.[2]

It has been proposed that many oral bacteria can produce proteolytic enzymes capable of destroying the dentine's organic matrix. However, until now, there has been no evidence that bacteria associated with the initiation and progression of the carious lesion can produce this type of enzyme. On the contrary, *in vitro* experiments have shown that cariogenic bacteria may only cause the demineralization of the dentine surface[3] and that they would be incapable of degrading collagen *in vitro*.[4]

The degradation of the organic matrix of mammalian tissues is mediated principally by proteases that belong to the family of matrix metalloproteinases (MMPs). In healthy dentine matrix, MMP-2, MMP-8, MMP-9 and MMP-20 have been identified,[1,5–9] and current evidence suggests that these host enzymes are responsible for the degradation of the dentine matrix during or after demineralization by bacterial acids.[1,2,10]

Through Western blot analysis and zymography, the gelatinases MMP-2 and MMP-9 have been identified in their latent and active forms in carious lesions in softened human crown dentine.[1] An *in vivo* study in rats demonstrated that the inhibition of MMPs by synthetic inhibitors reduced the progression of caries under fissures.[10]

Chlorhexidine (CHX) is a broad spectrum antimicrobial agent that is frequently used in dentistry to inhibit bacterial growth and in the control of periodontal disease.[11,12] Its mechanism mainly involves its antibacterial effect, as well as its interference with the proteolytic activity of some periodontal pathogens.[13] A study of the action of CHX on purified human MMPs isolated from human fibrosarcoma cells and mammalian cells determined that it had a dose-dependent inhibitory action with regard to the activities of MMP-2 and MMP-9. This inhibitory effect is associated with a chelating mechanism.[11] However, no experimental evidence exists regarding the capacity of this antimicrobial to inhibit carious human dentine tissue gelatinases. Therefore, the objective of this study was to determine if the gelatinase activity of carious dentine would be inhibited through the use of CHX in clinical concentrations.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the School of Dentistry at the University of Chile. After patient authorization was obtained through informed consent, permanent molars with caries were extracted and collected. The extracted teeth had extensive crown destruction due to carious lesions.

Isolation of carious dentine tissue

With the goal of visualizing gelatinase activity in carious crown dentine samples, a pool of 30 teeth extracted because of caries was collected in health centres in the metropolitan region. The teeth were stored in phosphate buffered saline (PBS) and manipulated immediately after extraction. The visible plaque (e.g. tartar or organic material) that covered the lesions was carefully removed, and the teeth were thoroughly cleaned with sterile PBS.[1] The softened carious dentine was removed with a spoon excavator and combined with lysis buffer solution (50 mM Hepes, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton, and 2 mM MgCl2), as well as a cocktail of protease inhibitors (1 M NaF, 2 mM PMSF, 2 ug/ml pepstatine, 2 ug/ml leupeptin, and 1 mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO, USA)). The sample was then shaken and centrifuged at 15 000 rpm for 15 minutes at 4 ºC. The concentrations of proteins in the supernatant were determined using bicinchoninic acid (BCA) (Bio-Rad, Hercules, CA, USA), while the gelatinase activity was determined by zymography.

Zymography

To determine the gelatinolytic activity of the carious dentine samples, supernatant aliquots containing 6 μg of protein were analysed in SDS 10% polyacrylamide gels copolymerized with gelatine following the Laemmli method[14] in non-reductive denaturant conditions. After electrophoresis, the gels were cleaned in 2.5% (v/v) of Triton X-100 to remove SDS and incubated overnight in an activation buffer (50 mM/L Tris, pH 7,5; 5 mM/L CaCl$_2$; 1 μM/L ZnCl$_2$) at 37 ºC. The gels were fixed and dyed with 0.2% Coomassie Brilliant Blue and destained until clear bands were observed. Purified human MMP-9 (Chemicon, Temucula, CA, USA) were used as positive controls. The gelatinolytic activity was determined through densitometric analysis using the programmes Gel-Pro Analyzer 3.1 and/or ImageJ.[15]

Inhibition of metalloproteinase activity

To determine if CHX would inhibit the gelatinases present in the carious dentine, samples of the supernatants from the carious dentine were analysed by zymography in the presence of gelatine. After electrophoresis, the gels were incubated overnight at 37 ºC in activation buffer conditioned with CHX diacetate in the following concentrations: 0.01%, 0.04%, 0.08% and 1% (Sigma-Aldrich, St. Louis, MO, USA). The conditioned medium without the inhibitor and with EDTA was used as control.

Western blot

To determine the presence of MMP-2 and MMP-9 in the carious dentine, samples of the supernatants from the carious dentine were analysed by Western blot. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (PIERCE, 0.45 μm) and were blocked for one hour in 0.1% TBS-T buffer (Tris-HCl 20 mM pH 7.6, NaCl 137 mM and Tween 20 at 0.1%) with 3% albumin. The membrane then underwent four 15-minute washes in 0.1% TBS-T. The membrane was then incubated with the anti-human MMP-2 and MMP-9 primary antibody (R&D systems) at a concentration of 1:250 in TBS-T for 17 hours. Subsequently, it was washed four times with 0.1% TBS-T and incubated with the peroxidase conjugated secondary antibody anti-Mouse IgG (Pierce Biotechnology, Rockford, USA) at a concentration of 1:5000 in TBS-T for one hour. After washing, the positive reaction was identified by chemiluminescence (Pierce Biotechnology, Rockford, USA). The membrane was exposed to radiographic film (Kodak) and developed. The molecular size of the bands was compared with proteins of known size (Precision Plus protein standards, BIO-RAD). Negative controls were performed without the use of the primary antibody.

Statistical analysis

All experiments were performed in triplicate. The mean was calculated, and the relative enzymatic activity percentage and its standard deviation were determined in relation to the standard enzymatic activity of MMP-9.

RESULTS

Determination of the gelatinolytic activity in the carious dentine

In the carious dentine samples obtained from the pool of the extracted teeth, five bands with gelatinolytic activity were detected, with molecular sizes of 86, 75, 38, 33 and 32 kDa (Fig. 1, lane 2). The percentage of gelatinolytic activity corresponding to each band is shown in Table 1. On average, the 38 kDa band exhibited 2.7 times more activity than was observed with the rest of the bands. The molecular sizes of the
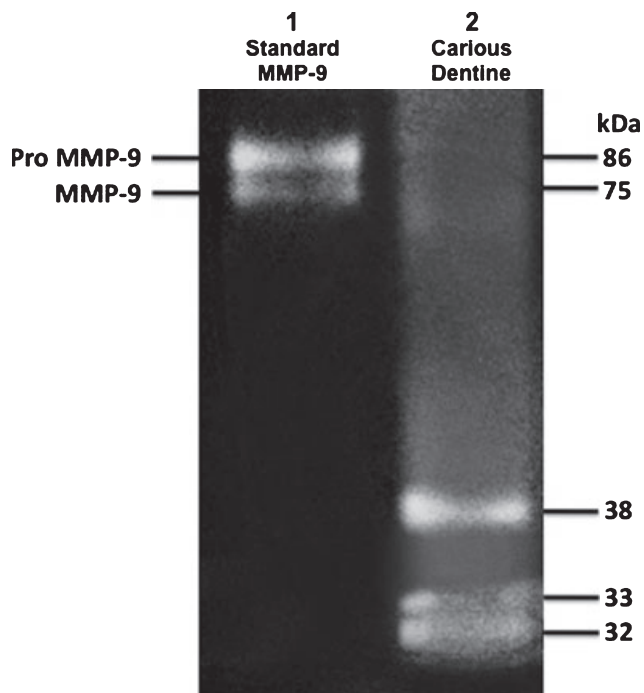
86 and 75 kDa bands corresponded to the molecular sizes of the latent and active MMP-9, respectively. The remainder of the observed gelatinolytic bands exhibited a smaller molecular size than those described for the MMP-2 or MMP-9 gelatinases. By analysing the carious dentine samples by Western blot assay, bands of molecular sizes of 86, 75, 33 and 32 kDa were identified as latent, active and truncated form of MMP-9 respectively (Fig. 3). When the samples were analysed with the anti-MMP-2 antibody, two new bands with molecular sizes of 67 and 25 kDa were observed (Fig. 3). The gelatinolytic band of 38 kDa was not identified with the antibodies used in this study.

In vitro inhibition of gelatinase activity by CHX in carious dentine

Figure 2 shows the results of the experiment that determined whether CHX could inhibit gelatinase activity in carious dentine *in vitro* using increasing concentrations of CHX. The gelatinase activity in carious dentine was completely inhibited by CHX concentrations greater than or equal to 0.04% (Fig. 2c–2e, lanes 6, 8 and 10), and a similar effect was observed for the enzymatic activity of the standard MMP-9 (Fig. 2c–2e, lanes 5, 7 and 9). All of the bands with gelatinolytic activity were inhibited at these concentrations of CHX, including the most prominent 38 kDa band. The percentages of the relative gelatinolytic activity observed in the inhibition assay with CHX are shown in Table 2. In relation to the control without CHX (Table 1), a CHX concentration of 0.01% partially lowered the enzymatic activity of the 86, 75, 38, 33 and 32 kDa bands 1.3, 1.5, 1.4, 3.0 and 3.2 times, respectively (Table 2). CHX concentrations of 0.04, 0.08 and 1% (Table 2) completely inhibited the relative enzymatic activities of all bands when compared to the control without CHX (Table 1).

DISCUSSION

Under the conditions of this experiment involving the use of carious human dentine tissues, it was possible to observe the presence of bands with gelatinolytic activity of different molecular sizes. Two of the bands

Fig. 1 Zymographic pattern of bands with gelatinolytic activity in carious dentine. Lane 1: carious dentine, lane 2: standard MMP-9.

Table 1. Profile of bands with gelatinolytic activity found in carious dentine

Band (kDa)	86 (Mean ± DS)	75 (Mean ± DS)	38 (Mean ± DS)	33 (Mean ± DS)	32 (Mean ± DS)
Percentage of relative enzymatic activity	45 ± 0.11	56 ± 0.13	116 ± 0.22	34 ± 0.22	45 ± 0.17

100% of relative enzymatic activity corresponds to the intensity of the 86 kDa band from the standard MMP-9. Percentage of relative enzymatic activity expressed as the mean ± standard deviation.
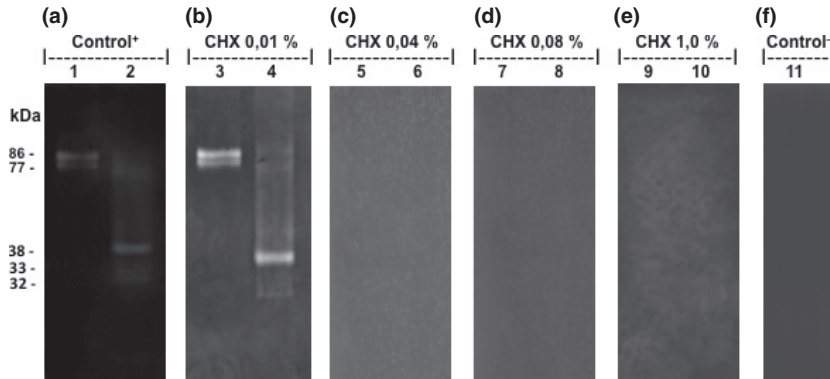
Fig. 2 The effect of increasing concentrations of chlorhexidine on the gelatinolytic activity in carious dentine. (a) Positive control: Incubation in an activation buffer. (b)–(e) Incubation in activation buffers with increasing concentrations of chlorhexidine. (f) Negative control: Incubation in an activation buffer in the presence of EDTA. Lanes 1, 3, 5, 7, 9 and 11: standard MMP-9. Lanes 2, 4, 6, 8, 10 and 12: carious dentine.

Table 2. The effect of increasing concentrations of chlorhexidine on the percentage of relative gelatinolytic activity in carious dentine

[Chlorhexidine]	Percentage of relative gelatinolytic activity Bands (kDa)				
	86	75	38	33	32
0.01	36 ± 0.32	38 ± 0.37	82 ± 0.72	11 ± 0.09	14 ± 0.12
0.04	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
0.08	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
1.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

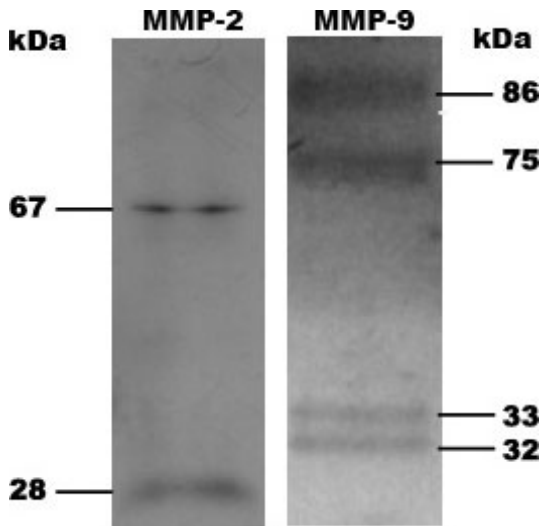Relative enzymatic activities expressed as the mean ± standard deviation.

Fig. 3 Western blot for MMP-2 and MMP-9 in carious dentine. Left: Truncated forms of MMP-2. Right: Latent, active and truncated forms of MMP-9.

corresponded to the sizes that have been previously described for MMP-9 and were identified as this metalloproteinase in the Western blot assay. The bands of molecular sizes of 33 and 32 kDa corresponded to truncated forms of MMP-9. Bands of similar molecular sizes have been described in prior studies using zymography.[1,16] Additionally, bands of MMP-2 of 67 and 28 kDa, without gelatinolytic activity were observed by Western blot. The gelatinolytic band of 38 kDa could correspond to the truncated form of MMP-8 described in other studies,[7,9] or to truncated forms of gelatinases which have lost the epitopes recognized by anti-MMP-2 and anti-MMP-9 antibodies. However, the mere presence of gelatinases in carious dentine does not indicate their origin. These enzymes could originate from the dental pulp, saliva, and/or dentine itself, as has been proposed by various authors.[1,5,7,10]

The presence of these molecules in the carious tissue supports the hypothesis that MMPs immersed in the mineralized dentine matrix could be activated and liberated after the demineralization process of this tissue during the formation of caries as a consequence of pH fluctuations within the lesion, either with or without the participation of cariogenic oral bacteria.[1] To address this issue, Toledano *et al.*[17] used immunofluorescence and found the carious process increases the activity of gelatinase MMP-2 at different expression levels in healthy dentine, affected dentine, and infected dentine samples. They observed the expression of MMP-2 was more intense in the affected dentine than in healthy dentine.

Our *in vitro* study demonstrates, for the first time, that CHX at concentrations of 0.04% or higher completely inhibits the gelatinases extracted from carious dentine. These results concur with the study by Gendron et al.[11] in which the inhibitory effect of CHX on MMP-2 and MMP-9 from human fibrosarcoma cells and mammalian cells was evaluated using similar concentrations as those described in our work.

The gelatinases and collagenases could also be activated by the acid conditioning of the dentine that results from the use of orthophosphoric acid or self-etching adhesives. The activation of these enzymes could cause the degradation of the tooth-restoration adhesive interface, or the hybrid layer, which is a phenomenon similar to what occurs in dentine caries.[8,18] In relation to this phenomenon, there is both *in vitro* and *in vivo* evidence that the use of CHX after acid etching lowers the rate of degradation of the hybrid layer associated with the action of these enzymes, which are present in dentine.[19–21]

Additionally, the *in vivo* study by Hebling et al.[18] demonstrated that the deterioration of the hybrid layer occurred very quickly. This was due to the adhesion process carried out on top of affected dentine, which exhibited a reduction in collagen undergoing the demineralization process resulting from caries. This accelerated degradation was associated with a greater increase in the collagenolytic activity of the affected dentine than in the caries-free tissue, and it was inhibited by the use of CHX after acid etching. Moreover, the study of Magalhães et al.[22] determined that the use of CHX *in situ* reduce dentine erosion and abrasion. In recent reports,[23,24] this protective effect of CHX was associated with their ability to reduced the degradation of organic matrix from dentine.

Our results support the use of CHX in topical applications, taking advantage of its inhibitory characteristics against gelatinolytic activity for use in the treatment and control of carious dentine. Dental CHX solutions formulated to control bacterial plaque are available in a range of concentrations from 0.1% to 0.2% and therefore may be used for their intended purpose. CHX could also be incorporated into the formulation of materials that come into contact with dentine in preventive or restorative processes to inhibit gelatinases that are activated by caries or therapeutic procedures.

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REFERENCES

1. Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesion. J Dent Res 1998;77:1622–1629.

2. Chaussain-miller C, Fioretti F, Goldberg M, Menashi S. The role of matrix metalloproteinases (MMPs) in human caries. J Dent Res 2006;85:22–32.

3. Katz S, Park KK, Palenik CJ. *In vitro* root surface caries studies. J Oral Pathol Med 1987;42:40–48.

4. van Strijp AJ, van Steenbergen TJ, ten Cate JM. Bacterial colonization of mineralized and completely demineralized dentine *in situ*. Caries Res 1997;31:349–355.

5. Martin-de Las Heras S, Valenzuela A, Overall CM. The matrix metalloproteinase gelatinase A in human dentine. Arch Oral Biol 2000;45:757–765.

6. Sulkala M, Larmas M, Sorsa T, Salo T, Tjäderhane L. The localization of metalloproteinase – 20 (MMP-20, enamelysin) in mature human theeth. J Dent Res 2002;81:603–607.

7. Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjäderhane L. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. Arch Oral Biol 2007;52:121–127.

8. Nishitani Y, Yoshiyama M, Wadgaonkar B, et al. Activation of gelatinolytic/collagenolytic activity in dentin by self-etching adhesives. Eur J Oral Sci 2006;114:160–166.

9. Shimada Y, Ichinose S, Sadr A, Burrow MF, Tagami J. Localization of matrix metalloproteinases (MMPs-2, 8, 9 and 20) in normal and carious dentine. Aust Dent J 2009; 54:347–354.

10. Sulkala M, Wahlgren J, Larmas M, et al. The effects of MMP inhibitors on human salivary MMP activity and caries progression in rats. J Dent Res 2001;80:1545–1549.

11. Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8 and 9 by chlorhexidine. Clin Diagn Lab Immunol 1999;6:437–439.

12. Azmak N, Atilla G, Luoto H, Sorsa T. The effect of subgingival controlled-release delivery of chlorhexidine chip on clinical parameters and matrix metalloproteinase-8 levels in gingival crevicular fluid. J Periodontol 2002;73:608–615.

13. Grenier D. Reduction of proteolytic degradation by chlorhexidine. J Dent Res 1993;72:630–633.

14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.

15. Mazzoni A, Mannello F, Tay FR, et al. Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. J Dent Res 2007;86:436–440.

16. Apajalahti S, Sorsa T, Ingman T. Matrix metalloproteinase -2, -8, -9, and -13 in gingival crevicular fluid of short root anomaly patients. Eur J Orthod 2003;25:365–369.

17. Toledano M, Nieto-Aguilar R, Osorio R, et al. Differential expression of matrix metalloproteinase-2 in human coronal and radicular sound and carious dentine. J Dent 2010;38: 635–640.

18. Hebling J, Pashley DH, Tjäderhane L, Tay FR. Chlorhexidine arrests subclinical degradation of dentin hybrid layers *in vivo*. J Dent Res 2005;84:741–746.

19. Carrilho MR, Carvalho RM, de Goes MF, et al. Chlorhexidine preserves dentin bond *in vitro*. J Dent Res 2007;86: 90–94.

20. Carrilho MR, Geraldeli S, Tay F, et al. *In vivo* preservation of the hybrid layer by chlorhexidine. J Dent Res 2007;86: 529–533.

21. Brackett WW, Tay FR, Brackett MG, Dib A, Sword RJ, Pashley DH. The effect of chlorhexidine on dentin hybrid layers *in vivo*. Oper Dent 2007;32:107–111.

22. Magalhães AC, Wiegand A, Rios D, Hannas A, Attin T, Buzalaf MA. Chlorhexidine and green tea extract reduce dentin erosion and abrasion *in situ*. J Dent 2009;37:994–998.

23. Kato MT, Leite AL, Hannas AR, *et al.* Impact of protease inhibitors on dentin matrix degradation by collagenase. J Dent Res 2012;91:1119–1123.

24. Buzalaf MA, Kato MT, Hannas AR. The role of matrix metalloproteinases in dental erosion. Adv Dent Res 2012;24: 72–76.

Address for correspondence:
Dr Irene Morales-Bozo
Department of Community and Basic Sciences
Faculty of Dentistry
University of Chile
Sergio Livingstone Pohlhammer 943
Independencia
Santiago
Chile
Email: irenemoralesbozo@gmail.com