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Sodium Fluoride Inhibits MMP-2 and MMP-9

ABSTRACT

The importance of fluoride (F) in preventing dental caries by favorably interfering in the demineralization-remineralization processes is well-established, but its ability to inhibit matrix metalloproteinases (MMPs), which could also help to prevent dentin caries, has not been investigated. This study assessed the ability of F to inhibit salivary and purified human gelatinases MMPs-2 and -9. Saliva was collected from 10 healthy individuals. Pooled saliva was centrifuged, and supernatants were incubated for 1 hr at 37°C and subjected to zymography. Sodium fluoride (50-275 ppm F) was added to the incubation buffer. The reversibility of the inhibition of MMPs-2 and -9 by NaF was tested by the addition of NaF (250-5,000 ppm F) to the incubation buffer, after which an additional incubation was performed in the absence of F. F decreased the activities of pro- and active forms of salivary and purified human MMPs in a dose-response manner. Purified gelatinases were completely inhibited by 200 ppm F (IC_{50} = 100 and 75 ppm F for MMPs-2 and -9, respectively), and salivary MMP-9 by 275 ppm F (IC_{50} = 200 ppm F). Inhibition was partially reversible at 250-1,500 ppm F, but was irreversible at 5,000 ppm F. This is the first study to describe the ability of NaF to inhibit MMPs completely.

KEY WORDS: fluorides, matrix metalloproteinase-2, matrix matalloproteinase-9, dentin, inhibition, extracellular matrix.

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INTRODUCTION

The importance of fluoride (F) in preventing dental caries, producing a favorable balance in tooth de- and remineralization, has been well-established (Buzalaf *et al.*, 2011). However, its action on the prevention of dentin erosion, when not associated with other potentially relevant cations such as tin, seems to be dependent on the maintenance of the demineralized organic matrix (DOM) (Ganss *et al.*, 2010).

When caries and erosion affect dentin, its mineral part is dissolved, and the DOM is exposed to breakdown by bacterially and host-derived enzymes, such as matrix metalloproteinases (MMPs) (Klont *et al*., 1991; Kleter *et al*., 1994; Dung *et al*., 1995; Tjäderhane *et al*., 1998; Chaussain-Miller *et al*., 2006; Boushell *et al*., 2011; Buzalaf *et al*., 2012). Since DOM acts as a mechanical barrier, hampering ionic diffusion into and out of the demineralizing area (Kleter *et al.*, 1994; Ganss *et al.*, 2004), the maintenance of the DOM during both cariogenic (Chaussain-Miller *et al*., 2006) and erosive challenges (Ganss *et al.*, 2004; Kato *et al.*, 2009; Kato *et al.*, 2010a,b) is able to reduce the rate of tissue loss. However, because of the presence of collagen-degrading MMPs in the oral cavity (Hannas *et al.*, 2007), the DOM may not persist on the erosive and cariogenic lesion surfaces *in vivo*. Inhibition of salivary MMPs eliminates the degradation of acid-exposed DOM (Tjäderhane *et al.*, 1998) and reduces the rate of dentin caries progression (Sulkala *et al.*, 2001). It has been assumed that if the DOM were preserved after erosive challenges, the rate of dentin loss would be reduced. Therefore, protease inhibitors have been added to solutions and gels, which were then able to reduce dentin loss under erosive challenges *in situ/ex vivo*. In these studies, F-containing products were included as controls and demonstrated a good protective effect against dentin loss (Kato *et al.*, 2010a,b; Magalhães *et al.*, 2009). More recently, 1.23% sodium fluoride (NaF) gel significantly reduced the DOM degradation (Kato *et al.*, 2012). This led us to hypothesize that, in addition to its established effect on the de-/remineralization cycle, F could also inhibit MMPs; this was evaluated in the present study.

MATERIALS & METHODS

The study was approved by the Institutional Review Board of Bauru Dental School, University of São Paulo, Brazil (Proc. no. 076/2011). Paraffin-stimulated whole saliva was collected from 10 healthy volunteers (aged 20-30 yrs) with no active caries lesions or periodontal disease. Pooled saliva was centrifuged (10,000 rpm, 10 min), and the supernatant stored frozen (-20°C) until used. The volunteers refrained from using fluoridated toothpastes for 4 days prior to collection. Despite their residence in a fluoridated area

(0.6-0.8 mg F/L) (Buzalaf *et al.*, 2013), fluoride concentration in the saliva pool was low (around 0.01 mg/L).

Gelatin Zymography

Saliva and purified human MMPs-2 and -9 were diluted in non-reducing sample buffer (10:3, sample:sample buffer), incubated for 1 hr at 37°C, and immediately subjected to zymography in quintuplicate. To assess the effect of F on MMPs-2 and -9 activities, we loaded human purified MMPs-2 and -9 or saliva on 10% SDS-PAGE containing 1% gelatin from porcine skin (Sigma Chemical, St. Louis, MO, USA). Electrophoresis was conducted at 120 V/25 A for 2 hrs, followed by incubation for 30 min in buffer I [50 mmol/L Tris HCl, 2.5% Tween 80, 0.02% (w/v) NaN_3, pH 7.5 at 22°C] and buffer II [50 mmol/L Tris HCl, 2.5% Tween 80, 0.02% (w/v) NaN_3, 1 µm $ZnCl_2$, 5 mmol/L $CaCl_2$]. The gels were cut into 2-cm strips, each containing both MMPs and saliva sample. Each strip was incubated separately at 37°C for 19 hrs in buffer III [50 mmol/L Tris HCl, 5 mmol/L $CaCl_2$, 1 µM $ZnCl_2$, 0.02% (w/v) NaN_3] containing (or not) (control) F (as NaF) at 50, 75, 100, 125, 150, 175, 200, 225, 250, or 275 ppm. Gels were incubated in 0.1% Coomassie Brilliant Blue R-250 for 60 min at 22°C on a shaker and destained in 10% acetic acid/10% methanol. After gels were destained, gelatinolytic activity was detected as clear bands in the background of uniform staining.

To quantify the relative inhibition of gelatinases by F, we scanned electrophoretic bands (Imagescanner, Amersham Biosciences, Uppsala, Sweden) and analyzed the band intensity with the Kodak Molecular Imaging software version 4.5 (Kodak, Rochester, NY, USA). To confirm that the activities represented MMPs, we incubated a representative gel loaded with MMPs in 3 mmol/L 1,10 phenanthroline, a specific MMP inhibitor. Percent inhibition of enzyme activity was plotted against F concentration. F inhibitory effect was determined from the concentration that inhibited 50% and 100% of the enzyme activity (IC_{50} or IC_{100}, respectively). The percentage inhibition of F was determined by comparison of the activity of MMPs with control reactions.

The reversibility of the inhibition of MMPs-2 and -9 by NaF was tested by the addition of NaF (250, 500, 1,500, and 5,000 ppm F) to the incubation buffer for 19 hrs, after which an additional incubation was performed for another 19 hrs in the absence of F.

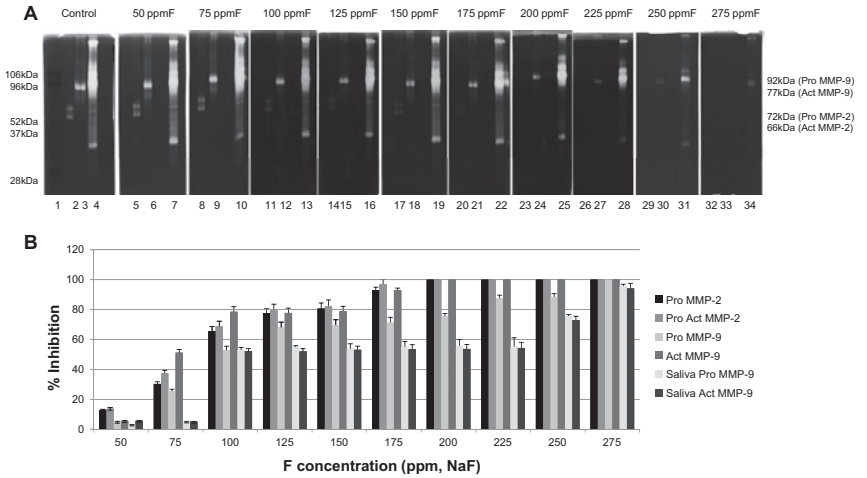

Figure 1. (A) The effect of NaF on gelatinolytic enzymes activities. Lane 1 – MWM, molecular-weight markers (kDa). Lanes 2 and 3 – Human purified MMP-2 and MMP-9 controls, respectively. Lane 4 – Saliva pool. Lanes 5 to 34: gel strips incubated in 50, 75, 100, 125, 150, 175, 200, 225, 250, and 275 ppm F (NaF) buffer, respectively. After incubation with NaF, the gelatin-degrading activity of pro- and active forms of MMP-2 or -9, as well as salivary MMP-9, decreased significantly and in a concentration-dependent manner (Lanes 5 to 34). **(B)** Dose-response of pro- and active forms of MMP-2 and -9 inhibition by incubation in buffer containing NaF, as observed in gelatin zymography. The scale represents mean (bars indicate SD) inhibition percentage when compared with untreated controls, when measured as electrophoretic bands of scanned gels (n = 5). F (as NaF) concentrations in buffer are shown below bars.

RESULTS

Four major bands were detected in the zymographic assays (Fig. 1A): 2 sharper bands with an approximate molecular mass of 72 and 66 kDa (corresponding to pro- and active MMP-2 M_r, respectively) and 2 broader bands migrating between 95 and 80 kDa (pro- and active MMP-9). These enzymes were characterized as MMPs, since their activities were inhibited by phenanthroline (data not shown).

NaF decreased the activities of pro- and active forms of salivary and purified human MMPs-2 and -9 in a dose-response manner. Purified forms of both MMPs-2 and -9 were completely inhibited by 200 ppm F (IC_{50} = 100 and 75 ppm F for MMPs-2 and -9, respectively). Salivary MMP-9 was completely inhibited by 275 ppm F (IC_{50} = 200 ppm F). Complete inhibition is shown in Fig. 1A by the absence of white bands and in Fig. 1B by the percentage of inhibition.

Regarding the reversibility experiments, the additional incubation of the gels in the absence of F demonstrated that the inhibition of MMPs-2 and -9 by NaF is partially reversible at F concentrations ranging between 250 and 1,500 ppm F, but is irreversible at 5,000 ppm F (Fig. 2).

DISCUSSION

The present study was designed to test the hypothesis that F could inhibit matrix proteases. This was evaluated by gelatin zymography, which confirmed our hypothesis, *i.e.*, that F was able to inhibit both MMPs-2 and -9 in a dose-response fashion. In gelatin zymography, F was added to the incubation buffer for 19 hrs, which is required by the protocol to allow for detection of the bands without inhibition. This incubation time is much higher than that expected to occur in the clinical situation, even

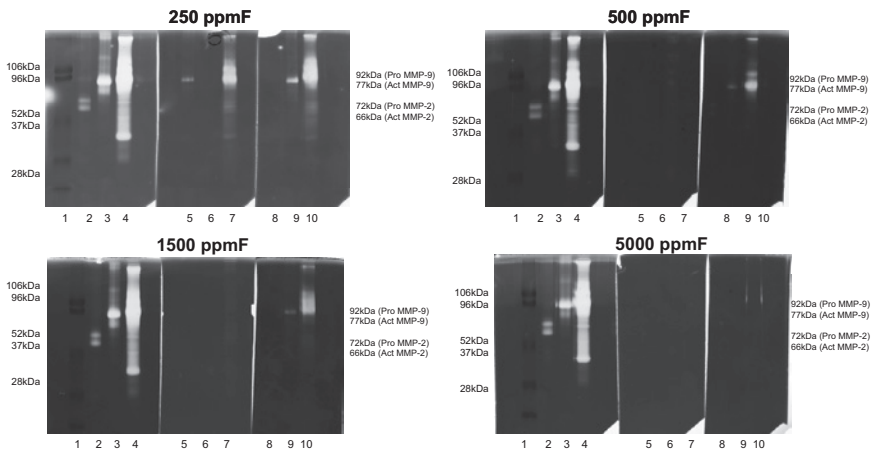

Figure 2. The effect of NaF reversibility on gelatinolytic enzyme activities. Lane 1 – MWM, molecular-weight markers (kDa). Lanes 2 and 3 – Human purified MMP-2 and MMP-9 controls, respectively. Lane 4 – Saliva pool. Lanes 5 to 7: gel strips incubated in 250, 500, 1,500, or 5,000 ppm F (NaF) buffer, 2 times at 19 hrs each. Lanes 8 to 10: gel strips incubated in 250, 1,500, or 5,000 ppm F (NaF) buffer for 19 hrs, and for another 19 hrs without NaF.

when F varnishes are used. However, it is possible that inhibition might occur in the clinical situation in shorter times, but this could not be tested by zymography, which requires overnight incubation. It is likely that, in the clinical condition, F alone is not sufficiently effective in MMP inhibition. One alternative would be the combination of F with more effective MMP inhibitors, which should be tested in further studies.

Many studies have shown that the degradation of the DOM increases the rate of demineralization of dentin lesions (Klont *et al.*, 1991; Kleter *et al.*, 1994; Schlueter *et al.*, 2010). The zymographic analyses, clearly showing the inhibition of salivary MMPs, identified by the characteristic molecular weight and inhibition by 1,10-phenanthroline (Toth *et al.*, 2012), support the results of a previous *in vitro* study that showed an F-related effect on the preservation of DOM (Kato *et al.*, 2012). However, the degradation of the demineralized dentin matrix *in vivo* does not depend only on the action of MMPs (Tjäderhane *et al.*, 2013). Other proteases, such as cysteine cathepsins, have more recently been suggested to be implicated in this process (Tersariol *et al.*, 2010; Nascimento *et al.*, 2011). Furthermore, these distinct proteases act in concert, since cysteine cathepsins can activate MMPs (Nagase, 1997). Thus, the impact of the inhibitory effect of NaF on MMPs, as shown in the present study on the progression of caries and erosive lesions, needs to be better investigated. This could be done with protocols that resemble these lesions and appropriate methodologies to distinguish the different collagen breakdown products released, such as analysis of the collagen C-terminal telopeptide release (Tjäderhane *et al.*, 2013).

The possible mechanism by which NaF inhibits the MMPs is not known. Considering that MMPs are Zn^{2+}- and Ca^{2+}-dependent enzymes, and F is highly electronegative, it seems logical that excess F could make these cations unavailable to participate in the catalytic process. Analysis of our data suggests that the inhibition of MMPs-2 and -9 by NaF is reversible at lower, but irreversible at higher (5,000 ppm), F concentrations. Again, the implication of this in the clinical condition is not known, since most of the forms of F delivery allow for much lower time of contact of the agent with the dentin surface. One noteworthy aspect is that in the study by Kato *et al.* (2012), a gel containing 1.23% F was applied to dentin blocks for only 1 min, and reduction of the degradation of the DOM by bacterial collagenase *in vitro* was achieved. Additional studies more closely resembling the clinical situation and using different response variables should be conducted to clarify this point. Another interesting aspect was that 50% inhibition of salivary MMP-9 was achieved by incubation with F at 100 ppm F, and this remained stable until 225 ppm F, increasing only thereafter (Figs. 1A, 1B). The reason for this is not apparent and is probably related to the mechanism of inhibition of salivary MMP-9 by F, which deserves further investigation.

Among the available F compounds used in dental products, NaF is the most widely used. Some alternative F compounds have additional ions that might present relevant activity against caries and/or erosion, such as stannous fluoride (SnF_2) (Ganss *et al.*, 2010), titanium tetrafluoride (TiF_4) (Magalhães *et al.*, 2008), and silver diamine fluoride (SDF) (Rosenblatt *et al.*, 2009). It is difficult to compare different salts and decouple the effects of fluoride from those of other ions, since their modes of action are different (Magalhães *et al.*, 2011). For this reason, we decided to test NaF in the present study, since Na^+ is not known to have an effect either on MMP activity or on the de-/remineralization process in dentin. Effects of SDF at different concentrations on MMPs have been evaluated with enzymatic fluorimetric assays kits for MMPs-2, -8, and -9 (Mei *et al.*, 2012). NaF at 3% and 10% served as controls, since they had the same F concentrations as those present in 12% and 38% SDF, respectively. However, even with these very high F concentrations (~ 15,000 and 50,000 ppm F, for 3% and 10% NaF, respectively), the inhibition of MMPs was in the range of 30% to 70% only. It should be noted that the maximum solubility of NaF in water is 4%. Thus, most of the F present in 10% NaF might not have been available in the reaction media. Conversely, 38% SDF inhibited 79% to 94% of MMP activities, which was probably due to the effect of silver. The higher degree of inhibition found in the present study might be due to the different type of assay used.

This is the first study to describe the ability of NaF, the most common F compound in oral hygiene products, to completely inhibit the activity of MMPs in clinically relevant concentrations. These findings might help to explain the observation that when the DOM is removed, the effect of F to prevent dentin erosion is remarkably reduced (Ganss *et al.*, 2010). In addition,

our results provide new insights into the mechanism of action of F, which needs to be studied further to evaluate its true importance in the prevention of caries and erosion in dentin.

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REFERENCES

Boushell LW, Nagaoka H, Nagaoka H, Yamauchi M (2011). Increased matrix metalloproteinase-2 and bone sialoprotein response to human coronal caries. *Caries Res* 45:453-459.

Buzalaf MA, Pessan JP, Honório HM, ten Cate JM (2011). Mechanisms of action of fluoride for caries control. *Monogr Oral Sci* 22:97-114.

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

Buzalaf MA, Moraes CM, Olympio KP, Pessan JP, Grizzo LT, Silva TL, *et al*. (2013). Seven years of external control of fluoride levels in the public water supply in Bauru, São Paulo, Brazil. *J Appl Oral Sci* 21:92-98.

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

Dung SZ, Gregory RL, Li Y, Stookey GK (1995). Effect of lactic acid and proteolytic enzymes on the release of organic matrix components from human root dentin. *Caries Res* 29:483-489.

Ganss C, Klimek J, Starck C (2004). Quantitative analysis of the impact of the organic matrix on the fluoride effect on erosion progression in human dentine using longitudinal microradiography. *Arch Oral Biol* 49:931-935.

Ganss C, Lussi A, Sommer N, Klimek J, Schlueter N (2010). Efficacy of fluoride compounds and stannous chloride as erosion inhibitors in dentine. *Caries Res* 44:248-252.

Hannas AR, Pereira JC, Granjeiro JM, Tjäderhane L (2007). The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand* 65:1-13.

Kato MT, Magalhães AC, Rios D, Hannas AR, Attin T, Buzalaf MA (2009). Protective effect of green tea on dentin erosion and abrasion. *J Appl Oral Sci* 17:560-564.

Kato MT, Leite AL, Hannas AR, Buzalaf MA (2010a). Gels containing MMP inhibitors prevent dental erosion *in situ. J Dent Res* 89:468-472.

Kato MT, Leite AL, Hannas AR, Oliveira RC, Pereira JC, Tjäderhane L, *et al*. (2010b). Effect of iron on matrix metalloproteinase inhibition and on the prevention of dentine erosion. *Caries Res* 44:309-316.

Kato MT, Leite AL, Hannas AR, Calábria MP, Magalhães AC, Pereira JC, *et al*. (2012). Impact of protease inhibitors on dentin matrix degradation by collagenase. *J Dent Res* 91:1119-1123.

Klont B, Damen JJ, ten Cate JM (1991). Degradation of bovine incisor root collagen in an in vitro caries model. *Arch Oral Biol* 36:299-304.

Kleter GA, Damen JJM, Everts V, Niehof J, ten Cate JM (1994). The influence of the organic matrix on demineralization of bovine root dentin *in vitro*. *J Dent Res* 73:1523-1529.

Magalhães AC, Kato MT, Rios D, Wiegand A, Attin T, Buzalaf MA (2008). The effect of an experimental 4% TiF_4 varnish compared to NaF varnishes and 4% TiF_4 solution on dental erosion in vitro. *Caries Res* 42:269-274.

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

Magalhães AC, Wiegand A, Rios D, Buzalaf MA, Lussi A (2011). Fluoride in dental erosion. *Monogr Oral Sci* 22:158-170.

Mei ML, Li QL, Chu CH, Yiu CK, Lo EC (2012). The inhibitory effects of silver diamine fluoride at different concentrations on matrix metalloproteinases. *Dent Mater* 28:903-908.

Nagase H (1997). Activation mechanisms of matrix metalloproteinases. *Biol Chem* 378:151-160.

Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, *et al*. (2011). Cysteine cathepsins in human carious dentin. *J Dent Res* 90:506-511.

Rosenblatt A, Stamford TC, Niederman R (2009). Silver diamine fluoride: a caries "silver-fluoride bullet". *J Dent Res* 88:116-125.

Schlueter N, Hardt M, Klimek J, Ganss C (2010). Influence of the digestive enzymes trypsin and pepsin in vitro on the progression of erosion in dentine. *Arch Oral Biol* 55:294-299.

Sulkala M, Wahlgren J, Larmas M, Sorsa T, Teronen O, Salo T, Tjäderhane L (2001). The effects of MMP inhibitors on human salivary MMP activity and caries progression in rats. *J Dent Res* 80:1545-1549.

Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, *et al*. (2010). Cysteine cathepsins in human dentin-pulp complex. *J Endod* 36:475-481.

Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T (1998). The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. *J Dent Res* 77:1622-1629.

Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, *et al*. (2013). Optimizing dentin bond durability: control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. *Dent Mater* 29:116-135.

Toth M, Sohail A, Fridman R (2012). Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography In: Metastasis research protocols. Dwek M, Brooks SA, Schumacher U, editors. Oxford, UK: Humana Press, pp. 121-136.