Effect of Sodium Fluoride on the endogenous MMP Activity of Dentin Matrices

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Results. Addition of NaF to the incubation media produced a progressive significant reduction (p<0.05) in the loss of mass of dentin matrices, with all concentrations demonstrating significantly less mass loss than the control group. Significantly less HYP release from the dentin beams was found in the higher fluoride concentration groups, while fluoride concentrations of 75 and 150 ppm significantly reduced rhMMP-9 activity by 6.5% and 79.2%, respectively. Conclusions. The results of this study indicate that NaF inhibits matrix-bound MMPs and therefore may slow the degradation of dentin matrix by endogenous dentin MMPs. Journal of Nature and Science, 1(6):e118, 2015

Dentin | matrix metalloproteinases | storage solution | NaF | dry mass loss

Introduction

Remineralization of partially demineralized enamel and dentin is facilitated by the presence of fluoride in remineralizing solutions [1]. When dentin is demineralized by acids, the preforms of matrix metalloproteinase (MMPs) bound to collagen are uncovered and activated [2]. These gelatinases (MMP-2 and MMP-9) and collagenases (MMP-8) [3] slowly begin to degrade the collagen fibrils, which are the substrate for remineralization. A race develops between how rapidly remineralizing effects can occur versus how rapidly collagen can be destroyed and solubilized by MMPs. In the absence of MMP inhibitors (Metastat, CMT-3, chemically modified tetracycline), the demineralized matrix is destroyed, preventing future attempts to remineralize cavitated carious lesions. Sukala et al fed rats a cariogenic diet with and without a synthetic MMP inhibitor [4]. Those rats who consumed the MMP inhibitor exhibited reduced rates of caries progression. Recently, 1.25% sodium fluoride (NaF) gel was added to demineralized dentin matrices to reduce degradation of dentin by matrix metalloproteinases [5].

Thus, it is possible that NaF may inhibit the endogenous MMPs in demineralized dentin matrices. Kato et al [6] recently isolated and separated MMP-2 and -9 from human saliva by SDS-PAGE. They incubated their zymography gels with increasing concentrations of NaF to see if it could inhibit the MMPs. Both MMPs showed significant reductions in activity with increasing F concentrations with an IC₅₀ of 100 and 75 ppm for MMPs-2 and -9, respectively. No one has yet determined if NaF can inhibit the endogenous MMPs of dentin matrices while they remain collagen bound as they are in vivo.

The purpose of this in vitro study was to test the null hypothesis that fluoride does not prevent collagen degradation of dentin matrices or the solubilization of collagen peptides from dentin matrices. Solubilization of collagen was quantitated gravimetrically by measuring the loss of dry mass of demineralized dentin matrices over time, and by measuring the amount of hydroxyproline (HYP) released from the solubilized collagen into the incubation media [7-9]. Increasing concentrations of fluoride were also evaluated for their ability to inhibit a specific rhMMP-9 using the Sensolyte Generic MMP kit (AnaSpec, Inc. San Jose, CA, USA).

Methods and Materials

Specimen Preparation and Treatment

Fifteen extracted human third molars were obtained with patient’s informed consent under a protocol approved by the Human Assurance Committee of the Georgia Regents University. The teeth were stored at 4°C in 0.9% NaCl supplemented with 0.02% sodium azide (to inhibit antimicrobial growth) for no more than one month before use. The enamel and superficial dentin of each tooth were removed from the crown using a diamond-encrusted copper disk (Isomet saw, Buehler Ltd., Lake Bluff, IL, USA), by a horizontal section 1 mm below the deepest central groove. One-half millimeter thick disks of mid-coronal dentin were then prepared by moving the blade slightly less than 1 mm apical to the first section. Two dentin beams approximately 5 mm × 5 mm × 0.5 mm were obtained from each dentin disk to yield a total of thirty beams.

For complete demineralization, the beams were tumbled in 10 wt % phosphoric acid for 18 h at 4°C. Complete demineralization was determined by subjecting the beams to 3-point flexure. Mineralized beams have a stiffness of 19-20 GPa while completely beams have stiffness of 3.5 MPa [10]. The completely demineralized beams were then rinsed in deionized water (DW) for 2 h at 3-4°C, replacing the water solution every 30 min. To determine the initial dry mass, the beams were transferred to a 96-well plate and placed in a sealed plastic box containing anhydrous calcium sulfate (Drierite, W.A. Hammond Drierite Company, Xenio, OH, USA). The beams were desiccated to a constant weight within 24 h. The initial dry mass was measured to the nearest 0.001 mg on an analytical balance (MettlerXP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA).

After dry mass measurements, they were rehydrated for 1 h in deionized water and divided into six groups. Each beam (n=10) was then separately incubated in one of six different fluoride concentrations in incubation media, in individually labeled capped propylene tubes containing 500 μL of incubation media as follows: 1) Incubation media (IM, Control): 5 mM HEPES, 2.5 mM CaCl₂, 2H₂O, 0.02 mM ZnCl₂, pH 7.4; 2) IM with 75 ppm NaF; 3) IM with 150 ppm NaF; 4) IM with 300 ppm NaF; 5) IM with 450 ppm NaF and 6) IM with 600 ppm NaF. The capped tubes were placed in a shaking
water bath (Reciprocal Shaking Bath, Thermo Scientific, Marietta, OH, USA) (60 cycles/min) at 37°C for seven days. The pH of all six groups was measured before and after incubation, and was shown to remain at pH 7.4.

**Dry Mass Loss and Analysis of Incubation Media for Solubilized Collagen Peptides**

After seven days, each beam was removed from its test tube, rinsed with deionized water to remove buffer salts, and the dry mass re-determined following the same procedure described for baseline. A second index of matrix degradation over time was obtained by measuring the amount of collagen peptide fragments that were solubilized from the dentin beams into the media over seven days of incubation. Aliquots of 100 \( \mu \)L of each of the incubation media were collected from each tube, placed into individually labelled ampules, and diluted with an equal volume of 12 N HCl to give a final concentration of 6 N HCl. Ampules were flame-sealed using an ampule sealer (Ampulmatic, Biosciences Inc., PA, USA). The media was hydrolyzed by heating the ampules to 120 °C in an oil bath for 18 h. After hydrolysis, the ampules were opened and placed in large glass desicicators containing anhydrous calcium sulfate and sodium hydroxide pellets to trap the HCl vapor released as the hydrolysate evaporated to dryness in a mild vacuum. When dry, the hydroxyproline (HYP) content of the hydrolysate was analyzed using the method of Jamall et al.\(^{11}\) in a spectrophotometer (Model UV-A180, Shimadzu, Tokyo, Japan) at 558 nm. The measured amount of HYP was used to estimate the amount of solubilized (i.e. degraded) collagen, assuming that 90% of the dry mass of the demineralized dentin beams consisted of type I collagen and that the dentin collagen contains 9.6 mass % of HYP. For each specimen, the solubilized collagen was expressed as \( \mu \)g of HYP/mg dry dentin.

**Inhibition of rhMMP-9 Activity**

This assay involved measurement of rhMMP-9 enzyme activity at various fluoride concentrations, and employed rhMMP-9 proenzyme (EMD Millipore; catalog No. PFO 38) and the SensoLyte Generic MMP colorimetric assay kit (Cat#72095) from AnaSpec, Inc. (San Jose, CA, USA) for determining the potential screening anti-MMP activity of NaF. The assay involves incubating a constant concentration of rhMMP-9 with a proprietary chromogenic substrate. The latter is a thiopeptolide that is cleaved by the MMPs and collagenases to release a sulfhydryl group. The sulfhydryl group reacts with 5,5'-dithiobis(2-nitrobenzoic acid) to produce the colored reaction product (2-nitro-5-thiobenzoic acid) which can be detected at 412 nm.

The thiopeptolide substrate solution was diluted to 0.2 mM with the supplied assay buffer in a 1:50 volume ratio. The 92-kDa rhMMP-9 was activated with 10 \( \mu \)g/mL of trypsin at 37°C for 2 h immediately before the experiment to generate its 68-kDa active form. The trypsin was then inactivated with 100 \( \mu \)g/mL trypsin inhibitor. In a 96-well-plate, fifty microliters of assay buffer were added to 4 background wells and 10 ul to experimental background wells. Ten microliters of activated rhMMP-9 enzyme was added to 4 positive control wells and experimental wells. Then, 40 ul of the five different concentrations of fluoride media were added to the appropriate wells, followed by 50 ul of substrate to each well. After placement of the substrate in each well, the MMP activity was determined by measuring their absorbance at 412 nm in a plate reader for 1 h, taking readings every 10 min (Synergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) against appropriate blanks.

**Statistical Analysis**

Dry mass loss, HYP release, and MMP inhibition data were analyzed separately. Dry mass loss and MMP inhibition were analyzed using a one-way ANOVA, followed by a Student-Newman-Keuls-post-hoc test. Because the HYP release data did not pass the normality test, it was log transformed and then analyzed by a one-way ANOVA, followed by a Holm-Sidak post-hoc test. Regression analysis was used to test the correlations between % loss of dry mass and F- concentration in the medium, and between hydroxyproline release and F- concentration. SigmaStat version 3.11, 2004 (Systat Software, Inc. San Jose, CA USA) was used for data analysis. The significance level was set to p<0.05 for all tests.
Results
The loss of dry mass (%) from dentin beams incubated in control media versus increasing concentrations of fluoride-containing media over seven days is shown in Figure 1. All concentrations of fluoride showed a significant reduction in mass loss relative to the control (p<0.05). The control beams incubated in fluoride-free media lost 17.7% of their dry mass over 7 days. When 75 ppm of fluoride was added to the incubation medium for 7 days, the loss of dry mass was only 10.2%, a significant reduction in mass loss compared to the control group (p<0.05). As the concentration of fluoride in the media continued to increase, the mass loss continued to decrease with 150, 300, 450 and 600 ppm demonstrating 9.1, 6.5, 5.7 and 4.5% dry mass loss, respectively.

When each group was compared to the other, concentrations of fluoride from 75 up to 450 ppm decreased mass loss over 7 days to 10.2, 9.1, 6.5, and 5.7%, respectively. Beams stored in 600 ppm F lost the least dry mass, 4.5%, of all groups (p<0.05). However, based on the statistical analysis, 600 ppm only differed from 75 ppm. While 600 ppm was statistically comparable to the other concentrations, they were not significantly different because of their relatively large standard deviations.

The release of HYP from solubilized collagen peptides is shown in Figure 2. Addition of fluoride to the incubation medium produced a significant decrease in HYP release (p<0.001). When the media was hydrolyzed to release all of the amino acids from solubilized collagen peptides, the concentration of HYP (11.09 μg/mg dentin) was significantly lower than all of the other groups. All other incubation groups (i.e. 75 to 600 ppm F) released less HYP than the control group, indicating that the higher the concentration of fluoride, the less release of HYP as a marker for the solubilization of collagen peptides. Fluoride concentrations above 300 ppm did not significantly lower HYP release any further than did 300 ppm F. Only the two lowest concentrations of fluoride could be tested for rhMMP-9 inhibition using the SensoLyte kit (Figure 3). While the 75 ppm only inhibited rhMMP-9 by 6.5%, the 150 ppm concentration inhibited rhMMP-9 by 79.2%. The 300 ppm, 450 ppm, and 600 ppm F concentrations precipitated the MMP substrate from solution, thus these results could not be included.

Regression analysis revealed a significant (p<0.005) negative exponential correlation between loss of dry mass and media F concentration (Figure 1, insert). Similarly, a highly significant negative experimental relationship was seen between the release of HYP by dentin beams and F concentration in the medium (Figure 2, insert).

Discussion
Because increasing concentrations of media fluoride inhibited loss of dry mass, solubilization of insoluble collagen and rhMMP-9 activity, the test null hypothesis that “fluoride does not prevent collagen degradation of dentin matrices or the solubilization of collagen peptides from dentin matrices” must be rejected. The loss of dry mass of completely demineralized dentin beams is a simple but sensitive method for quantifying the ability of endogenous dentin proteases to solubilize the insoluble collagen [7] in the dentin matrix.

Dentin matrices contain MMP-2, MMP-3, MMP-8, and MMP-9 [3, 12-14]. Dentin matrix also contains cysteine cathepsins [15-18]. Of the many cathepsins, only cathepsin K has been shown to have telopeptidase and collagenase activity [19]. Of the MMPs in dentin, only MMP-8 is a true collagenase, although MMP-2 can cleave type I collagen under some conditions. MMP-2 and 9 are both telopeptidases that can solubilize ICTP telopeptide fragments from insoluble dentin collagen [20, 21]. Cathepsin K is both a telopeptidase (it releases CTX telopeptide fragments [21] and a true collagenase [22].

Kato et al reported that 200 ppm F completely inhibited both pro and active forms of MMP-2 and 9 from human saliva, but that the inhibition was time-dependent [6]. They inhibited MMPs while they were bound to SDS-PAGE gels. In the current study, the endogenous dentin matrix MMPs remained bound to the matrix as they are in vivo. Thus, F inhibits both soluble and matrix-bound MMPs. Our work, using demineralized dentin beams known to contain MMP-2, 8, and 9, revealed that 150 ppm F inhibited the loss of dry mass up to by 51.3% (Figure 1, compare IM versus 150 ppm F mass loss). When rhMMP-9 was incubated with 150 ppm F, the activity of the enzyme was inhibited 79% within a few minutes (Figure 3). This confirms the work of Kato et al and extends it to collagen-based extracellular matrices. Thus, this confirms work by Mai et al that fluoride must be added to the list of MMP inhibitors [23].
Figure 3. Percent inhibition of rhMMP-9 activity by increasing concentrations of fluoride using the Sensolyte Generic MMP assay kit. Heights of bars are the means; error bars indicate ± 1 SD. N = 10. Groups identified by different letters are significantly different (p<0.05).

Nakajima et al [24] compared the durability of resin-dentin bonds made with ClearFil SE Bond to those made by what is now marketed as ClearFil Protect SE. The difference between them is that ClearFil SE Bond contains no antimicrobial MDPB in its primer and it contains no F– in its adhesive. In that study, they bonded some specimens with SE primer plus Protect adhesive containing F-. That group showed no change in microtensile bond strength (μTBS) of SE primer plus Protect adhesive over 6 or 12 months, while in the SE primer and SE adhesive (Fluoride-free) group, the μTBS fell significantly over time. At that time, the authors concluded that decreases in bond strength were water-dependent, but could be prevented using the fluoride-containing resins. Similar results were obtained by Shinohara et al [25, 26], but the mechanism responsible was never identified. Today, we speculate that it is due to F release from the adhesive of Clearfil Protect SE that may diffuse into the underlying hybrid layer inhibiting the MMPs in dentin, thereby protecting the collagen fibrils in the hybrid layer.

Matrix metalloproteinases depend on calcium (Ca++) to maintain their three dimensional configuration for enzymatic activity. Ethylenediamine tetraacetic acid (EDTA) is commonly used as an MMP inhibitor by chelating calcium/zinc to inactivate MMPs [27]. It is possible that 75-600 ppm (4-32 millimoles/L Fluoride) could complex not only with ionized calcium bound to the enzyme, but also with calcium in the incubation medium to form tightly complexed CaF2. The incubation medium contained 2.5 millimoles/L of Ca++ and 0.02 millimoles of Zn++ plus, 5 mM HEPES (pH 7.4). The highest concentration of fluoride used in the medium, 600 ppm = 31.6 millimoles/L was far in excess of the amount of calcium 2.5 millimoles/L in the media. Therefore, 600 ppm F may have formed CaF2 complexes with all the calcium in the media and the dentin beams. We speculate that most of the MMP activity was completely inhibited at F concentration greater than 600 ppm. Kato et al [6] showed that the inhibition of MMPs by fluoride is partially reversible at 250-1500 ppm F. If additional calcium ions had been added to the incubation medium, we might have been able to reverse the F– induced inhibition. This is a feature of the experiments that may be remedied in future studies.

When we attempted to measure ICTP and CTX telopeptides in the incubation medium by ELISA assays as an index of total MMP activity, the assay did not work. Fluoride apparently interferes with both ICTP and CTX ELISAs (data not shown).

Since EDTA does not inhibit cathepsin activity, fluoride ions may not inhibit dentin matrix cathepsin activity. Further research should test that idea.

Conclusion
Within the limitations of this in vitro study, increased sodium fluoride concentrations in incubation medium significantly decreased degradation of demineralized dentin over 7 days. This may explain the increased durability of resin-dentin bonds seen when fluoride-containing adhesive are used [24, 25].

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