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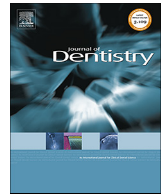

Stannous chloride and stannous fluoride are inhibitors of matrix metalloproteinases

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ABSTRACT

Objectives: Matrix metalloproteinases (MMPs) in dentin and saliva can degrade collagen. Divalent metals are known inhibitors of MMPs, but stannous – such as in the form of stannous chloride ($SnCl_2$) or stannous fluoride (SnF_2) – is yet to be tested for a possible inhibitory effect. In this study, we tested the inhibitory effect on the proteolytic activity of MMP-2 and MMP-9.

Methods: Sodium chloride (NaCl), sodium fluoride (NaF), and chlorhexidine (CHX) were used as controls. Gelatin zymography was performed with recombinant human MMP-2 and MMP-9. $SnCl_2$, SnF_2, NaF, NaCl, and CHX were included either in the incubation buffer (M1) or added to the recombinant MMPs (M2) before the MMPs were analyzed using zymography. Furthermore, the effect of $SnCl_2$, SnF_2, and NaF on the enzymatic activity of MMP-2 and MMP-9 was measured in human dentin either before or after acid etching using 37% phosphoric acid. The effect of $SnCl_2$, NaF, and CHX on the viability and of $SnCl_2$ and NaF on the proliferation of human gingival fibroblasts and L929 mouse fibroblasts was also determined.

Results: For M1, inhibitory concentrations (w/v%) of $SnCl_2$ 0.5% and 0.5%, SnF_2 0.25% and 0.12%, NaF 0.12% and 0.5%, CHX 0.012% and 0.05%, were observed for MMP-2 and MMP-9, respectively. NaCl had no inhibitory effect. For M2, $SnCl_2$ 0.007% and 0.12%, and SnF_2 0.03% and 0.5%, inhibited MMP-2 and MMP-9, respectively. NaF, NaCl and CHX had no effect. The enzymatic activity was slightly reduced when $SnCl_2$ and NaF were applied on dentin before the acid attack. Regarding cell viability and proliferation of the cells after stimulation with the respective substances, NaF showed almost no effect, $SnCl_2$ appeared to increase viability and proliferation of the cells, and CHX decreased the viability of cells.

Conclusions: Stannous ions caused a direct inhibition of the matrix metalloproteinases, whereas F^- only had an inhibitory effect when added to the zymography buffer.

Clinical significance: Inhibition of MMPs using $SnCl_2$ and SnF_2 could play an important role in the prevention of dental erosion and caries. However, the clinical relevance of these findings needs to be proven.

1. Introduction

Acid attacks due to caries processes or dental erosion result in demineralized dental hard tissue [1,2]. Increasing the pH leads to the termination of the demineralization process; furthermore, remineralization can take place, especially in the presence of calcium and phosphate together with fluoride [3]. Maintaining the demineralized organic matrix until remineralization can occur reduces the amount of tissue loss in dentin. This applies both to erosion [4] and dental caries [5]. However, even if the demineralization of enamel and dentin by extrinsic acids has stopped, degradation of the exposed collagen in dentin can occur as a result of endogenous collagenases, including matrix metalloproteinases (MMPs) [6].

MMPs are zinc- and calcium-dependent enzymes that regulate the physiological and pathological metabolism of collagen-based tissues [7]. MMPs are secreted as inactive proenzymes and are activated in the tissue by cleavage of the propeptide [8]. MMPs from dentin or saliva are activated by a low pH of 4.5; nevertheless, they are not able to degrade the dentin organic matrix at acidic pH [9]. However, as soon as the pH returns to normal levels, MMPs are activated and degrade the demineralized collagen-rich organic matrix that remained on the dentin after the acid attack [10].

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MMP-2 and MMP-9 (gelatinases) [11], MMP-8 (collagenase) [12], MMP-3 (stromelysin) and MMP-20 (enamelysin) [13] have been found in dentin. The main proteolytic enzymes in dentin are the gelatinases MMP-2 and MMP-9 [14,15], and they are easily detected using the gelatin zymography assay, which has a detection sensitivity of up to 10 pg for MMP-2 and MMP-9 [15]. This method allows the detection of any inhibitory effect of various substances on MMP-2 and MMP-9. Several experimental approaches to inhibiting MMPs have already been investigated. MMP inhibitors, e.g. green tea's epigallocatechin-gallate, ferrous sulfate and chlorhexidine, in solutions, gels and dentifrices showed a protective effect against dentin erosion and erosion plus abrasion [10]. Furthermore, metal salts such as $ZnSO_4$ strongly inhibit MMP-2 and MMP-9 activity, and $CuSO_4$ has been shown to inhibit MMP-2 activity isolated from inflamed gingival tissue [16].

Polyvalent metal ions, such as stannous, are also frequently used in dentistry, but their inhibitory effect on MMPs has yet to be investigated. Stannous-containing substances show an anti-erosive [17], as well as anti-cariogenic [18] potential because stannous has a strong affinity for mineralized dental tissues. Consequently, it promotes a protective effect owing to the mechanical formulation of a surface layer with increasing acid resistance [19]. In addition to this layer, it has also been suggested that, when the acid attack exposes the demineralized collagen-rich organic matrix in dentin, stannous may also be partly retained by the organic matrix [20]. Because stannous remains, in part, on the organic matrix, any MMP-inhibitory effect it produces would also play a protective role against further dentin demineralization.

Therefore, the aim of this study was to investigate the effect of stannous chloride on MMP-2 and MMP-9 in comparison to the effects of the known MMP inhibitors NaF and CHX. To rule out the possibility that the effect is produced by fluoride or sodium, we also used SnF_2 and NaCl in the experimental procedure. Furthermore, when previous studies have analyzed the inhibitory effect of different substances, these substances were mainly added to the zymography buffer and not directly to the MMP-containing samples [16,21]. We therefore investigated the MMP-inhibitory effect of these compounds by adding them directly to the MMP samples, as well as to the buffer.

2. Materials and methods

2.1. Gelatin zymography

In one set of experiments (M1), the MMP inhibitors were added to the buffer, and in another set of experiments (M2), the MMP inhibitors were added directly to the supernatants containing the MMPs.

For M1, human recombinant matrix metalloproteinase-2 (MMP-2) and MMP-9 (both ProSpec, East Brunswick, NJ, USA) at a final concentration of 3 mM were diluted in Laemmli buffer at a 1:4 dilution and run on 10% SDS-PAGE gels containing 1% gelatin (Sigma-Aldrich, Buchs, Switzerland). Gels were incubated for 30 min in buffer A (50 mmol/L Tris HCl, 2.5% Tween 80, 0.02% (w/v) NaN_3, pH 7.5) at room temperature, followed by incubation for 30 min in buffer B (50 mmol/L Tris HCl, 2.5% Tween 80, 0.02% (w/v) NaN_3, 1 µm $ZnCl_2$, 5 mmol/L $CaCl_2$). Gels were cut into strips, each containing one lane of rhMMP-2 and rhMMP-9, and incubated in buffer C (50 mmol/L Tris HCl, 5 mmol/L $CaCl_2$, 1 µM $ZnCl_2$, 0.02% (w/v) NaN_3) at 37° overnight. Buffer C contained $SnCl_2$, NaF, SnF_2, or NaCl (1%, to 0.007%), CHX (0.1% to 0.0007%), or no additional substance. The molecular weight of the substances used is given in Table 1. The range of concentrations used for possible inhibition was determined in preliminary experiments. The next day, gels were stained for 60 min in 0.1% Coomassie Brilliant Blue in 10% acetic acid / 10% methanol at room temperature. After destaining, MMP-2 and MMP-9 appeared as a clearance zone within the stained gel. The experimental procedure is shown in Fig. 1A.

For M2, rhMMP-2 and rhMMP-9 at a final concentration of 3 mM were incubated with $SnCl_2$, NaF, SnF_2, NaCl (1%–0.007%), CHX (0.1%–0.0007%), or distilled water (negative control). Then, samples were diluted in Laemmli sample buffer and gelatin zymography was performed as described above. Buffer C did not contain the test substances. The experimental procedure is shown in Fig. 1B.

Table 1
Substances tested for their inhibitory potential on MMP-2 and MMP-9, their molecular weight and the molar concentration in a 1% weight per volume solution.

Substance	MW	1%w/v in mmol
$SnCl_2$	189,61	52,7
SnF_2	156,69	63,8
NaCl	58,44	171,1
NaF	41,99	238,2
CHX	505,446	2,0

2.2. MMP activity testing

To further simulate the clinical situation and to investigate also matrix bound MMPs, human dentin samples were treated with $SnCl_2$, NaF, or SnF_2 and enzyme activity was measured after a simulated acid attack (InnoZymeTM Gelatinase MMP-2/MMP-9 Activity Assay kit, Fluorogenic, Merck, Germany). Two methods were used in this experimental setting. In the first setting, etching the samples was performed first and then the treatment with $SnCl_2$, NaF, or SnF_2 was carried out and in the second setting the treatment with $SnCl_2$, NaF, or SnF_2 was performed first and then the etching was carried out. Human third molars were directly frozen after extraction and used for the experiments within one week. Informed consent by the donors was obtained (Ethics Committee of the Medical University Vienna; EK Nr. 1065/2013). Directly before the experiments, discs of two mm thickness were cut out of the molars and 20 round samples of dentin with a diameter of about 5 mm were prepared. Half of the samples were directly etched with 37% phosphoric acid for 15 s., rinsed with deionized water and put in a solution of $SnCl_2$, NaF, or SnF_2 (all 1%), or in deionized water for ten minutes. The other half of the samples were put in the solution first, rinsed with deionized water and etched thereafter. Dentin samples without any treatment acted as control. Directly afterwards, the dentin samples were placed in 96-well plates and 90 µl of Assay buffer was added in each well. After ten minutes 10 µl substrate solution was added to each well and samples were incubated at 37 °C. After two hours, the dentin samples were removed and the enzyme activity was measured using a fluorescence reader at an excitation wavelength of 340 nm and an emission wavelength of 405 nm.

2.3. Viability testing

Gingival fibroblasts, which may come in contact with any substance in the oral cavity, and a L929 cell line frequently used for cytotoxicity testing were used for experiments on cell viability. Human gingival fibroblasts (GF) were prepared from tissue grafts after approval (Ethics Committee of the Medical University Vienna; EK Nr. 631/2007) and after informed consent by the donor were obtained. Tissue explants were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Linz, Austria) and antibiotics (Invitrogen) at 37 °C, 5% CO_2, and 95% humidity. Fibroblasts that grew out from the explants were used for further experiments. Cells were suspended in growth medium at 30,000 cells/cm^2 and plated into culture dishes. The next day, cells were incubated in serum-free medium containing $SnCl_2$, NaF (both at 1%, 0.5%, 0.25%, 0.12%, 0.06%, 0.03%, 0.015% or 0.007%), CHX (at 0.1%, 0.05%, 0.025%, 0.012%, 0.006%, 0.003%, 0.0015% or 0.0007%), or no additional substance. After 10 min, cells were washed with phosphate-buffered saline (PBS), and serum-free media was added containing MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide, 0.5 mg/ml,

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