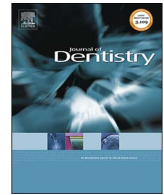
ARTICLE IN PRESS

Journal of Dentistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent



Full Length Article

Use of sodium trimetaphosphate in the inhibition of dentin matrix metalloproteinases and as a remineralizing agent

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A R T I C L E I N F O

Keywords:
Dentin
Matrix metalloproteinases
Protease inhibitors
Demineralization
Hardness
Tooth remineralization

A B S T R A C T

Objectives: Because of its ability to act as an antiproteolytic agent, the effect of sodium trimetaphosphate (STMP) against specific enzymes extracted from sound dentin and its performance under acidic challenge on demineralized dentin were investigated.
Methods: The antiproteolytic potential of STMP (0.5%, 1.0%, and 1.5%) was assessed in triplicate by zymography. For the evaluation of remineralization activity, 50 bovine-root dentin specimens were selected and randomly divided into 5 groups (n = 10). Three areas were determined for each specimen: 1) control (no treatment); 2) demineralized (artificial caries-like challenge); 3) treated (demineralized and subjected to pH-cycling for 7 days, and treated for 10 min with 1.5% STMP, 1.5% STMP + calcium hydroxide ($Ca[OH]_2$), 1.5% STMP + sodium fluoride (NaF), NaF, or deionized H_2O). The dentin specimens were analyzed for superficial hardness (SH) and cross-sectional hardness (CSH) at different depths (10, 30, 50, 70, 90, 110, and 220 µm) using a Knoop penetrator (10 g/10 s). Statistical analyses were performed with analysis of variance (ANOVA) and Tukey tests ($p < 0.05$).
Results: The zymographic analysis showed that 1.5% STMP promoted complete inhibition of gelatinolytic activity. Therefore, 1.5% STMP was investigated in association with supplemented calcium or fluoride; a combination of 1.5% STMP and $Ca(OH)_2$ significantly increased the mechanical properties of the treated dentin.
Conclusion: 1.5% STMP serves as an antiproteolytic agent against matrix metalloproteinases extracted from human dentin. Furthermore, when supplemented with $Ca(OH)_2$, 1.5% STMP may potentially induce remineralization.
Clinical significance: STMP can be introduced as a novel strategy that combines enzymatic inhibition and remineralizing potential, which can serve to strengthen dentin and improve stability. STMP may have potential in the treatment of demineralized dentin lesions, especially when supplemented with calcium.

1. Introduction

The dynamic changes in the balance between demineralization/remineralization processes can cause mineral loss from the enamel or dentin. Acids produced by oral microorganisms, followed by degradation of the collagen matrix by proteolytic enzymes, guide dental caries. The acid diffuses through the calcified dental tissue, resulting in dissolution of the apatite crystals, creating a partially demineralized zone of dentin, as well as activating endogenous enzymes known as matrix metalloproteinases (MMPs) [1,2]. MMPs are metallic ion-dependent enzymes with catalytic activity; they are able to degrade almost all extracellular matrix components, including collagen [1–3]. Therefore, MMP-2 and -9 activation is related to collagen degradation in dental caries lesions [1,2]. This scenario stimulated the search for strategies to minimize this undesirable effect, and the use of enzyme inhibitors has been one of the most-investigated areas in recent years [4–7]. The present investigation focused on the presence and activity of MMPs in mineralized tissues. These enzymes were extracted from human dentin powder, allowing a better understanding of the pathophysiological processes in which the degradation of the organic matrix of dentin

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http://dx.doi.org/10.1016/j.jdent.2017.10.009
Received 12 July 2017; Received in revised form 20 October 2017; Accepted 23 October 2017
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occurs, as well as its inhibition.

The preservation and stability of the collagen chain in dentin is essential during the remineralization process [8,9], as it serves as a scaffold for mineral deposition in the presence of noncollagenolytic proteins (dentin matrix protein 1 [DMP1], dentin phosphophoryn [DPP], and DMP2) [10]. These proteins are necessary for the regulation of dentin mineralization and to control the dimension and order of the deposition of apatite on the organic matrix, since they bind to collagen fibrils and may contribute to dentin biomineralization [10,11]. This approach is mediated by specific bioactive agents that enhance and reinforce dentin by localized modification of biochemical and biomechanical properties [12].

It has been speculated that sodium trimetaphosphate (STMP, $Na_3P_3O_9$), which has been widely used as chemical phosphorylation reagent in the alimentary industries [13] has the potential to phosphorylate collagen type I. One hypothesis for this activity is that the introduction of one phosphate group on the collagen surface of the demineralized dentin could induce its remineralization [14]. Li and Chang [14] have demonstrated that chemical phosphorylation of collagen is a possible strategy for the biomimetic-directed growth of apatite crystals. In a previous study, polyvinylphosphonic acid (PVPA), one biomimetic analogue of STMP used in mineralization strategies [15,16], showed anti-MMP-9 potential activity [17].

Moreover, Zhang et al. [18], studying the formation of calcium phosphate crystals on type I collagen, observed that treatment with STMP supplemented with calcium hydroxide ($Ca[OH]_2$) can be a feasible method to remineralize type I collagen from eggshell membrane. Therefore, surface treatment of demineralized dentin with $Ca(OH)_2$ might also induce remineralization, once Ca^{2+} promotes the development of calcium phosphate precursor. The calcium phosphate precursor, in turn, promotes conditions conducive to mineral crystal growth that are necessary for remineralization [19,20]. An in vitro study [21] demonstrated that STMP can act by contributing to the selective permeability of enamel by favoring F^- diffusion and, consequently, remineralization.

Therefore, the use of the STMP may be an innovative strategy to stabilize and strengthen the dentin by its interaction with noncollagenolytic proteins and by remineralization, decreasing the biodegradation rate and increasing mineral nucleation [11–13,22]. The purpose of this study was to investigate (1) the STMP antiproteolytic potential against human-purified MMPs-2 and -9, and enzymes extracted from sound dentin; and (2) its capacity to promote caries-like dentin remineralization. The null hypotheses to be tested were that STMP (1) could not inhibit the gelatinolytic activity of these enzymes; and (2) could not enhance the mechanical properties of demineralized dentin.

2. Material and methods

2.1. Experimental design

This study was approved by the Institutional Review Board of Bauru Dental School, University of São Paulo, Brazil (process number 49810315.1.0000.5417). The antiproteolytic potential of STMP (Sigma-Aldrich Co., St. Louis, MO, USA) against MMPs was assessed by zymographic analysis using human purified MMP-2 and MMP-9, and proteins extracted from sound human radicular dentin. STMP was studied at four concentrations: 0%; 0.5%; 1.0%, and 1.5%, and the variable response was the antiproteolytic effect of STMP against dentin gelatinolytic activity. The STMP concentrations that had the capacity to completely inhibit the enzymes' activities were further analyzed for their remineralizing potential according to changes on the mechanical properties. Five solutions were then studied: 1.5% STMP: 1.5% STMP + $Ca(OH)_2$; 1.5% STMP + sodium fluoride (NaF); NaF; and distilled water; the variable responses were superficial hardness (SH) and cross-sectional hardness (CSH).

2.2. Protein extraction from human dentin

Proteins were extracted from human dentin to assess the effect of STMP as a potential MMP inhibitor. This study used purified human MMP-2 (Calbiochem, Millipore Corp., Billerica, MA, USA) and purified human MMP-9 (Abcam, Cambridge, MA, USA), as well as proteases extracted from sound dentin. Samples were subjected to zymography, in triplicate. Ten freshly extracted human molars were obtained from young individuals (18–30 years of age), cleaned, and the crowns completely separated from the roots. The teeth were then ground free of cementum and pulpal soft tissue, frozen, and the dentin fragments were triturated to fine powder in a ball mill (MM401; Retsch, Newtown, PA, USA) at 30 Hz for 3 min. The resultant powder was demineralized in 1% aqueous H_3PO_4 for 10 min, centrifuged for 20 min at 4 °C (20,800*g*) and then resuspended in 1 mL extraction buffer (50 mM Tris-HCl, pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, and 0.1% Triton X-100) for 24 h [23]. The sample was then sonicated for 10 min (approximately 30 pulses) and centrifuged for 20 min at 4 °C (20,800*g*); the supernatant was removed and recentrifuged. The protein content was further concentrated by means of Amicon tubes (Amicon Ultra – 15 Centrifugal Filter Units; Merck Millipore, Tallagreen, Ireland) at 4 °C. Total protein concentration of the dentin extract was determined by Bradford assay [24].

2.3. Gelatin zymography

Samples were diluted in Laemmli sample buffer in a 4:1 ratio and electrophoresed under nonreducing conditions on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/mL gelatin from porcine skin (Sigma Chemical, St. Louis, MO, USA). Prestained, low-range molecular weight SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular weight markers (MWMs). After electrophoresis, the gels were washed for 1 h in 2% Triton X-100 and were incubated for 18 h at 37 °C in activation solution (50 mM Tris HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% [weight/volume] NaN_3; pH 7.4) containing 0.0% (positive control), 0.5%, 1.0%, or 1.5% STMP, or 2 mM 1,10-phenanthroline [25] (specific MMP inhibitor; negative control). After incubation, the gels were stained in 0.1% Coomassie Brilliant Blue R-25 for 30 min and destained in a solution of 30% methanol, 10% acetic acid, and 60% water. Gelatinolytic activity was detected as clear bands. The gels were scanned (Image Scanner; Amersham Biosciences, Uppsala, Sweden) and evaluated by densitometry using the software ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). STMP inhibition was expressed as a percentage according to the activity of MMPs without pretreatment with STMP (control). Experiments were performed in triplicate.

2.4. Specimen preparation and caries-like lesions

Only the STMP concentration that completely inhibited enzyme activity (1.5% STMP) was used for hardness tests. In total, 100 dentin specimens (4.0 × 4.0 × 6.0 mm) were prepared from buccal cervical root bovine incisors using a diamond saw (Isomet 1000; Buehler, Lake Bluff, IL, USA) under water cooling. Surfaces were wet-polished with 600- and 800-grit SiC paper (Extec Corp, Enfield, CT, USA) at low speed and with 1200-grit SiC paper at high speed using a polishing machine (AROPOL E; Arotec Industria e Comércio Ltda, Cotia, SP, Brazil). The final polishing was performed with 1-μm diamond paste and wet felt wheels (Extec Corp., Enfield, CT, USA).

The baseline SH was measured to select 50 dentin specimens with 32 ± 2 KHN for the experiments. These specimens were distributed among 5 groups (n = 10).

To maintain reference surfaces for analysis, each specimen was divided into three areas (Fig. 1):

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