UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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# The use of sodium trimetaphosphate for matrix metalloproteinase inhibition, remineralization and bonding to dentin

# O uso de trimetafosfato de sódio sobre a inibição de metaloproteinases e na remineralização e adesão à dentina

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Tese apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Dentística.

Orientadora: Profa. Dra. Linda Wang

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## ABSTRACT

**The use of sodium trimetaphosphate for matrix metalloproteinase inhibition, remineralization and bonding to dentin**

The adhesive process to dentin substrate depends on the condition determined by the combined action of the mineral loss and the endogenous enzymes activity. Thus, considering a more complete therapeutic approach, sodium trimetaphosphate (STMP) may be a novel strategy that conciliates the remineralization potential to the promotion of dentin strengthening and its stability, possibly directing mineral nucleation and controlling the rate of biodegradation. In this study, the effect of STMP was evaluated in 2 studies. In study 1, different concentrations of STMP (0.5, 1.5, 3.5 and 5%) were investigated to assess their anti-proteolytic capacity on human purified MMPs-2 and -9 by zymography. Afterwards, only the concentrations (1.5, 3.5 and 5%) that showed total inhibition of both MMPs were used to evaluate their remineralizing capacity in dentin substrate submitted to artificial cariogenic challenge, through surface hardness (SH) and cross-sectional hardness (CSH). In study 2, based on the previous results, the capacity of the 1.5% STMP associated or not with NaF or $Ca(OH)_2$ solutions in improving the dentin bond strength of a universal adhesive system was evaluated by the microtensile test . Thus, these studies suggest that 1.5% STMP is an effective inhibitor of collagen degradation mediated by purified human MMPs-2 and -9. In addition, demineralized and treated dentin with 1.5% STMP supplemented with $Ca(OH)_2$ may induce remineralization. Thus, the use of STMP can be introduced as a new strategy that combines enzymatic inhibition and remineralization potential, reestablishing favorable conditions to affected dentin. These evidences support perspectives of therapies to restructure dentin and propose feasible and promising clinical strategies.



**Key-words:** Dentin. Matrix metalloproteinase. Protease inhibitor. Hardness. Tooth remineralization

## RESUMO

O processo adesivo ao substrato dentinário depende da condição determinada pela ação combinada da perda mineral e atividade de enzimas endógenas. Deste modo, considerando uma abordagem terapêutica mais completa, o trimetafosfato de sódio (STMP) pode ser uma estratégia inovadora que concilia o potencial remineralizador à promoção do fortalecimento da dentina e sua estabilidade, possivelmente direcionando a nucleação mineral e controlando a taxa de biodegradação. Neste trabalho, o efeito do STMP foi avaliado em 2 estudos. No estudo 1, diferentes concentrações de STMP (0,5; 1,5; 3,5 e 5%) foram investigadas para avaliar sua capacidade anti-proteolítica sobre as MMPs-2 e -9 purificadas humanas, por zimografia. Posteriormente, somente as concentrações (1,5; 3,5 e 5%) que apresentaram capacidade de inibição total de ambas MMPs foram utilizadas para avaliar sua capacidade remineralizadora em substrato dentinário submetido ao desafio cariogênico artificial, através da dureza de superfície (DS) e longitudinal (DL). No estudo 2, baseado nos resultados anteriores, foi avaliada a capacidade do STMP à 1,5% associado ou não a soluções de NaF ou $Ca(OH)_2$ em melhorar a resistência de união à dentina de um sistema adesivo universal pelo teste de microtração. Desta forma, estes estudos sugerem que o STMP à 1,5% apresenta-se como um inibidor eficaz da degradação do colágeno mediada por MMPs-2 e -9 humanas purificadas. Além disso, a dentina humana desmineralizada e tratada com STMP à 1,5% suplementada com $Ca(OH)_2$ pode induzir à remineralização. Assim, o uso de STMP pode ser introduzido como uma nova estratégia que combina inibição enzimática e potencial de remineralização, reestabelecendo condições favoráveis a partir de uma dentina afetada. Estas evidências sustentam perspectivas de terapias para reestruturar a dentina e propor estratégias clínicas factíveis e promissoras.



**Palavras-chave:** Dentina. Metaloproteinases da Matriz. Inibidores de Proteases. Dureza. Remineralização Dentária.

# SUMMARY

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# 1 INTRODUCTION

# 1 INTRODUCTION

Dental caries disease determine the development of lesions, as a result of two main stages distinctly observed microscopically: acidic dissolution of dentin minerals promoted by bacterial biofilm followed by the degradation of dentinal matrix with continuous infiltration of bacteria or their products into the intertubular dentin (Ten Cate, 1998; Buzalaf et al., 2015; Tjaderhane et al., 2015).

This scenario is a consequence of the disequilibrium in the dynamic process of de- and remineralization of the oral environment on enamel and dentin. The use of fluoride is a well-established method reported in the literature able to prevent and inhibit caries lesions specially for enamel and in early stagies (Featherstone, 1994; Mukai et al., 2001). $H^+$ ions can penetrate into a porous substrate even in the presence of high amount of fluoride, indicating the complexity of different mechanisms that can occur simultaneously in dentin caries establishment, specially in dentin tissue (Ten Cate et al., 1995; Moron et al., 2013).

Dentin is a complex mineralized tissue composed (in weight) of 70% of mineral, 20% of organic materials and 10% of fluids, approximately (Linde, 1989). Ninety per cent of the organic matrix is made up by collagen type I fibrils and the rest consist in phosphoproteins and proteoglycans (non-collagenolytic proteins) (Linde, 1989).

According to recent evidences, the isolated contribution of bacteria-derived enzymes to dentin matrix degradation during the carious process may be less important than initially thought (Tjaderhane et al., 2015). Knowlegde regarding mechanisms of caries-affected dentin degradation highlight the importance of collagenous and non-collagenous proteins and specific proteolytic enzymes in this process (Vidal et al., 2014; Padovano et al., 2015; Tjaderhane et al., 2015; Scaffa et al., 2017). This attractive area of research is stimulated by the evolution of the understanding of caries physiopathology, which present robust evidences of promissory clinical approaches based on the ability of dentin recovery even in deep caries lesions. These investigations support the minimal intervention dentistry (Frencken et al., 2012; Bjorndal et al., 2017).

The lactic acid produced by cariogenic microorganisms diffuse through the calcified dental tissue resulting in dissolution of the apatite crystals (Kim et al., 2010). This acid works dissolving some of interfibrillar crystals creating a partial demineralized zone of the dentin as well as activating endogenous enzymes that are able to degrade collagen, knowing as matrix metalloproteinases (MMPs) (Magalhaes et al., 2009; Kim et al., 2010; Toledano et al., 2010; Buzalaf et al., 2012).

In this process, endogenous enzymes known as intrinsic proteolytic enzymes of dentin like MMPs are activated, which favor even more the degradation of extracellular matrix (Tjaderhane et al., 1998; Santos et al., 2009; Toledano et al., 2010). Metalloproteinases, mainly the collagenolitic MMP-2 and -9, and the cysteine cathepsins (CCs) are the most investigated host-derived enzymes that are metal-ion–dependent (calcium and zinc) for its catalytic activity (Tjaderhane et al., 1998; Santos et al., 2009; Toledano et al., 2010; Nascimento et al., 2011; Vidal et al., 2014).

Overall, host-derived enzymes can hydrolyze most of the extracellular matrix protein (Aimes et al., 1995), specially highly cross-linked triple-helical collagen (type I) (Visse et al., 2003). As type I collagen is the most prevalent organic component of mature dentin, its degradation during caries progression can lead to a greater impact on the structural integrity of this tissue, even compromising the resin-dentin-bonded interface (Carrilho et al., 2007; Carrilho et al., 2007; Vidal et al., 2014). Among these enzymes, the MMP-2 and -9 activation are the most investigated ones related to collagen degradation in caries lesions (Tjaderhane et al., 1998; Santos et al., 2009; Toledano et al., 2010). In this way, enzyme inhibitors that minimize these constituents should also worth.

These evidences stimulated the search for strategies to minimize this effect. The use of inhibit agents of theses enzymes had been the most investigated area in the last years such as the use of chlorhexidine (Carrilho et al., 2007; Magalhaes et al., 2009; Giacomini et al., 2017). In this purpose, other agents were also investigated such as epicatechin gallate (ECG), dimethyl sulfoxide (DMSO) (Stape et al., 2016; Mehtala et al., 2017) and others (Tjaderhane et al., 2013; Vidal et al., 2014). The most agents used up to now aimed to inhibit the action of the reactivated enzyme and/or modify the collagen fibrils, protecting them (Mehtala et al., 2017; Stape et al., 2016; Carrilho et al., 2007b; Giacomini et al., 2017; Magalhaes et al., 2009). As caries induce significant alterations in dentin, investigations have been performed to address strategies to minimize the permanent damage to this tissue

(Gu et al., 2011; Scaffa et al., 2012; Zhang et al., 2012; Machado et al., 2015; Padovano et al., 2015).

Thus, the preservation and stability of dentin is not only important for providing its physiological structural framework (viscoelastic properties) but also defining ordered mineral deposition in the presence of non-collagenous proteins (NCPs), such as dentin matrix protein-1 (DMP-1) (Kuboki et al., 1977; Fusayama, 1979; Kuboki et al., 1981; Nijhuis et al., 2014; Padovano et al., 2015). It is well established that type I collagen matrix does not have the capacity to induce matrix-specific mineral formation by itself (Kawasaki et al., 1999; George et al., 2008). Furthermore, *in vitro* studies have demonstrated a lack of ordered mineralization of apatite on collagen fibril without additives (Saito et al., 1997; Bradt et al., 1999; He et al., 2004).

Based on the role of NCPs on dentin mineralization, biomimetic approaches to induce dentin remineralization have been proposed in which the preservation of the collagen fibrils and fostering of tissue remineralization in a structured way are the fundamental mechanisms (Liu et al., 2011). For that, the incorporation of phosphate groups into the collagen fibrils may stimulate nucleation and growth of hydroxyapatite by attracting calcium ions into this negatively charged dentin matrix.

DMP-1 is an acidic non-collagenous protein (George et al., 1993) known to be present in the matrix of mineralized tissue (dentin and bone) and to present high affinity for hydroxyapatite (HA) (Hohling et al., 1995; He et al., 2003b). In the dentin matrix, it has been identified as a key NCP in biomineralization (He et al., 2004). In this way, contemporary concept considers DMP-1 to exert an important role during this process (He et al., 2004; Nijhuis et al., 2014), as the preservation of the collagen fibrils and fostering of dentin remineralization in a structured way characterize the biomimetic strategy, improving the support for remineralization (Liu et al., 2011).

Within the dentin matrix, DMP-1 has highly phosphorylated serine and threonine residues, in which phosphate group could stabilize calcium ions resulting in prenucleation clusters (George et al., 2008). Consequently, guiding mineral deposition to the desired gaps regions areas within the collagen matrix, enhance the mechanical properties of the dentin extracellular matrix (He et al., 2003a; Demichelis et al., 2011; Nijhuis et al., 2014).

In this way, it is speculated that sodium trimetaphosphate (STMP, $Na_3P_3O_9$) (Figure 1), which has been widely used as chemical phosphorylation reagent in the food industry (Zhang et al., 2007; Leone et al., 2008) has potential to phosphorylate

type I collagen. STMP phosphorylates serine and threonine proteins present in DMP-1 (George et al., 2008). Since the phosphate group has high binding affinity to calcium ions (order of binding affinity of some functional groups to $Ca^{2+}$: phosphate> carboxyl> amide> hydroxyl) (Wang et al., 2001), its presence on the surface of organic template could trap calcium ions by electrostatic force and direct apatite nucleation within the gap zones of collagen fibrils (Wang et al., 2001; George et al., 2008). Thereby, one hypothesis would be that the introduction of one phosphate group on the collagen surface of the demineralized dentin could induce its remineralization. Li and Chang (Li et al., 2008), 2008, have been demonstrated that the chemically phosphorylation of the collagen is a possible strategy to the biomimetic directed growth of apatite crystals.

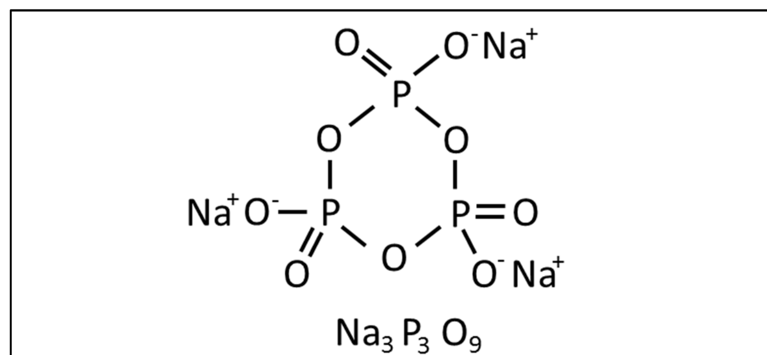


**Figure 1-** Sodium trimetaphosphate molecule structure (STMP-$Na_3P_3O_9$)

Due to all these modifications provoked by caries and other demineralization process on dentin, the therapeutic approach based on dentin bonding systems also can be impacted. Therefore, the analysis of the use of different agents on this purpose also need to be investigated along the performance on dentin bonding (Komori et al., 2009; Giacomini et al., 2017).

According to the exposed above, the purpose of this study was to investigate the STMP anti-proteolytic potential against MMP-2 and MMP-9 and its capacity to promote dentin remineralization in comparison and/or association to some other agents, exploring possible phenomena associated in these processes.

# 2 ARTICLES

# 2 ARTICLES

The articles below were written according to the Caries Research and Journal of Dentistry instructions and guideline for article submission, respectively.

- Article 1 - Sodium trimetaphosphate as a novel strategy for matrix metalloproteinase inhibition and dentin remineralization

- Article 2 - Effect of dentin biomodification using sodium trimetaphosphate on the microtensile bond strength.

## 2.1 Article 1

This article has been accepted by Caries Research (18-Oct-2017) (DOI: 484486), that is given all credits. This article receive the permission to be used as part of my Thesis (Annexe 3)

**Title Page**

Sodium trimetaphosphate as a novel strategy for matrix metalloproteinase inhibition and dentin remineralization

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Short title: Sodium trimetaphosphate as dentin remineralization agent

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Keywords: Dentin. Matrix metalloproteinases. Protease inhibitor. Hardness. Tooth remineralization.

**Declaration of Interests**

All authors affirm that there are no financial and personal relationships with other people or organizations that could inappropriately influence this work.

**Abstract**

The effect of sodium trimetaphosphate (STMP) as anti-proteolytic and remineralizing agent on demineralized dentin was evaluated in vitro. The inhibitory potential of STMP at 0.5, 1.5, 3.5 and 5% over recombinant matrix metalloproteinases (MMPs) MMP-2 and -9 was assessed by zymography. To investigate its remineralization potential, 40 bovine root specimens were obtained and subjected to a demineralization protocol to produce caries-like dentin lesions. After that, dentin surfaces were divided in 3 areas: 1-mineralized- no treatment; 2-demineralized; and 3-treated with STMP- demineralized and submitted to a pH-cycling associated or not with STMP (1.5, 3.5 or 5% STMP, 10 min treatment). After that, superficial hardness (SH) and cross-sectional hardness (CSH) were determined. Polarized light microscopy (PLM) was used to qualitatively evaluate mineralization within the caries-like lesions. The zymographic analysis showed that STMP solution is a potent inhibitor of the gelatinolytic activity of MMP-2 and -9 depending on the dose, since the lowest concentration (0.5%) partially inhibited the enzyme activity, while the higher concentrations completely inhibited their activities. Regarding remineralization effect, only 1.5% STMP solution enhanced both the SH and CSH. PLM showed that the area treated with 1.5% STMP presented similar birefringence compared to mineralized sound dentin. In conclusion, 1.5% STMP solution is effective as MMPs anti-proteolytic agent and to promote dentin remineralization.

**Introduction**

Alternative approaches to arrest caries and repair enamel and dentin have gained attention recently, especially strategies to minimize the permanent damage to the tissues and to stimulate their recovery [Gu et al., 2011; Padovano et al., 2015; Zhang et al., 2012]. This attractive area of research is stimulated by the evolution of the understanding of caries physiopathology. The isolated contribution of bacteria-derived enzymes to dentin matrix degradation during the carious process may be less important than initially thought [Tjäderhane et al., 2015]. Evidence regarding mechanisms of caries-affected dentin degradation highlight the importance of collagenous and non-collagenous proteins and specific proteolytic enzymes in this process [Padovano et al., 2015; Tjäderhane et al., 2015; Vidal et al., 2014].

Dentin endogenous enzymes like matrix metalloproteinases (MMPs) can hydrolyze most of the extracellular matrix content, including highly cross-linked triple-helical collagen (type I) [Aimes and Quigley, 1995; Visse and Nagase, 2003]. As type I collagen is the main organic component of mature dentin, its degradation during caries progression can lead to a greater impact on the dentin structural integrity.

The preservation and biostability of dentin is not only important for providing its physiological structural framework (viscoelastic properties) but also defining ordered mineral deposition in the presence of non-collagenous proteins (NCPs) [George and Veis, 2008; Nijhuis et al., 2014]. These NCPs are very acidic and contain multiple phosphorylation sites, which allow them to exert an important role during the biomineralization process by stabilizing calcium and phosphate ions and guiding mineral deposition within dentin matrix [George and Veis, 2008; He and George, 2004; Nijhuis et al., 2014]. Based on the role of NCPs on dentin mineralization, biomimetic approaches to induce dentin remineralization have been proposed in which the preservation of the collagen fibrils and fostering of tissue remineralization in a structured way are the fundamental mechanisms [Liu et al., 2011]. For that, the incorporation of phosphate groups into the collagen fibrils may stimulate nucleation and growth of hydroxyapatite by attracting calcium ions into this negatively charged dentin matrix.

Sodium trimetaphosphate (STMP, $Na_3P_3O_9$) has been widely used as chemical phosphorylation reagent in the food industry [Zhang et al., 2007]. In dentistry, it could serve as a potential chemical phosphorylation agent to mimic matrix phosphoproteins-induced remineralization once several in vitro studies have demonstrated that phosphate groups can be introduced onto type I collagen surface using STMP [Gu et al., 2011; Xu et al., 2010; Zhang et al., 2012]. Previous in vitro studies have demonstrated that STMP aids enamel remineralization possibly by creating a barrier that protects tissue surface and avoids acid diffusion as well as contributing to enamel selective permeability by favoring calcium and

fluoride diffusion [Favretto et al., 2013; Takeshita et al., 2011]. On the other hand, there is little information in the literature about the use of STMP on dentin [Gu et al., 2010; Liu et al., 2011] and no data regarding its potential to inhibit collagen degrading enzymes.

The study of new therapies to prevent and reduce caries progression in dentin should be more explored in order to avoid the organic matrix degradation and promote its remineralization, developing potential reparative clinical therapies. Considering the biomimetic approach, the use of STMP can be a novel strategy to stabilize and strengthen dentin matrix by interactions with NCPs to induce remineralization, decrease of biodegradation rates and increase the mineral nucleation [Liu et al., 2011; Zhang et al., 2012]. Therefore, the aim of this in vitro study was to investigate (1) the anti-proteolytic potential of STMP against MMP-2 and -9, and (2) its ability to enhance dentin remineralization. The null hypotheses to be tested were that STMP: (1) could not potentially inhibit the gelatinolytic activity of MMP-2 and -9; and (2) could not favor remineralization by enhancing the mechanical properties of superficial and deep demineralized dentin.

## Material and Methods

*Experimental design*

The MMPs anti-proteolytic potential of STMP was assessed by zymographic analysis. The factor in study was the STMP concentration in four levels: 0.5, 1.5, 3.5 and 5% and the variable response was the anti-proteolytic potential of STMP against MMP-2 and -9 activities. The STMP concentrations that had the capacity to completely inhibit the gelatinolytic activity of both proteases were selected to be further used to analyze dentin remineralization by changes on its mechanical properties. The factor in study was the treatment in four levels: 1.5% STMP, 3.5% STMP, 5% STMP and distilled water (DW) (control); and the variable responses were the superficial and cross sectional hardness.

*Gelatin Zymography*

To assess the effect of STMP (Sigma-Aldrich Co., St. Louis, MO, USA) as a potential MMPs inhibitor, purified human MMPs -2 (Calbiochem, Millipore Corp., Billerica, MA, USA) and -9 (Abcam, Cambridge, MA, USA) were diluted in sample buffer at a 4:1 ratio and electrophorized under non-reduced conditions on 10% sodium dodecyl sulfate-polyacrylamide gel (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 Laboratories, Hercules, CA, USA) were used as molecular-weight markers (MWM). After electrophoresis, the gels were washed for 1 h in 2% Triton X-100 and were incubated for 18 h at 37ºC in activation solution (50 mM Tris HCl, 5 mM $CaCl_2$, 1 μM

$ZnCl_2$, 0.02% (w/v) $NaN_3$, pH 7.4) containing 0.5, 1.5, 3.5 or 5% STMP. Positive control gel was incubated in similar conditions with activation solution containing 2 mM/L of 1,10 phenanthroline (specific MMPs inhibitor) [Toth et al., 2012]. After incubation, gels were stained in 0.1% Coomassie Brilliant Blue R-25 for 30 min and destained (30% methanol, 10% acetic acid diluted in DW). The gelatinolytic activity could be detected as clear bands. The gels were scanned (Imagescanner, Amersham Biosciences, Uppsala, Sweden) and bands evaluated by densitometry (arbitrary units) using the software ImageJ (Research Services Branch, National Institutes of Health, Bethesda, Maryland, USA). Experiments were performed in triplicate and the STMP inhibition was expressed as percentage according to the activity of MMPs without pretreatment with STMP (negative control).

*Microhardness Analysis*

This methodology was performed to evaluate the remineralization ability of STMP solutions that presented complete inhibition of MMPs -2 and -9 in zymography analysis. Forty dentin blocks (4.0 x 4.0 x 6.0 mm) were obtained from buccal cervical root bovine incisors using a slow speed 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, EUA) in low speed and 1,200-grit SiC paper in high speed using a polishing machine (AROPOL E - Arotec Industria e Comércio Ltda, Cotia, SP, Brazil). The final polishing was performed with 1µm diamond paste and wet felt wheels (Extec Corp. Enfield, CT, USA). The surface microhardness (SH) was measured at baseline to select specimens with 32 ± 3 KHN for further experiments. The measurements were performed using a microhardness tester (Instron 3342; Buehler, Chicago, IL, USA) and the software BlueHill Lite (version 2.25, Buehler), with a Knoop indenter set with 10 g static load for 10 seconds.

*Caries-like lesion formation*

One third of each surface of the specimens were covered with two consecutives layer of acid-resistant varnish (nail polish - Revlon International Corp, NY, USA) and dentin specimens were subjected to demineralization (Figure 1). To demineralize the dentin and produce caries-like lesions, the specimens were immersed in 30 mL of 50 mM acetate buffer solution containing 2.2 mM $CaCl_2$, 2.2 mM $KH_2PO_4$, at pH 5.0 for 7 days [Moron et al., 2013; ten Cate and Duijsters, 1982]. A subsurface dentin demineralization with 90 µm (±30) in deep was obtained, which was confirmed by transversal microradiographic analysis (PW2233/20; Philips, Kassel, Germany). After that, dentin surface was covered again to leave only 1/3 of its area exposed and specimens were treated with different concentrations of STMP. Therefore, each surface was divided in 3 areas: 1- mineralized - 1/3 of the area covered with

acid resistant varnish and not exposed to the demineralization solution nor STMP treatment; 2- demineralized – after demineralization this area was covered with acid resistant varnish in order to avoid contact with the STMP treatment solutions and, 3 - demineralized and treated with STMP – after demineralization, the dentin surface was submitted to STMP treatment solutions as described as follows (Figure 1).

*pH Cycling*

After demineralization and covering 2/3 of dentin surface with acid resistant varnish, specimens were randomly distributed according to the treatment (N = 10): 1.5, 3.5, 5% STMP solutions or deionized water (DW). All specimens were submitted to pH cycling alternating the demineralization (1.5 mM CaCl2, 0.9 mM KH2PO4, 50 mM lactic buffer, pH 5.0, 8 h) and remineralization solutions (5 mM $CaCl_2$, 0.9 mM $KH_2PO_4$, 130 mM KCl, 20 mM Hepes, 5 mM $NaN_3$, pH 7.0, 16 h) for 7 days [Lagerweij and ten Cate, 2006]. During the pH cycling, prior to the incubation with the remineralization solution, dentin surfaces were treated with 30 mL of one of the STMP solutions or DW for 10 min. The STMP solutions were freshly prepared. Since protein phosphorylation with STMP requires alkaline hydrolysis into linear form, the STMP was hydrolyzed at pH 12 for 5 h followed by neutralization to pH 7.4 [Shen, 1966]. The pH was monitored periodically to assure its stability necessary to validate the test conditions. In addition, after the exposure to the STMP solution, dentin specimens were immersed in a saturated solution of $Ca(OH)_2$ (30 mL, for 10 min).

*Dentin Hardness Analysis*

After the pH cycling, the remineralization potential of STMP was assessed by modifications on dentin microhardness. Hardness was performed as described above for selection of dentin specimens (baseline). For the superficial hardness (SH) assessment, acid resistant varnish was removed and all the three areas of each specimen were tested in triplicate and addressing the indenter in the center of the area, with a distance of 100 µm between each indentation. Results were expressed as difference in KHN between mineralized surface × demineralized and mineralized surface × STMP treatment. After SH evaluation, dentin specimens were longitudinally sectioned, using a double-sided floppy diamond disk Ø 22 mm (KG Sorensen Ind. and Com., Cotia, SP, Brazil) at low speed and under intense water cooling and each half-block was used for cross sectional hardness (CSH) measurements. For this assessment, dentin specimens were embedded in acrylic resin and gradually polished as described above. Three series of indentations were done at 7 different depths from the dentin surface (10, 30, 50, 70, 90, 110 and 220 µm) in the central region of each area. The indentations were spaced 100 µm from each other. SH and CSH

data were calculated and statistically analyzed with a statistics software (Statsoft®, Tulsa, OK, USA). The assumptions of normal distribution and of equality of variances were evaluated using Kolmogorov-Smirnov and Levene test, respectively. As the assumptions were satisfied, data was analyzed by repeated measure two-way ANOVA and Tukey post-hoc tests ($p<0.05$).

*Polarized light microscopy*

Two half-specimens of each tested solution were cut into slices (approximately 600 mm-thick), ground and polished to a thickness of 100 µm. They were placed on glass microscope slides with a film of DW and covered with a cover glass. The effect of the different STMP concentrations on caries-like lesion remineralization was qualitatively observed using a polarized light microscope (PLM; Axio-Phot; Zeiss, Oberkochen, Germany) at 5X magnification.

**Results**

*Gelatin Zymography*

Zymograms of gelatinolytic activity and bands intensities determined by densitometry are shown in Figure 2 A and B. In the negative control gel (no STMP), it is possible to verify the 72 kDa and 66 kDa bands, which correspond to pro- and active forms of MMP-2 (Figure 2A-I, lane 2), respectively. In addition, 92 and 77 kDa bands were detected, corresponding to pro- and active forms of MMP-9, respectively (Figure 2A-I, lane 3). Reduced activity of MMPs is clearly seen regardless of STMP concentration while inhibition potential depends on the dose. At 0.5%, STMP partially inhibited MMPs -2 and -9 (Figure 2A-II), however, interestingly, in this condition, the MMP-2 showed to be more sensitive to the presence of STMP compared to the MMP-9 (96% and 10% of inhibition, respectively) (Figure 2A-II and B). At higher concentrations (1.5 – 5%), STMP was able to completely inhibit both MMPs-2 and -9 (Figure 2A-III and B).

*Artificial Caries-like Lesion*

Dentin artificial caries-like lesions created by demineralization were validated by transverse microradiography (Figure 3 and Table 1). Subsurface and deep dentin demineralization (90 µm ± 30, in depth) (Figure 3 - asterisk) as well as maintenance of mineralized outer surface (11 µm ± 5; Figure 3 - arrow) were confirmed.

*Dentin Hardness Analysis*

Dentin SH results are presented on Table 2. The RM two-way ANOVA test revealed that the factors concentration (p=0.0001), depth (p<0.0000) and dentin condition (p=0.0069) were statistically significant as were their interaction (p<0.0000). In the mineralized dentin, average SH was 31.9 ± 2.9 KHN, without significant differences among the groups (p>0.05). When the dentin specimens were submitted to the artificial caries challenge, a mean mineral loss rate of 65% was detected for all groups, without significant differences among them (Table 2). Only 1.5% STMP treatment was able to reduce mineral loss (p<0.05), which was not detected for any other tested concentration. No significant change in SH was verified when dentin specimens were treated with 3.5% or 5% STMP, DW (control) or when compared to the demineralized condition (Table 2).

Regarding the CSH analysis, similar values of lesion depth were observed for all specimens in the demineralized condition, showing a homogeneous subsurface lesion depth. When the specimens were treated with DW, it is notable that there is an overlap of the lines (demineralized and treated condition) (Graphic 1). This performance attests that DW had no effect in reversing the lesion produced. On the other hand, regardless of STMP concentration used, all treated areas showed potential to aid the remineralization of the lesion in relation to the demineralized condition. However, only 1.5% SMTP was able to significantly revert the lesion formed, in which it can be observe in Graphic 1 up to a depth of 90 µm. However, in the 3.5% and 5% groups, surprisingly, these concentrations impaired the remineralization process (Graphic 1). Moreover, no statistically significant differences were found between 3.5% and 5% STMP for changes in lesion depth.

*Polarized light microscopy*

Figure 4 shows representative photomicrographs obtained using PLM. The images confirm the presence of the subsurface lesion after demineralization (asterisk) when compared to the mineralized area. The profile of CSH shows a substantial mineral loss until the depth of 90 (±30) µm. No difference between treated and demineralized area can be seen when DW was used, which is observed a similar pattern of demineralization with higher birefringence area when compared to the mineralized condition (Figure 4A). When dentin was treated with 1.5% STMP, a smaller area of birefringence can be observed indicating a substantial mineral deposition, showing a visual condition similar to the mineralized area (Figure 4B). However, when higher concentration of STMP (3.5% or 5%) was used, mineral loss within the lesion is seen, similar to the demineralized area (Figure 4C) and to the control group (DW), presenting a more pronounced birefringent area. These images are in accordance with the CSH assessment.

**Discussion**

During the caries process, bacterial acids dissolve the mineral content of the dentin exposing the organic matrix and activating host-derived enzymes, such as matrix metalloproteinases (MMPs) [Martin-De Las Heras et al., 2000; Sulkala et al., 2002; Tjäderhane et al., 1998]. Investigations in Biology and Oral Biochemistry have clarified the events that lead to the carious lesion progression and undermined the traditional concept that cariogenic bacteria enzymes are determinant for the destruction of dentin [Caufield and Griffen, 2000; Scaffa et al., 2017; Tjäderhane et al., 1998]. Different members of MMPs family were localized in sound and carious-affected dentin, both in latent and active forms, including MMP-2 and -9 [Kato et al., 2011; Tjäderhane et al., 1998] with increased activity in carious condition [Liu et al., 2011]. In this way, multi-mechanism reparative and preventive therapies for biomimetic remineralization and endogenous proteolytic enzymes degradation seem to be promising strategies [Giacomini et al., 2017]

In this in vitro study, the anti-proteolytic potential of different concentrations of STMP was investigated against recombinant MMPs. According to our results, STMP was able to inhibit MMPs-2 and -9, so our first null hypothesis was rejected. The inhibition of recombinant MMPs by STMP is explained by its chelating mechanism. Due to its molecular structure, STMP has the capability of chelating metal cations and rejecting anions. Metallic ions are important for MMPs to maintain their three dimensional configuration as well as conserving their active functional site for enzymatic activity [Visse and Nagase, 2003]. Since phosphate groups have high binding affinity to calcium ions (order of binding affinity of some functional groups to Ca2+: phosphate> carboxyl> amide> hydroxyl group) [Wang et al., 2001] probably the anionic group attracts calcium ions by electrostatic force resulting in calcium phosphate crystals. The formation of complexes with calcium was also proposed to explain MMPs inhibition by sodium fluoride [Brackett et al., 2015]. Thus, the sequestration of Ca2+ from the incubation medium by the phosphate group could have kept MMPs in inactive state (zymogen) once they are zinc- and calcium-dependent enzymes. Further studies should explore the inhibitory potential and mechanisms of inhibition of dentinal MMPs by STMP. Moreover, these MMPs inhibitory potential depended of the dose, and low concentrations such as 0.5% was able to inhibit partially MMP-2 and -9 activities. Probably these results are due to the low concentration of STMP on the incubation medium and, consequently, a small amount of phosphate groups available. This event led to a greater amount of free Ca2+ in the microenvironment allowing the activation of the enzymes. Nevertheless, it is interesting to investigate the role of additional calcium ions added to the incubation medium to reverse the STMP induced inhibition. Interestingly, MMP-2 was more sensitive to the presence of 0.5% STMP, while 90% of MMP-9 remained when incubated with the same concentration of STMP. Although presenting similar molecular structure and substrate specificity, differential

inhibition of MMP-2 and MMP-9 by other reagents was reported and might be related to slight differences on their collagen-binding domains and length of their hinge region [Breschi et al., 2010; Silva Sousa et al., 2016]. Specific mechanisms of inhibition as well as inhibition of different members of the MMPs family by STMP should be clarified in future studies.

From the concentrations of STMP tested in zymographic analysis, only those that completely inhibited the gelatinolytic activity (1.5, 3.5 and 5%) were selected to investigate the dentin degradation and remineralization potential through the mechanical tests. The induction of artificial carious lesions (subsurface lesion) in bovine dentin was an important tool to investigate the use of STMP as a strategy to stabilize and strengthen dentin matrix to induce remineralization, decrease of biodegradation rates and increase the mineral nucleation. According to our results, the second null hypothesis must be rejected either, once the use of STMP enhances the mechanical properties of demineralized dentin. The results suggested that 1.5% STMP was able to induce recovery of dentin mineral loss, when applied during a pH cycling model. SH was improved about 29% compared to demineralized condition, control group (DW) or when the dentin specimens were treated with higher concentration of STMP (3.5% or 5%). Similarly, CSH indicated a significant reduction on dentin demineralization within the lesion by 1.5% STMP when compared to the other treatment conditions. According to Gu et al. 2010 [Gu et al., 2010], phosphophoryn, that is the most abundant NCPs in dentin, is deposited directly at the advancing mineralization front of dentin during tooth formation [Weinstock and Leblond, 1973].This fact may be taken into account as in the hardness model used in this simulation, constant acid challenge occurs during a cariogenic challenge and thus, which might allow lower content of NCP available favoring remineralization.

In PLM, it was possible to observe that 1.5% STMP treatment favored the recovery of the lesion depth showing a similar aspect between the treated and sound areas, differently when compared to the demineralized condition, which presented a greater area of birefringence indicating demineralization zone (Figure 4B). Dentin remineralization by STMP associated with a calcium solution by the formation of calcium phosphate crystals was previously reported [Zhang et al., 2012] and is also supported by our results. The combination of STMP with calcium hydroxide [Ca(OH)2] might also favor dentin remineralization by the formation of calcium phosphate precursor that promotes conditions for the minerals crystal growth required for remineralization [Featherstone, 2000; Kawasaki et al., 1999]. The phosphorylation of proteins present on the surface of organic template by STMP could trap calcium ions by electrostatic force resulting in prenucleation clusters [George and Veis, 2008; Wang et al., 2001]. Such clusters may guide mineral deposition to the desired gaps regions areas within the collagen matrix [Demichelis et al., 2011; He et al., 2003; Nijhuis et al., 2014]. These mechanisms partially explain the improvement in the

mechanical properties of the dentin extracellular matrix observed herein and might be related to a high binding affinity of phosphate groups from STMP to calcium ions.

However, similar remineralization potential was not observed for high concentrations of STMP. Actually, lower mineral content shown as higher birefringence can be seen within caries-like lesions by PLM (Figure 4C). It can be speculated that the addition of high concentrations of STPM in the medium may have supersaturated the dentin tissue leading to sequestration of Ca2+ from the dentin. [Anbar et al., 1979; Changgen and Yongxin, 1983; da Camara et al., 2016]. Supposedly, the concentration of 3.5% and 5% were able to decrease the Ca/P ratio of these groups, once this is the main reason of calcium loss, which impaired remineralization.

Exponential decrease of Ca2+ in enamel in the presence of STMP has been reported [McGaughey and Stowell, 1977; Souza et al., 2013]. When used at high concentrations, SMTP has the ability to supersaturate the enamel surface and further sequester Ca2+ from hydroxyapatite due to its strong ability to complex metal ions, resulting in mineral loss [da Camara et al., 2016]. Although, this evidence was reported for enamel, similar mechanism is expected for dentin. Further research needs to establish ideal concentrations for STMP use to promote remineralization of both enamel and dentin carious lesions.

Moreover, for dentin, the role of organic matrix content may also have a relevant effect on STMP remineralization mechanism. Within the dentin matrix, DMP-1 has highly phosphorylated serine and threonine residues, in which phosphate group could stabilize calcium ions resulting in prenucleation clusters [George and Veis, 2008]. Consequently, guiding mineral deposition to the desired gaps regions areas within the collagen matrix, enhance the mechanical properties of the dentin extracellular matrix [Demichelis et al., 2011; He et al., 2003; Nijhuis et al., 2014]. Considering a biomimetic approach, it is possible to mimic this natural mechanism in an in vitro scenario by inserting certain functional molecules onto the DMP-1 as nucleation sites to trigger initial nucleation and mineralization. In this way, it is speculated that sodium trimetaphosphate has potential to phosphorylate type I collagen, once it probably could phosphorylates serine and threonine proteins present in DMP-1.

Despite of the limitations of this study, the potential of STMP of a simple and easily available agent to be used in attempt to reverse dentin caries-like lesion was evidenced, taking into account the biological mechanisms involved.

**Conclusion**

According to the conditions of this study, it can be concluded that:

(1) STMP presents anti-proteolytic effect against both MMP-2 and -9 activities, being this inhibition dose-dependent and not observed for concentrations lower than 1.5%,

(2) The use of 1.5% STMP is able to act as a biomimetic agent to promote remineralization of dentin previously submitted to acidic challenge.

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**Legends**

Table 1 - Mean (±SD) of some caries-like subsurface lesion parameters obtained by transverse microradiography (n = 8).

Table 2 Mean (SD) of dentin surface hardness in different dentin substrates and treatment solutions.

Different capital letters indicate significant difference between the substrates. Different small letters indicate differences among treatments within the same substrate (Tukey test, n = 10, $p < 0.05$).

**Figure 1-** Dentin specimens preparation for pH cycling method. Schematic shows areas protected with acid resistant varnish before demineralization and treatment with STMP.

**Figure 2-** Representative image of zymograms (A) and gelatinolytic enzymes activities (percentage of inhibition) (B) obtained after eletrophoresis showing the effect of different concentration of STMP against the gelatinolytic activity of MMP-2 and -9.

I – MMPs incubated without STMP (control). II - The effect of 0.5% STMP on gelatinolytic enzymes activities. III- Gelatinolytic activity when incubated with 1.5% STMP. In all gels: lane 1- standard molecular-weight marker (MWM); lanes 2 and 3 - Purified human MMP-2 (66 KDa) and MMP-9 (67 KDa), respectively. Incubation with 3.5 or 5% STMP resulted in similar zymograms as 1.5% STMP (data not shown). Negative Control - MMPs activities without pretreatment with STMP; Positive Control - MMPs activities with pretreatment with 1,10 phenanthroline (specific MMPs inhibitor).

**Figure 3 -** Subsurface dentin lesion verified in transverse microradiography. Arrow- Surface layer preserved (11 μm). Asterisk- subsurface lesion (90 μm).

**Figure 4 -** Polarized light photomicrograph (5X) of the three different areas according to the dentin condition (mineralized; demineralized; demineralized + treated) with:(A) DW (control); (B) 1.5% STMP; (C) 3.5%/5% STMP – representative image. Asterisk – subsurface lesion.

**Graphic 1 -** Cross-sectional hardness (mean, %) at different depths in dentin blocks demineralized and treated or not with different concentrations of STMP.

**Tables**

Table 1

| Parameters | Mean ±SD |
|---|---|
| Surface layer thickness (µm) | 11 ± 5 |
| Lesion depth (µm) | 165 ± 30 |
| Mineral loss over the lesion depth (vol %) | 24 ± 4 |

Table 2

| Substrates / Treatment | Mineralized/Demineralized | Mineralized/Treated |
|---|---|---|
| DW | -62.77 (10.76)<br>**Aa** | -59.53 (8.07)<br>**Aa** |
| 1.5% STMP | -66.22 (6.95)<br>**Aa** | -37.15 (8.31)<br>**Bb** |
| 3.5% STMP | -66.82 (8.97)<br>**Aa** | -63.76 (7.43)<br>**Aa** |
| 5% STMP | -64.74 (8.48)<br>**Aa** | -61.48 (7.86)<br>**Aa** |

**Figures**

Figure 1

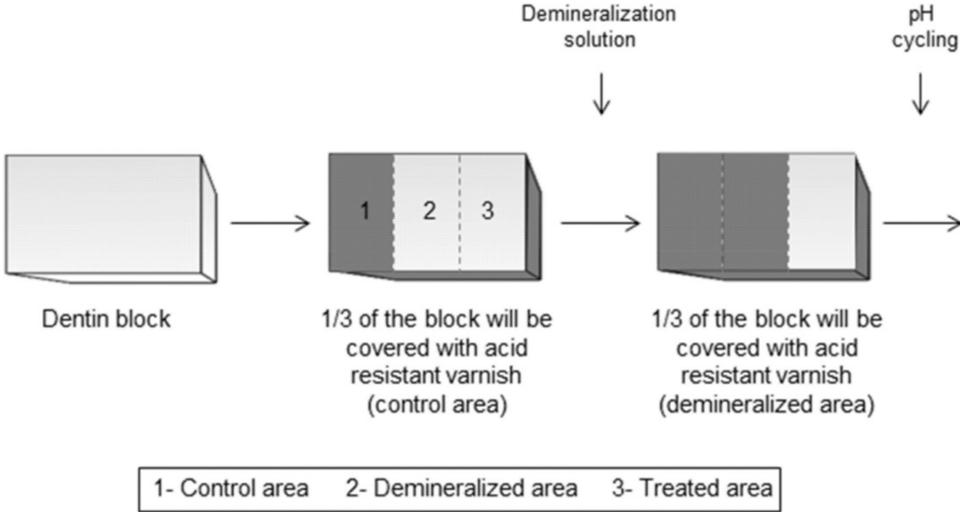


Figure 2

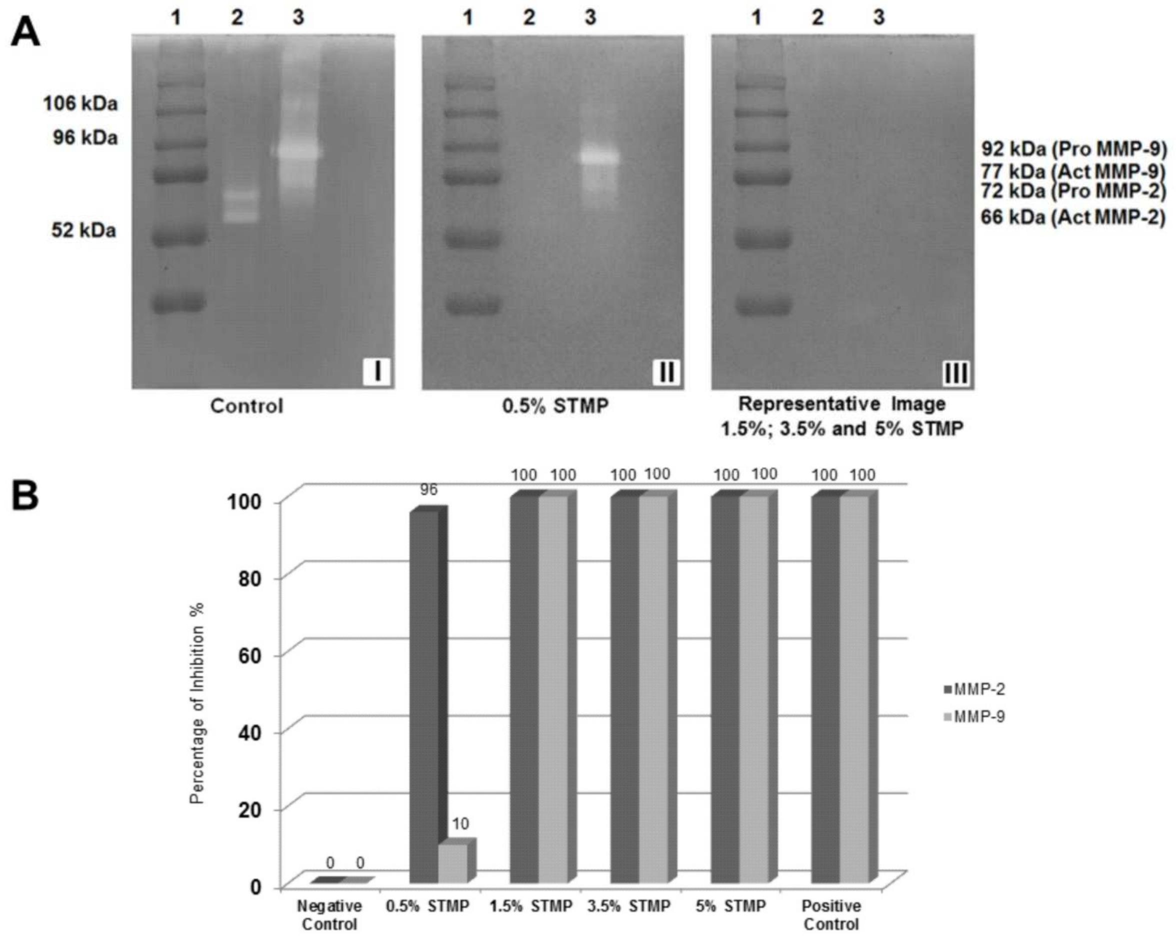


Figure 3

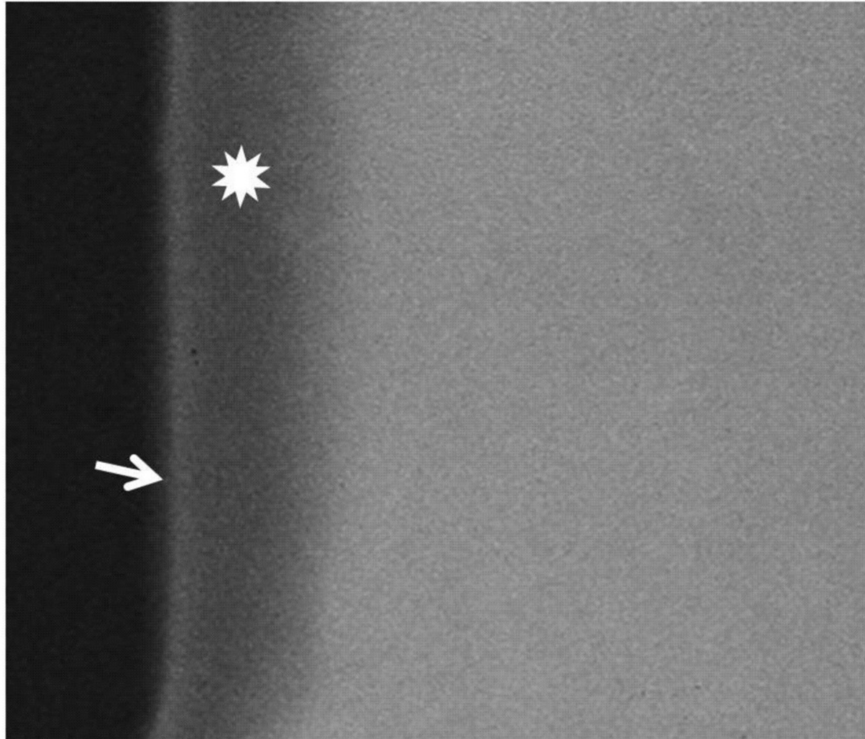
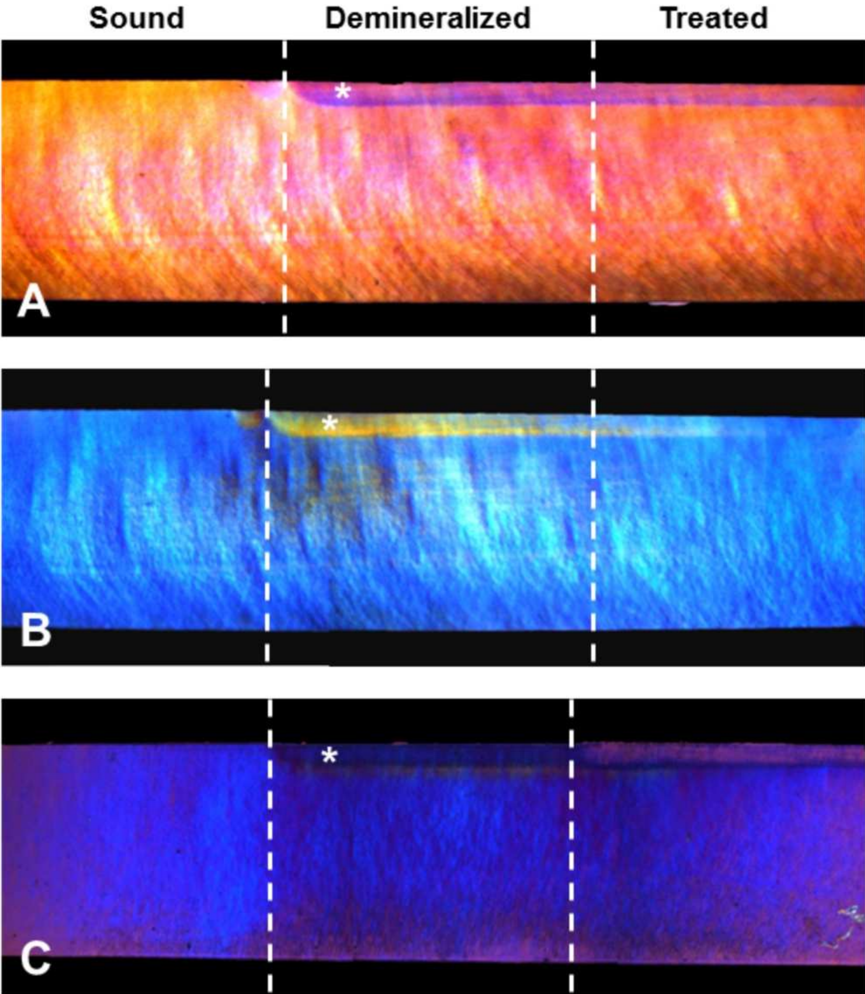
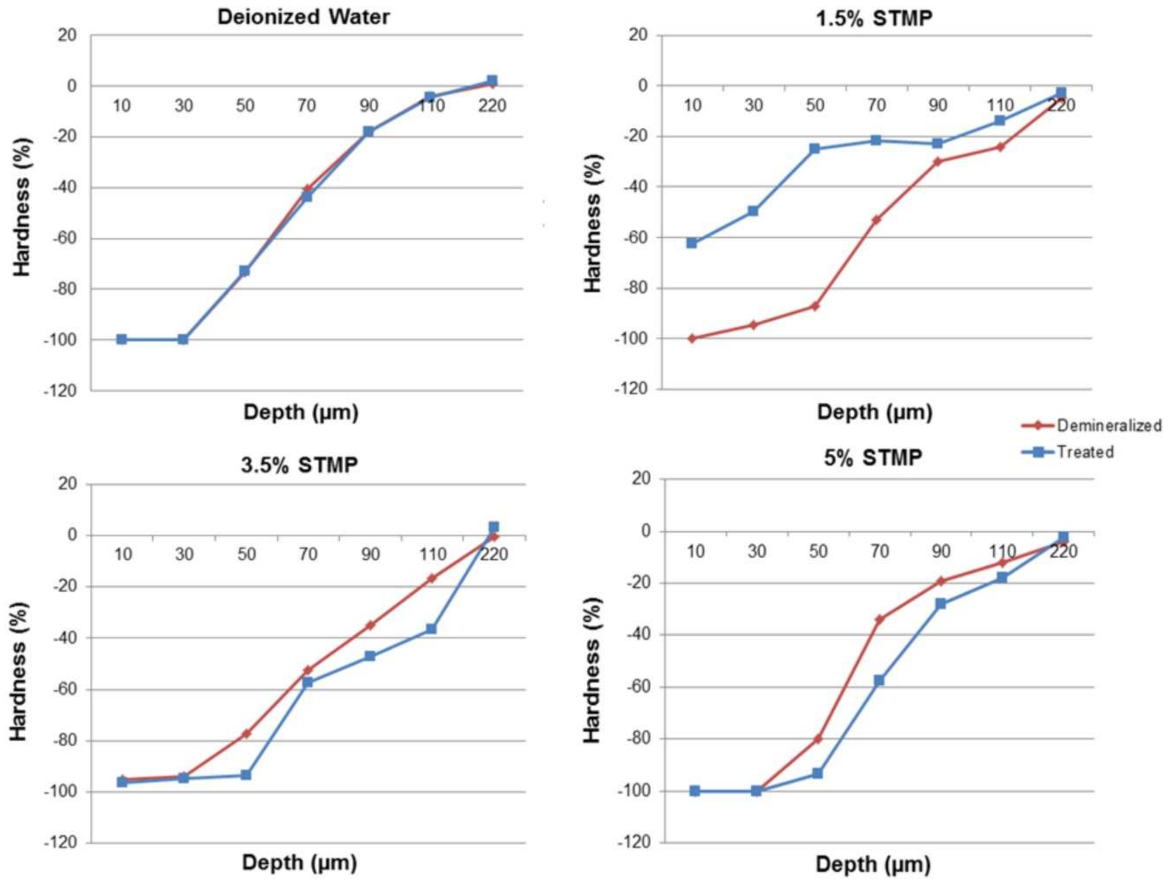


Figure 4



Graphic 1



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**Role of each author**

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Polliana Mendes Candia Scaffa

- contributed substantially with experimental design, experimental development and discussion and proofread the manuscript

Marina Ciccone Giacomini

- Contributed with the experimental development and discussion of the manuscript

Cristina de Mattos Pimenta Vidal

- Contributed substantially to discussion and proofread the manuscript

Heitor Marques Honório

- Prepared the experimental design and performed statistical analysis and the interpretation of data

Linda Wang

- Experimental design and contributed substantially to discussion and proofread the manuscript. She is the advisor of this study

**2.2 Article 2**

Sodium trimetaphosphate: impact on the improvement on dentin bonding up 12 months

Short title: Sodium trimetaphosphate improves dentin bonding

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Keywords: Key words: Adhesive; Dental caries; Dentin; Tensile strength; Tooth remineralization.

**Abstract**

Objective: This study aimed to evaluate the effect of STMP at 1.5% as biomimetic analog of dentin matrix on the dentin microtensile bond strength (μTBS), over time. Material and Method: Sixty sound human third molars were prepared by exposing medium dentin. Ten molars were maintained sound (GI) and fifty were submitted to artificial cariogenic challenge (7d/37ºC) (GII-GVI), and divided in 6 groups (n=10) according to the treated solution purposed: GI- deionized water/sound dentin, GII- deionized water/demineralized dentin, GIII-STMP, GIV- STMP + $Ca(OH)_2$, GV- STMP + NaF, and GVI- NaF. After the treatments (24h), the specimens were restored (Adper Single Bond Universal + Filtek Z250), to obtain resin–dentin sticks with a cross sectional area of $0.64mm^2$. 2/3 of these sticks were stored in artificial saliva (37°C) for analyzis after 6 months (1/3) and 12 months (1/3). The 1/3 remains were subjected to μTBS test (baseline). μTBS data were individually analyzed by two-way ANOVA and Kruskal-Wallis test ($p<0.05$). Multiple comparisons were made using Dunn's test. Results: In general, the highest μTBS values were obtained in sound condition while the demineralized condition obtained the minimum values. Groups treated with NaF with or without STMP were not able to improve adhesion over time. Only the use of STMP + $Ca(OH)_2$ improved the μTBS. The adhesive failure pattern was predominant in all time. Conclusion: The use of the STMP associated with $Ca(OH)_2$ seems to be a viable therapeutic strategy conciliating the biomimetizing capacity to the adhesive process satisfactorily.

Clinical Significance: STMP can potentially be used as biomimetic analog of dentin matrix improving the dentin bond strength. Its use can be a novel strategy to stabilize and strengthen the dentin substrate by the interaction with non-collagenolytic proteins and through remineralization, decreasing the biodegradation rate and increasing mineral nucleation optimizing the adhesives procedures.

**Introduction**

As a biofilm and sugar dependent disease, dental caries determines lesions due to two simultaneously processes: acidic dissolution of dentin minerals promoted by bacterial agents and the degradation of dentinal matrix with continuous infiltration of bacteria into the intertubular dentin [1].

In this process, endogenous enzymes known as intrinsic proteolytic enzymes of dentin are activated, which intensify even more the degradation of extracellular matrix including collagen [1-4]. Metalloproteinases (MMPs), mainly the collagenolitic MMP-2 and -9, and the cistein cathepsins (CCs) are the most investigated host-derived enzymes that are metal-ion–dependent (calcium and zinc) for its catalytic activity [2, 3, 5, 6]. All these events promote significant impact on the substrate in distinct levels, impairing the adhesive restorative procedures [5, 7-9].

Previous studies of Mazzoni et al., 2006 [10] and Nishitani et al., 2006 [11] indicated the increasing of the gelatinase activity from dentin demineralized with phosphoric acid or self-etch adhesives compared to not exposed substrate, which in turn, contribute to the adhesive interface degradation. Also, Giacomini et al., 2017 [9] evidenced the different susceptibility of degradation according to dentin substrate conditions, wherein caries affected dentin is more prone to degradation overtime in comparison to eroded or sound dentin.

In this scenario, strategies that minimize this effect have been widely investigated, especially mostly regarding the association of chlorhexidine as proteolytic inhibitor [7, 12]. Other synthetic agents with this purpose has also been investigated [13, 14]. Natural antibacterial agents have recently employed with the ability to increase collagen synthesis [15,16]. New proposals aim to preserve the collagen fibrils and favor for structured dentin remineralization, which characterizes the biomimetic strategy [14].

As sodium trimetaphosphate (STMP - $Na_3P_3O_9$), extensively investigated as a remineralization agent [14, 17, 18], also presents the potential for collagen type I phosphorylation, it seems to induce the directional biomimetic growth of apatite resulting in a strengthen dentin structure [17, 19]. This process is based on the phosphorylation of serine and threonine residues present in the non-collagenous proteins as the dentin matrix protein-1 (DMP-1). This protein regulates dentin mineralization, by controlling the size and orientation of the apatite nucleation in the organic matrix [20]. As a result, the phosphate group, which has high affinity for $Ca^{2+}$ ions, can trap these ions to form inorganic crystals that are essentials for dentin remineralization [20, 21].

Furthermore, the preservation and stability of collagen fibrils would be positive for the dental adhesive procedure, since it depends on the mechanical interaction of the hydrophilic monomer of adhesive systems to the collagen fibrils for forming the hybrid layer [22].

With the perspective of the use of STMP as a biomimetic agent, its association with $Ca(OH)_2$ could lead to the development of calcium phosphate precursors, which would favor for conditions to the growth of mineral crystals needed for remineralization [23, 24].

Considering the beneficial, the aim of this study was to evaluate the effect of STMP as biomimetic analog of dentin matrix on the demineralized dentin bond strength over time.

## Materials and Methods

Sixty extracted, caries-free human molars were used in this study, after approval of the Committee of Ethics in Research from the Bauru School of Dentistry, University of São Paulo (FOB-USP) (protocol no. 48755115.0.0000.5417).

The teeth were cleaned of adhering soft tissues and the occlusal surfaces were transversally sectioned with a lowspeed diamond saw (Extec Co., Einfield, CT, USA) under water irrigation in a metallographic cutter (Buehler, Lake Bluff, IL, USA). The root portion was sectioned 2 mm below the CEJ and discarded. The exposed coronal dentin surfaces were further polished (APL, Arotec, Cotia, SP, Brazil) using wet #600-grit SiC paper (Extec Co., Einfield, CT, USA) for 60 s to standardize the smear layer.

### *Experimental design*

This *in vitro* study involved two factors: treatment in 6 levels and time in 3 levels. The main response variable was the bond strength by means of microtensile bond strength (μTBS).

### *Specimen preparation and Groups distribution*

The occlusal enamel of sixty human sound third molars was removed horizontally (perpendicular to the long axis of the tooth) using a water-cooled diamond disc (Extec Corp, Enfield, CT, USA) coupled to a cut machine (Isomet 1000, Buehler, Lake Bluff, IL, USA) to expose flat dentin surface. The dentin surface was standardized using 600-grit SiC paper under running water for 30 s (Politriz APL-4 Arotec, Cotia, São Paulo, Brazil). Fifty units were varnished with acid-resistant varnish (Revlon International Corp, New York, NY, USA), except for the occlusal surface, and exposed in 30 mL of 50 mM acetate buffer solution containing 2.2 mM $CaCl_2$, 2.2 $mMKH_2PO_4$, at pH 5.0, for 7 days in order to produce a caries-like lesion in dentin [25, 26]. The remain ten teeth were kept sound as control group. Therefore, they were randomly divided into six groups (n=10), resulting from the combination of the dentin demineralization and treatment solution. After, specimens were individually immersed in 40 mL of treatment solution for 24 h, at room temperature, according to their groups division as described below (Table 1).

*GI- Deionized water/Sound dentin:* no demineralization and further treatment was performed on dentin surface, being considered the positive control group.

*GII- Deionized water/Demineralized dentin:* specimens were demineralized and then immersed in deionized water, being a negative control group.

*GIII- 1.5% STMP solution:* protein phosphorylation with STMP requires alkaline hydrolysis into linear form. Thus, STMP was hydrolyzed at pH 12 for 5 h followed by neutralization to pH 7.4 with minimal reduction in its phosphorylation potential [27, 28]. The STMP (Sigma-Aldrich Co., St. Louis, MO, USA) solution was prepared at a concentration of 1.5%.

*GIV- 1.5% STMP solution + saturated solution of $Ca(OH)_2$:* after the exposure of the dentin slabs in the 1.5% STMP solution, as described previously, the specimens were treated with saturated solution of $Ca(OH)_2$ in the same way and time treated with STMP solely.

*GV- 1.5% STMP solution + sodium fluoride (NaF):* STPM solution was performed in the same way previously described, however sodium fluoride was added in its composition. The fluoride solution was made in a 280 µg F/mL concentration, which was selected to simulate the dilution (1:3 weight/weight) that occurs in the oral cavity when 1,100 ppm dentifrices are used [29]. Fluoride solution was made from the salts NaF.

*GVI- NaF solution:* Fluoride solution was prepared as described previously.

*Restorative procedure and sticks obtainment*

The “multi-mode” adhesive system (Adper Scotchbond Universal - 3M ESPE, St. Paul, MN, USA) was applied as self-etching system in wet-bonding technique in accordance with the manufacturer’s instructions described in Table 2. After the bonding procedures, all teeth were restored with a microhybrid composite restoration Filtek Z250 (3M ESPE - St. Paul, MN, USA- A2 shade) in three increments of 2 mm. Each increment was light polymerized for 40 s using a LED light curing unit set at 1,200 $mW/cm^2$ (Radii-cal, SDI Limited, Bayswater, Victoria, Australia). After, the restored teeth were stored in artificial saliva at 37 ºC for 24 h.

The specimens were sectioned longitudinally in the mesio-distal and buccal-lingual directions across the bonded interface, using a slow-speed diamond saw to obtain resin–dentin sticks with a cross sectional area of approximately 0.8 mm width (±0.2 mm) measured with a digital caliper (Digimatic Calliper, Mitutoyo, Tokyo, Japan).

After sticks random selections, 2/3 of these were stored in artificial saliva at 37 °C for analyzis after 6 months (1/3) and 12 months (1/3). The 1/3 remain specimens was subjected

to baseline mechanical test through microtensile bond strength test (24 h after the restorative procedure).

*Microtensile bond strength test (mTBS) and failure mode*

The specimens were fixed with cyanoacrylate glue (Super Bonder Flex Gel, Henkel Loctite; SP, Brazil) to a jig, which was mounted on a universal testing machine (Instron, Norwood, MA, USA) and subjected to tensile forces at a crosshead speed of 0.5 mm/min until deboning. µTBS (MPa) was calculated by dividing the peak force (N) by the cross-sectional area of the failed interface ($mm^2$), measured by a digital caliper.

The failure mode of the sticks was classified as cohesive (failure exclusive within dentin or resin composite), adhesive (failure at resin/dentin interface), or mixed (failure at resin/dentin interface). The classification was performed under a stereomicroscope at 200x magnification (Stemi SV11, Carl Zeiss, Jena, German). Specimens with premature failures were included in the tooth mean calculation.

Data of µTBS mean and standard deviation were collected for each group and analyzed by two-way ANOVA and Kruskal-Wallis tests. Multiple comparisons were made using Dunn's test. All statistical analyzes were carried out using Statistica software (Statsoft®, Tulsa, OK, USA). Statistical significance was established at $\alpha = 0.05$.

**Results**

The overall values of the µTBS according to the substrate condition and time of analyzes are shown in Table 3. The two-way ANOVA test revealed that the factors treatment ($p<0.0000$) and time ($p<0.0000$) were statistically significant as were their interaction ($p<0.0000$).

Regarding to the dentin treatment, in initial time (24 h storage), it can be observed that sound condition, serving as positive control, showed the highest dentin bond strength. On the other hand, the demineralized condition showed the lower result (negative control), which did not differ when compared to the demineralized dentin treated with NaF or 1.5% STMP solutions only. Among the demineralized groups, only1.5% STMP supplemented with $Ca(OH)_2$ was statistically higher compared to the all other treatments, excepted for the group treated with 1.5% STMP associated with NaF, even the µTBS showed lower values compared to the sound condition ($p> 0.05$).

After 6-month storage, 1.5% STMP associated with $Ca(OH)_2$ on demineralized dentin performed similarly to immediate time, being effective overtime. Differently, on the others conditions, the µTBS showed lower values compared to the immediate time. In this time, the highest µTBS values still was observed to sound dentin followed by the group treated with 1.5% STMP + $Ca(OH)_2$, with statistical difference between them ($p< 0.05$). Again,

demineralized dentin treated with water showed the lowest μTBS values statistically different compared to sound and demineralized/1.5% STMP + $Ca(OH)_2$ groups, even statistically similar to the other conditions ($p > 0.05$).

After 12-month storage, the sound condition and the demineralized specimens that were treated with 1.5% STMP + $Ca(OH)_2$ was not able to stabilize the bond strength overtime. In the analyzes of the other conditions, the μTBS showed similar values compared to the results yielded after 6 months of storage. Despite this performance, the sound condition kept showing the highest μTBS values followed by the specimens treated with 1.5% STMP + $Ca(OH)_2$. The groups treated or not with 1.5% STMP supplemented or not with NaF or only NaF showed the lowest results, with no statistical significant differences between them.

In general, the majority of the specimens (88.3%) showed adhesive failures. Cohesive failures were observed in 5.2% of the specimens and mixed failures in 1.4%. A small number of premature failures (5.2%) were observed in the present study (Table 4).

**Discussion**

Evidences has robustely proven the role of dentin intrinsic enzymes on the degradation process of dentin on carious process revealing their presence and/or activity [3, 30, 31]. These enzymes are bound to the organic matrix or prevenient from the saliva and may lead to the degradation of both the collagen and other organic components of the dentin matrix. In this way, all dentin proteins are targets of the action of these proteases [3].

MMP-2 and -9 have been identified as the major responsible for the destruction of the organic matrix of the dentin tissue during the carious process [32]. These proteases are capable of degrading almost all components of the extracellular matrix, especially type I collagen fibrils [2-4].

In the present in vitro study, dentin from freshly extracted third human molars were used to evaluate the microtensile bond strength on artificial demineralized dentin and the effect of treatments to reverse or minimize demineralization effect over time.

The focus of this study relied on STMP as this agent aid to reverse demineralization, induce remineralization, inhibit proteolytic enzymes and also exert potential for dentin biomimetization [14, 17, 18, 33, 34]. To explore its potential with different combinations, it was associated or not with NaF or $Ca(OH)_2$ solution. The 1.5% concentration was chosen based on previous studies carried out in our laboratory [34]. It showed to be the best concentration to act both as a remineralizing agent and as an inhibitor of the gelatinolytic activity of MMPs [34].

In general, the data showed higher μTBS values for the group treated with STMP associated with saturated solution of $Ca(OH)_2$. The addition of saturated $Ca(OH)_2$ solution

increased the potential of STMP by approximately 16% compared to STMP group or with the other demineralized groups.

The more plausible explanation is associated with the role of this solution on the phosphorylation of the type I collagen fibrils. The treatment of the dentin surface with calcium hydroxide likely contributed to the formation of inorganic calcium phosphate crystals necessary for dentin remineralization [20, 21]. Thus, this ability to stabilize calcium and phosphate, guiding mineral deposition into gaps of the collagen matrix certainly contributes to improve the mechanical properties of the demineralized dentin [17].

The abovementioned performance indicates that the use of STMP supplemented with $Ca(OH)_2$ may be more effective in promoting the remineralization of dentin. Its use is based on the specific phosphorylation of serine and threonine residues present in DMP-1 proteins that the phosphate group can obtain $Ca^{2+}$ ions to promote the formation of inorganic crystals necessary for the remineralization of dentin [20, 21]. In event that involves calcium and phosphate as in this investigation, STMP acts as a stabilizer for $Ca^{2+}$ and induces hierarchical remineralization of hybrid layer and may increase the durability of resin-dentin bond [35].

As reported by Kawasaki et al., 1999 [24], the remineralization of dentin occurs neither by mineral nucleation in the organic matrix nor by its spontaneous precipitation, but by the growth of residual inorganic crystals in the body of the lesion. In consequence, this remineralization should be responsible for the improvement on its mechanical proprieties related to the observed results.

Based on the μTBS values, it has been shown that the use of the STMP solution by itself was not sufficient to improve the bond strength of a universal adhesive system to the dentin. In addition, when this solution was associated with NaF or when only the NaF solution was used, no difference was also observed in the values of μTBS compared to the demineralized group and between these groups.

In this study, the use of fluoride does not seem to be effective in improving dentin bond strength. The effective role of $F^-$ ions depends on the amount of $CaF_2$ deposited on tooth surface, which did not occur in this study perhaps due to the lack of association of calcium ions with the NaF solution.

Based on the methodology and results of this study, it is suggested that the use of STMP may have altered the nanostructure of the collagen fibrils, since the introduction of the phosphate group on the surface of type I collagen (DMP-1) could serve as nucleation sites to trigger the mineralization of the dentin [20]. Thus, with the additional use of $Ca(OH)_2$ solution, the phosphate group could have stabilized calcium ions resulting in pre-nucleation clusters [23, 24]. Consequently, guiding the deposition of minerals to the desired areas within the collagen matrix, increasing the mechanical properties of the extracellular matrix of the dentin

[36-38] as it could be seen in the improvement of the µTBS values of the group treated with STMP + $Ca(OH)_2$ of this study.

However, long term success of this material is limited by the influence of oral dynamic conditions. The 6-month performance highlighted the potential of 1.5% STMP + $Ca(OH)_2$ on comparison to the other tested treatment. However, none yielded the performance showed by the control sound group. After 12 months, all groups showed a decrease in µTBS values compared to initial condition. Not even the presence of SMTP supplemented with $Ca(OH)_2$ was able to maintain the µTBS values over time, even it was more effective in comparison to other tested treatments.

Even the use of 1.5% STMP + $Ca(OH)_2$ addressed to improve bonding strength to dentin, it is far from the expected potential as described during the analyzes of previous studies regarding their remineralization and anti-enzymatic roles [33, 34]. Therefore, once applied in dentin, it is relevant to consider its interaction to the substrate and to the tested bonding agent. Dentin substrate itself can serve as a mechanical barrier and dilute this impact. Also, 10-MDP is the main ingredient of this multimode adhesive system, which mechanism of action depends on the consumption of calcium to bond to dentin. When 1.5% STMP + $Ca(OH)_2$ are used, Ca ions are available from this solution and probably MDP and STMP can compete for this ion and so, a negative interaction can be established. Chemical analyzes of this prepared substrate could be tested to clarify this scenario.

The adhesive/dentin interface degradation is still a challenge in Dentistry [39, 40]. The durability of bonding to dentin depends on the interaction of monomers with the network of collagen fibrils [39, 41]. Probably, insufficient adhesive penetration results in exposed fibers, where degradation of the adhesive interface may begin [39]. Degradation may also start breaking the covalent bonds between polymers by water addition, leaving exposed collagen fibrils [39, 42]. Consequently, collagen network degradation reduces the adhesive bond strength to dentin, compromising the durability of adhesive procedures, over time [39, 40, 42].

Therefore, the results of this study strength the evidences that STMP in fact contribute to improve the conditions for the bonding performance to dentin. However, its effect also seems partially effective overtime. The association with $Ca(OH)_2$ is able to optimize STMP benefits.

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Tables

| Table 1- Groups division (n=10) according to the treatment pourposed | | |
|---|---|---|
| **Groups** | **Dentin Demineralization** | **Treatment Solution** |
| **I** | No | Deionized water ($H_2Od$) |
| **II** | Yes | Deionized water ($H_2Od$) |
| **III** | Yes | 1.5% STMP |
| **IV** | Yes | 1.5% STMP + $Ca(OH)_2$ |
| **V** | Yes | 1.5% STMP + NaF 1,100ppm |
| **VI** | Yes | NaF 1,100ppm |

| Table 2- Composition and protocol of use of the tested universal dentin bonding system. | | |
|---|---|---|
| **Material** | **Composition*** | **Manufacturer's Instructions** |
| Adper Scotchbond Universal<br>3M ESPE<br>(Lot:579967) | MDP 10-metacriloyxidecil dihydrogen phosphate, dimethacrylate resins, HEMA 2-hydroxyethyl methacrylate, methacrylate modified polyalkenoic acid copolymer, filler, ethanol, water, initiators, silane | Self-etch strategy<br>1. Scrub adhesive for 20 s on dentin<br>2. Air thin for 5 s<br>3. Light cure for 10 s |

* Information provided by the manufacturer

Table 3- Means values µTBS in MPa (SD) according to substrate condition and time of analyzes.

| **Substrate Condition** | *µTBS (MPa)* | | |
|---|---|---|---|
| | **Initial** | **6-month** | **12-month** |
| Sound (Positive Control) | 45.28 (1.35) **Aa** | 45.96 (3.65) **Aa** | 39.06 (4.73) **Ba** |
| Demineralized (Negative Control) | 8.72 (0.54) **Ad** | 5.19 (2.87) **ABc** | 3.37 (0.93) **Bc** |
| Demineralized + 1.5% STMP | 8.35 (1.52) **Ad** | 5.64 (2.18) **ABc** | 4.11 (2.81) **Bc** |
| Demineralized + 1.5% STMP + $Ca(OH)_2$ | 16.01 (1.50) **Ab** | 12.78 (1.16) **Ab** | 9.11 (2.50) **Bb** |
| Demineralized + 1.5% STMP + NaF | 13.25 (2.67) **Abc** | 6.16 (2.96) **Bc** | 2.82 (3.38) **Bc** |
| Demineralized + NaF | 10.14 (1.82) **Acd** | 5.95 (1.56) **Bc** | 4.73 (2.50) **Bc** |

For each line, different capitals letters indicate significant differences of µTBS values between the times of analyzis. For each column, different lowercase letters indicate differences between the dentin condition for each time (Dunn's test, n = 10, $p < 0.05$).

Table 4- Distribution of failure mode (%) before 12 month of storage

| **Types of failure** | *Time* | | |
|---|---|---|---|
| | **Initial** | **6-month** | **12-month** |
| Adhesive | 89.0 % | 87 % | 89.0 % |
| Cohesive in Dentin | 1.9 % | 3.4 % | 2.3 % |
| Cohesive in Resin | 3.4 % | 2.1 % | 2.0 % |
| Mixed | 2.3 % | 1.0 % | 0.9 % |
| Premature | 3.4 % | 6.5 % | 5.8 % |

# 3 DISCUSSION

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Investigations in the Biology and Oral Biochemistry fields have been essential for the comprehension of carious lesions development, progression and therapies. From these evidences, translational researches may be performed to support the clinical conducts (Zerhouni, 2005).

Therefore, in this process, the role of the organic matrix decomposition has been playing more significant relevance than was supposed years ago. During a carious process, studies have shown that bacterial acids from the metabolism of sucrose promote both the exposure of the matrix and the activation of host enzymes hampering more besides its degradation only (Van Strijp et al., 1994; Tjaderhane et al., 1998; Sulkala et al., 2001).

These enzymes, especially the matrix metalloproteinases - MMPs, bound to the organic matrix or from the saliva, may lead to the degradation of both the collagen and other organic components of the dentin matrix. Also, all the dentin proteins are targets of the action of proteases, whether endogenous and/or exogenous (Tjaderhane et al., 1998).

In this way, MMPs -2 and -9 have been identified in dentin tissue during the carious process, being the main responsible for the destruction of the organic matrix during this event (Mazzoni et al., 2015). These proteases are capable of degrading almost all components of the extracellular matrix, especially type I collagen fibrils, which is the most abundant organic component in dentin tissue (Visse et al., 2003). As this component is part of the constitution of the hybrid layer for adhesive restorative procedures, its physical and chemical integrity has a greater impact on the longevity of these approaches overtime (Carrilho et al., 2007; Carrilho et al., 2007; Vidal et al., 2014).

In this scenario, the use of STMP can be seen as a new strategy that has the capacity to stop the progression of the carious process as well as to act on dentin recovering in terms of remineralization and resistance. Also, its easy way of application would also favor for its indication as an aqueous solution.

Clinical trials and systematic reviews have addressing that the focus for more effective and conservative approaches are possible and desirable, as on the

recovering of early dentin lesions determined by events as dental caries and erosion (Frencken et al., 2012; Bjorndal et al., 2017). For instance, based on the current concept of caries selective partial removal, strategies that can provide conditions to not solely remineralize the affected dentin but also provide its strengthen with easy and low cost therapies are certainly welcome (Schwendicke et al. 2016; Innes et al. 2016).

The initial thoughts to investigate the use of STMP to potentially recover dentin was inspired on the extensive evidences about its use to treat early caries affected or eroded enamel, with the perspectives of a widely clinical applications (Manarelli et al., 2011; Favretto et al., 2013; Manarelli et al., 2013; Manarelli et al., 2014). This event certainly raised evidences aided to support the comprehension of the STMP mechanism of action and its interaction with dentin.

So, the purposed investigations of the use of STMP in demineralized dentin addressed for different aspects related to two main functions that this agent could determine simultaneously: 1- protect against enzymatic degradation and 2- support remineralization. Both beneficial effects are due to its functional phosphate group, specially associated with calcium ions.

Considering all tested conditions, the performance of 1.5% STMP containing $Ca(OH)_2$ can be regarded as the most promising strategy since it yielded the better performance in all tested challenges. Likely, it can strengthen the damaged dentin while promoting anticaries effect, taking $Ca(OH)_2$ to participate in this process organizing the nucleation of calcification (Kawasaki et al., 1999; Featherstone, 2000; Xu et al., 2010; Zhang et al., 2010; Gu et al., 2011; Zhang et al., 2012).

As caries-affected and eroded dentin represent the most altered substrate in clinical challenges (Komori et al., 2009; Giacomini et al., 2017), dentin presents less content of calcium due to the demineralization. Therefore, the additional supplementation of calcium is relevant as dentin itself could not serve as a natural resource for STMP. In this investigation, 1.5% STMP seems to be a feasibly concentration that can inhibit both purified human MMPs-2 and -9. Furthermore, this solution in associated with $Ca(OH)_2$ showed evidences to be effective in reduce demineralization in dentin tissue. However, based on the analyzes regarding bond strength, even the supplementation of $Ca(OH)_2$ did not promoted the expected beneficial. Likely, two main factors must be considered: 1- the use of self-etching mode of this multi-mode bonding systems and 2- the presence of 10-MDP.

Regarding the first factor, self-etching mode is the current most indicated bonding strategy discarding the use of phosphoric acid as pretreatment, and so preserving more content of calcium. It could favor for the STMP process, however, based on the second factor, the presence of 10-MDP and its mechanism of action which strongly depends on the calcium can create a competition for the calcium. Additionally, it is also notable that we tested artificially demineralized dentin substrate, which presents less reactive calcium and phosphate. Therefore, the actual available of ionized calcium and the interaction between them, 10-MDP and STMP calls for more investigation to elucidate it.

Finally, all these investigations may determine new pathways for therapeutic approach based on dentin bonding systems. So, the use of STMP may leads a great impact in adhesives procedures since it can be considered a biomimetic analog and has the capacity to foster dentin remineralization in a structured way. Its use can enhance for minimize the clinical limitations related to adhesion as marginal infiltration and secondary caries, for example.

So, the use of STMP can be introduced as a novel strategy that combines enzymatic inhibition and remineralizing potential, which can serve to the strengthening of the dentin and their stability, being considered a promising method in the treatment of demineralized dentin lesions, especially when associated with supplemented calcium.

# 4 FINAL CONSIDERATIONS

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Based on the methodology and results of this study, it is suggested that the use of 1.5% STMP may be the concentration ideal to use in demineralized dentin. This concentration could be able both to inhibit the MMPs activities and to alter the nanostructure of collagen fibrils enhancing its mechanical properties.

If in one hand, the chelating action of the phosphate group present in the STMP can act inhibiting the action of proteases, on the other hand the introduction of the phosphate group on the surface of type I collagen (DMP-1) could serve as nucleation sites to trigger the dentin mineralization (He et al., 2003a). Furthermore, with the addition of saturated solution of $Ca(OH)_2$, the phosphate group could have stabilized calcium ions resulting in pre-nucleation clusters. Consequently, guiding mineral deposition to the desired areas within the collagen matrix, increasing the mechanical properties of the extracellular matrix of the dentin (Balooch et al., 2008; Bertassoni et al., 2011; Niu et al., 2014). It was not so expressive for the bonding strength as for the other tested applications, likely due to the combination of the MDP-based system, the self-etching strategy mode and due to the presence of the dentin substrate itself as a mechanical barrier. However, increase the calcium supplementation or even the concentration of STMP could also be an alternative, allowing STMP to be an agent with perspectives of an easy and reliable clinical use.

In summary, within the limitations of this in vitro methodology, it can be concluded that the STMP at 1.5% serves as an effective inhibitor of collagen degradation mediated by both purified human MMPs-2 and -9 as well as by proteases extracted from sound dentin. In addition, dentin affected by caries treated with 1.5% STMP supplemented with $Ca(OH)_2$ may induce remineralization, whereas the surface without this treatment cannot induce detectable remineralization. Bonding ability to dentin has also been proven the benefit role of STMP, specially combined with $Ca(OH)_2$ overtime.

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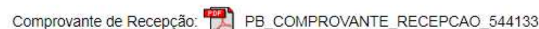
# ANNEXES

**Annexe 1**

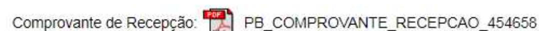
**Approval of the Committee of Ethics in Research from the Bauru School of Dentistry, University of São Paulo (FOB-USP) (protocol no. 49810315.1.0000.5417)**

**Título da Pesquisa:** O uso de TMP sobre enzimas endógenas dentinárias
**Pesquisador Responsável:** Rafael Simões Gonçalves
**Área Temática:**
**Versão:** 2
**CAAE:** 49810315.1.0000.5417
**Submetido em:** 05/11/2015
**Instituição Proponente:** Universidade de Sao Paulo
**Situação da Versão do Projeto:** Aprovado
**Localização atual da Versão do Projeto:** Pesquisador Responsável
**Patrocinador Principal:** Financiamento Próprio

Comprovante de Recepção: 


**Approval of the Committee of Ethics in Research from the Bauru School of Dentistry, University of São Paulo (FOB-USP) (protocol no. 48755115.0.0000.5417)**

**Título da Pesquisa:** Efeito do TMP como biomimetizador na resistência de união à dentina de um sistema adesivo universal
**Pesquisador Responsável:** Linda Wang
**Área Temática:**
**Versão:** 2
**CAAE:** 48755115.0.0000.5417
**Submetido em:** 28/09/2015
**Instituição Proponente:** Universidade de Sao Paulo
**Situação da Versão do Projeto:** Aprovado
**Localização atual da Versão do Projeto:** Pesquisador Responsável
**Patrocinador Principal:** Financiamento Próprio

Comprovante de Recepção: 

**Annexe 2**

**DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS**

We hereby declare that we are aware of the article “Sodium trimetaphosphate as a novel strategy for matrix metalloproteinase inhibition and dentin remineralization” will be included in (Dissertation/Thesis) of the student Rafael Simões gonçalves was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, September 26th, 2017.

Rafael Simões Gonçalves
Author

Signature

Polliana Mendes Candia Scaffa
Author

Signature

Marina Ciccone Giacomini
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Cristina de Mattos Pimenta Vidal
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Linda Wang
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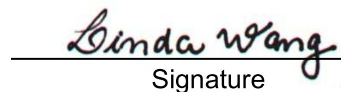
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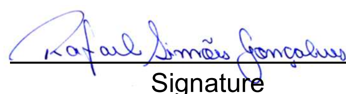
Rafael Simões Gonçalves
Author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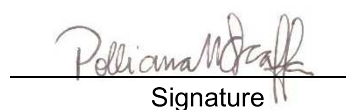
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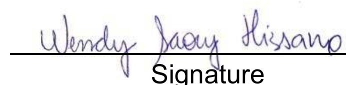
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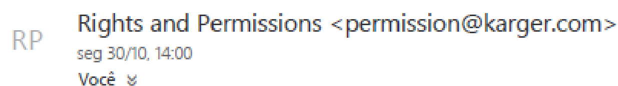
## Annexe 3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**Simões Gonçalves, R. et al: Caries Research DOI: 484486**

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**Annexe 4**

The article "Use of sodium trimetaphosphate in the inhibition of dentin matrix metalloproteinases and as a remineralizing agent" (No. JJOD-D-17-00702R1) was accepted for publication at Journal of Dentistry (23-Oct-2017).

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Corresponding author: Dr. Linda Wang
First author: Dr. Rafael Simões Gonçalves
Online publication complete: 31-OCT-2017
DOI information: 10.1016/j.jdent.2017.10.009

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27/10/2017 Clarification of our policy on prior publication

# Clarification of our policy on prior publication

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**By Gemma Hersh Posted on 27 January 2016**

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**Title Page**

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

Short title: Sodium trimetaphosphate acts as dentin MMPs inhibiton and remineralizing agent

Rafael Simões Gonçalves[a], Polliana Mendes Candia Scaffa[b], Marina Ciccone Giacomini[c], Marília Afonso Rabelo Buzalaf[d], Heitor Marques Honório[e], Linda Wang[f].

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Keywords: Dentin. Matrix metalloproteinases. Protease inhibitors. Demineralization. Hardness. Tooth remineralization.

**Manuscript**

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

**Abstract**

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.

**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 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 potential anti-MMP-9 activity [17].

Moreover, Zhang et al. [18], in 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.

**Material and Methods**

***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 against 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).

***Extraction of MMPs from Human Dentin***

MMPs 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 ºC (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].

***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 ºC 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.

***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 x 4.0 x 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 1,200-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 (Figure 1):

1- Sound area (positive control area): One-third of the surface area was covered with acid-resistant varnish to avoid contact with the demineralizing solution and with any of the proposed treatments.
2- Demineralized area (negative control area): The specimens were immersed in demineralizing solution to produce a subsurface dentin lesion. Subsequently, they were covered with acid-resistant varnish to avoid contact with the treatment solutions.
3- Treated area (experimental area): After demineralization, the dentin surface was subjected to one of the proposed treatment solutions.

To demineralize the dentin, the specimens were immersed in 50 mL of 50 mM acetate buffer solution containing 2.2 mM $CaCl_2$ and 2.2 mM $KH_2PO_4$, at pH 5.0 for 7 days [26, 27]. Subsurface dentin demineralization 90 ± 30 µm deep was achieved and verified by transversal microradiographic analysis (PW2233/20; Philips, Kassel, Germany). To avoid any shrinkage artifacts during X-ray exposure due to desiccation, the specimens were immersed in ethylene glycol (Sigma-Aldrich, Steinheim, Germany) 24 h before the transversal microradiographic analysis [26, 28].

***Treatments and pH Cycling***

Fifty dentin specimens were randomly divided into five groups (n = 10) using Microsoft Excel (Redmond, WA, USA), according to the treatment proposed and subjected to a 7-day pH-cycling regimen at 37 ºC, as follows:

STMP solution: A 1.5% STMP-based solution (Sigma-Aldrich Co.) was freshly prepared. Protein phosphorylation with STMP requires alkaline hydrolysis into linear form. Thus, STMP was hydrolyzed at pH 12 for 5 h, followed by neutralization to pH 7.4 with minimal reduction in its phosphorylation potential [29].

STMP solution + NaF: The STPM solution was prepared as previously described, with NaF added to the composition. The fluoride solution was made in a 280-µg F/mL concentration, which was selected to simulate the dilution (1:3 weight/weight) present in the oral cavity when 1,100 ppm dentifrices are used [30]. Fluoride solution was made from the NaF salt (Sigma-Aldrich Co., St. Louis, MO, USA).

<u>*STMP solution + saturated solution of $Ca(OH)_2$:*</u> After exposure of the specimens in the STMP solution as described above, the specimens were treated with a saturated solution of $Ca(OH)_2$ (Sigma-Aldrich Co., St. Louis, MO, USA). All specimens were immersed for 10 minutes in each solution.

<u>*NaF solution:*</u> Fluoride solution was prepared as previously described.

<u>*Deionized water ($H_2Od$):*</u> The dentin blocks were immersed in deionized water, constituting a negative control group.

The pH cycling process was performed for 7 days at 37 ºC. For each completed cycling, the samples were immersed in one of the treatment solutions (50 mL) for 10 min and subsequently stored in remineralizing solution (5 mM $CaCl_2$, 0.9 mM $KH_2PO_4$, 130 mM KCl, 20 mM HEPES, and 5 mM $NaN_3$; pH 7.0). After 16 h, the specimens were transferred to the demineralization solution (1.5 mM $CaCl_2$, 0.9 mM $KH_2PO_4$, and 50 mM lactic buffer; pH 5.0) for 8 h. Deionized water rinses were performed between all steps.

***Dentin Hardness Analysis***

After the pH cycling, SH was measured using a microhardness tester (Buehler, Chicago, IL, USA) and a Knoop diamond under a 10-g load for 10 s. Three indentations, 100 μm apart from each other, were made in the center of the sample. For CSH measurements, dentin specimens were longitudinally sectioned through their centers, embedded in acrylic resin with the cut face exposed, and then gradually polished. Three series of 7 indentations at different depths from the dentin surface (10, 30, 50, 70, 90, 110, and 220 μm) were assessed in the central region of each area and spaced 100 μm apart. The mean values of all 3 series at each distance from the surface were averaged. Results for SH and CSH were expressed as differences between mineralized surface (baseline) × demineralized (negative control) and mineralized surface × treated.

***Statistical Analysis***

SH and CSH data were statistically analyzed; the data showed normal distribution (Kolmogorov-Smirnov) and satisfied the equality of variances (Levene test). As the assumptions were satisfied, data were analyzed by repeated two-way analysis of variance (ANOVA) and Tukey post hoc tests ($p < 0.05$). Analyzes were performed using Statistica statistical software (Statsoft®, Tulsa, OK, USA) with a significance level set at 5%.

**Results**

***Gelatin Zymography***

The zymographic analysis detected gelatinolytic activity in the control gel (no STMP) for MMP-2 purified enzymes in both the pro- and active forms (72 and 66 kDa, respectively) and for pro-MMP-9 (92 kDa), and indicated the presence of MMP-2 pro- and active forms and the active-form of MMP-9 (86 kDa) in dentin extract (Figure 2A). MMP activities were efficiently inhibited by different concentrations of STMP (Figure 2B–D). At a concentration of 0.5%, STMP partially inhibited MMPs-2 and MMP-9 activities, and reduced the dentin extract activity by approximately 50% (Graphic 1). At a concentration of 1.0%, STMP inhibited 60% of the purified MMP-2 and -9 enzymes' activity, and reduced the dentin extract activity by more than 70% (Graphic 1). At a concentration of 1.5%, STMP completely inhibited the gelatinolytic activity (Figure 2D). Zymograms incubated with 2 mM 1,10-phenanthroline showed no enzymatic activity.

***Hardness Analysis***

The statistical analysis revealed interaction between the dentin condition (sound/demineralized and sound/treated) and treatments ($p < 0.0001$). In the demineralized dentin (artificial caries-like challenge), the mean mineral loss (± standard deviation) was 78% (±5.60) (sound/demineralized), with no significant differences among the groups. After the treatment (sound/treated), no enhancement of mechanical properties was observed in any group. Only 1.5% STMP combined with $Ca(OH)_2$ resulted in a significantly higher %SH when compared with other treatments (Table 1).

**Table 1.** Mean* (SD) of dentin surface hardness (%) in different phases and treatment solutions.

| Phase / Treatment | Sound/Demineralized | Sound/Treated |
|---|---|---|
| $H_2O_d$ | -77.60 (4.70)<br>**Aa** | -78.59 (4.02)<br>**Ab** |
| 1.5% STMP | -78.05 (7.33)<br>**Aa** | -75,77 (9.10)<br>**Ab** |
| 1.5% STMP + $Ca(OH)_2$ | -78.41 (5.85)<br>**Ba** | -59.78 (6.45)<br>**Aa** |
| 1.5% STMP + NaF | -77.84 (5.91)<br>**Aa** | -72.46 (4.90)<br>**Ab** |
| NaF | -77.68 (4.94)<br>**Aa** | -75.21 (4.35)<br>**Ab** |

*Negative values indicate loss of hardness compared with the sound condition. Different capitals letters indicate a significant difference between the phases of each treatment. Different lowercase letters indicate differences between the same phase among different treatments (Tukey test, n = 10, $p < 0.05$).

The same capital letters indicate no statistically significant differences between columns. Different small letters indicate no statically significant differences between rows

The CSH analysis showed similar values among the groups (± 90 μm) after the caries-like challenge (sound/demineralized) (Graphic 2). STMP + $Ca(OH)_2$ yielded significant enhancement of the mechanical properties when compared with the demineralized condition and with other groups (10–30 μm) (Graphic 2). From a depth of 50–70 μm, the use of STMP + $Ca(OH)_2$ or NaF resulted in a significant decrease in mineral loss compared with the demineralized condition (caries-like challenge). The use of STMP in association with $Ca(OH)_2$ or NaF, and NaF alone yielded similar results that were significantly higher than those of the other groups. Moreover, no significant differences were observed in %CSH at a depth of 90 μm–220 μm in any conditions.

**Discussion**

MMPs were the first family of proteases implicated in organic matrix destruction within dentin caries lesions [1]. Collectively, they are capable of degrading almost all components of the extracellular matrices, which mainly comprise highly cross-linked triple-helical collagen, thereby reducing the possibility of intrafibrillar remineralization [1]. The study of new therapies to prevent and reduce caries progression should be further explored to verify new beneficial effects of STMP since it is important to discover innovative therapies to avoid

organic matrix degradation and to promote its remineralization. The present study was designed to test the hypotheses that STMP, in different concentrations, could inhibit both human purified MMPs and MMPs extracted from radicular human dentin. The study was also intended to verify whether STMP could promote remineralization of caries-affected dentin.
In the first part of the study, the zymographic analysis showed the antiproteolytic potential of STMP against purified MMP-2 and MMP-9, and extracted dentin enzymes, but this effect was dose-dependent. Only 1.5% STMP completely inhibited gelatinolytic activity, which may be explained by STMP's chelating mechanism. STMP's phosphate groups are thought to attract calcium ions by electrostatic forces since they have high binding affinity to each other [31]. MMPs are zinc- and calcium-dependent endopeptidases and, consequently, the depletion of $Ca^{2+}$ from the incubation medium could have kept these enzymes in an inactive state (zymogen). Thus, the presence of $Ca^{2+}$ is important for maintaining the three-dimensional configuration necessary for enzymatic activity [32]. In addition, the presence of $F^-$ could form a complex not only with ionized calcium bound to the enzyme, but also with calcium in the incubation medium to form a tightly complexed $CaF_2$ [33]. On the other hand, the lower concentrations (0.5% or 1.0%) of STMP did not yield complete inhibition of MMPs. These results are likely due to both an insufficient concentration of STMP in the incubation medium and the small number of phosphate groups available. Lower concentrations of STMP were unable to cause depletion of all calcium ions present in the incubation medium, thereby allowing activation of the proteases.

Of the concentrations of STMP tested in zymographic analysis, only the 1.5% concentration completely inhibited the gelatinolytic activity. Therefore, that formulation was selected for combination with $Ca^{2+}$ or NaF to investigate its remineralization capacity in caries-affected dentin by SH and CSH tests. Based on SH values, the use of STMP solution alone was not sufficient to reduce mineral loss. Moreover, when this solution was associated with NaF or when NaF-only solution was used, no differences in hardness values were observed compared with the demineralized condition.

Conversely, the addition of a saturated solution of $Ca(OH)_2$ increased the potential of STMP by approximately 16% compared with the group that used only 1.5% STMP and the other groups. According to Chesnutt et al. [34], $Ca(OH)_2$ treatment led to the development of calcium phosphate precursors. Consequently, this treatment could create favorable conditions for the growth of mineral crystals, which could be a possible explanation for the results seen in our study. The CSH analysis demonstrated the positive effect of calcium on lesions, which was more pronounced for 1.5% STMP + $Ca(OH)_2$, which resulted in a lesion area that was reduced by 50% when compared to the others groups. Moreover, at a depth of 50 µm, this effect did not differ from that of NaF or NaF + STMP.

The above findings indicate that the use of STMP supplemented with $Ca(OH)_2$ can be effective in promoting dentin remineralization. Its use is based on the serine and threonine residues of phosphorylation present on the DMP-1 proteins; the phosphate groups can get $Ca^{2+}$ ions to promote inorganic crystal formation necessary for dentin remineralization [10, 31]. In addition, surface treatment of the demineralized dentin with $Ca(OH)_2$ may have favored its remineralization, since this condition could lead to the development of a calcium phosphate precursor that would have created positive conditions for the mineral crystal growth that is necessary to remineralization [19, 20]. As reported by Kawasaki et al. [20], dentin remineralization does not occur because of nucleation of minerals on the organic matrix or by spontaneous precipitation, but by the growth of residual inorganic crystals in the lesion body.

Based on the methodology and results of this study, it is suggested that the use of STMP may have altered the nanostructure of collagen fibrils once the introduction of phosphate groups on the surface of type I collagen (DMP-1) could serve as nucleation sites to trigger dentin mineralization [18]. Therefore, with the additional use of a saturated solution of $Ca(OH)_2$, the phosphate groups could have stabilized calcium ions, resulting in prenucleation clusters [10]. Consequently, guiding mineral deposition to the desired gap regions areas within the collagen matrix enhanced the mechanical properties of the dentin extracellular matrix [35–37].

In this study the use of fluoride did not appear to be effective in promoting dentin remineralization. The role of $F^-$ depends on the amount of $CaF_2$ deposited, which did not occur, as calcium ions were not used in combination with NaF solution. Furthermore, the presence of STMP in the fluoride solution reduces the availability of fluoride, so the results observed for the STMP + NaF and NaF-only groups would be expected to reduce the availability of this ion.

Despite being an in vitro study, the evidence elucidates STMP's mechanism of action and its interaction with dentin. STMP could be part of new clinical applications to minimize dentin matrix degradation through the caries process, as well as potential reparative clinical therapies. Therefore, the use of STMP may constitute a new strategy with simultaneous benefits. In particular, because of the current concept of the partial removal of caries, easy and low-cost strategies that can strengthen dentin are certainly welcome.

The 1.5% STMP solution containing $Ca(OH)_2$ can be regarded as a promising strategy for clinical use since it combines the potential to strengthen damaged dentin while promoting anticaries effects. This may lead to advances in adhesive procedures since STMP can be considered a biomimetic analog and has the capacity to foster dentin remineralization in a structured way. In consequence, its use can optimize dentin bonding procedures over

time, improving problems related to adhesion—including marginal infiltration and secondary caries.

In summary, within the limitations of this in vitro methodology, it may be concluded that 1.5% STMP can serve as an effective inhibitor of collagen degradation mediated by both human purified MMP-2 and MMP-9, as well as proteases extracted from sound dentin. Furthermore, caries-affected dentin treated with 1.5% STMP supplemented with $Ca(OH)_2$ may induce remineralization.

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**Figure's Legends**

**Figure 1-** Sequence of dentin specimen preparation for the sequence of acid challenges and treatments.

**Figure 2-** Representative image of zymography assay gel showing the effect of the presence of STMP against the gelatinolytic activity of human purified MMP-2 and -9 and proteases extracted from sound dentin. Line 1- MWM - molecular-weight marker (kDa). Line 2 and 3 - Purified human MMP-2 and MMP-9, respectively. Line 4 - Proteases extracted from sound dentin (MMP-2). A- Gel incubated without the presence of STMP, control. B- The effect of 0.5% STMP on gelatinolytic enzymes activities. C- The effect of 1.0% STMP on gelatinolytic enzymes activities. D- The effect of 1.5% STMP on gelatinolytic enzymes activities.

**Graphic 1-** Gelatinolytic enzymes activities (%) according to different concentration of STMP used.

Negative control – without any enzyme inhibitor; Positive control – use of a specific MMP inhibitor (1,10 phenanthroline).

**Graphic 2-** Graphical representation of mean hardness as a function of depth according to the artificial caries-like challenge and treatment

**Figures**

Figure 1

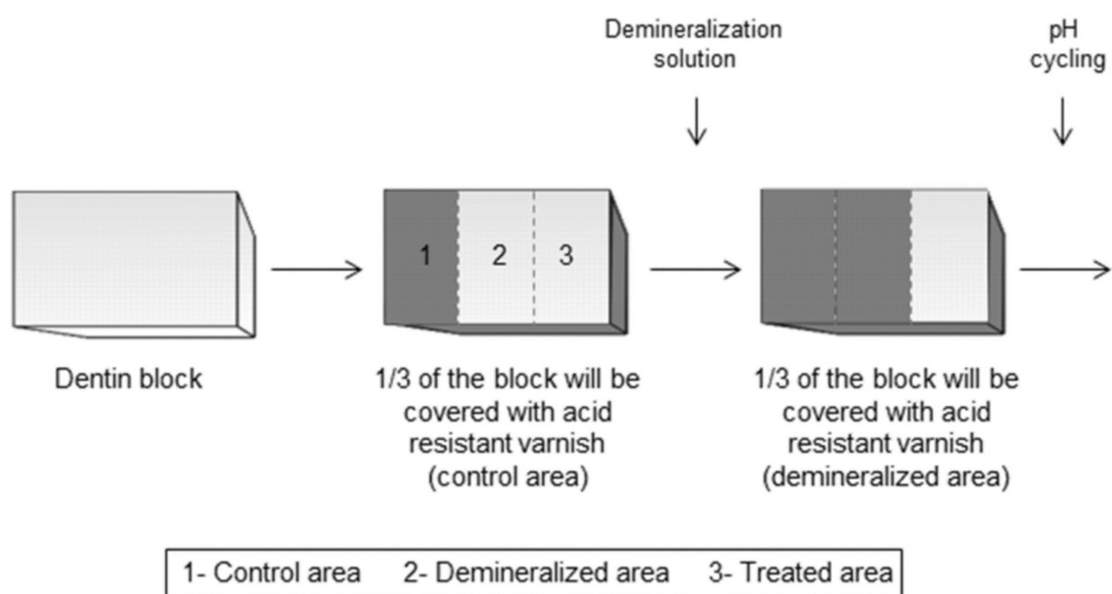
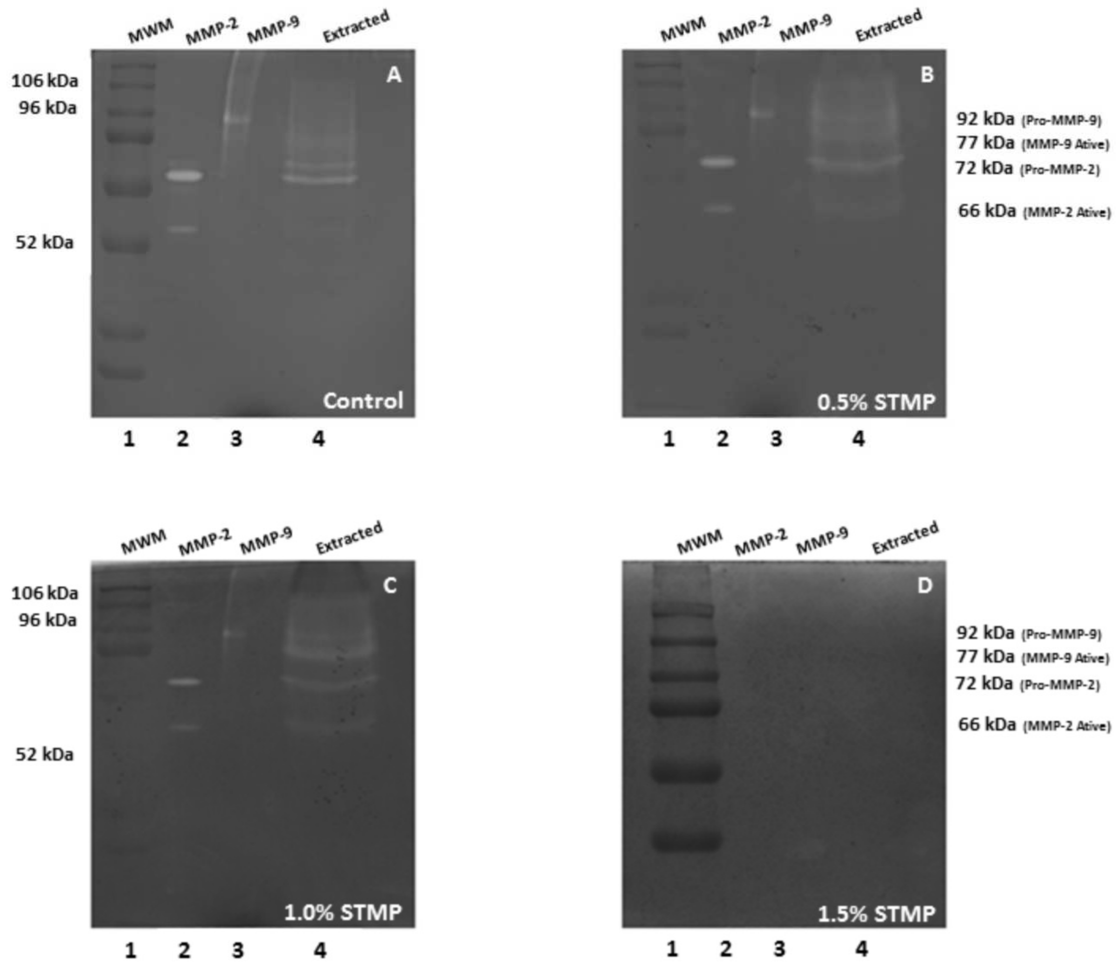
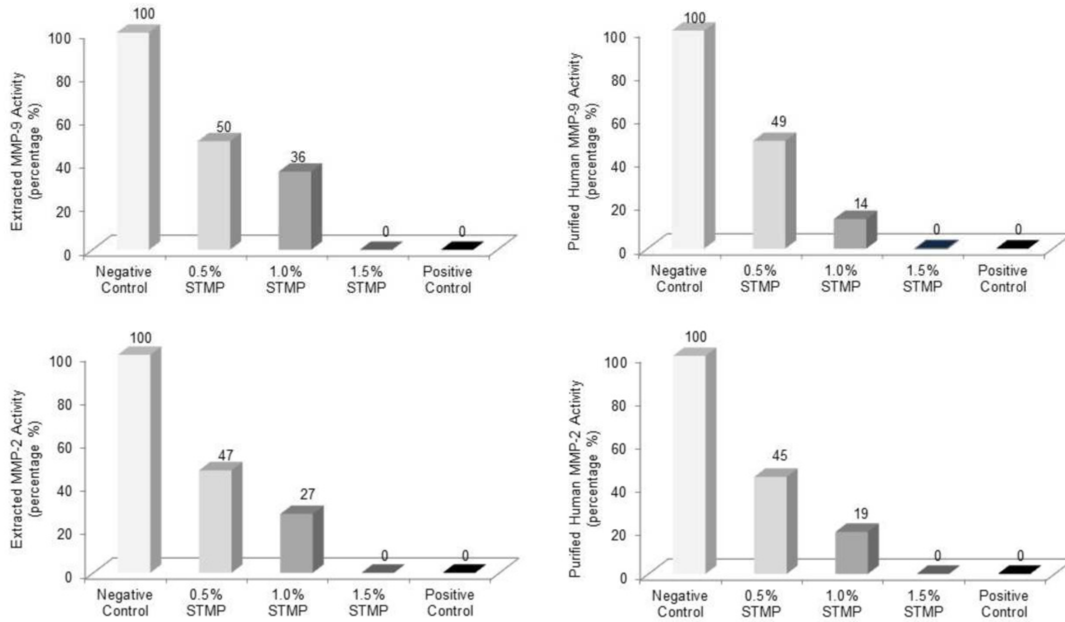


Figure 2



Graphic 1



Graphic 2