Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

MICAELA CARDOSO

Ação de agentes remineralizantes bioativos na redução da desmineralização de lesões de cárie em esmalte

Demineralization of the enamel caries-like lesions decreased by bioative remineralizing agents

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Tese apresentada *à* Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Odontologia, na Área de Odontopediatria.

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Orientadora: Profª Drª Regina Maria Puppin Rontani

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RESUMO

A cárie dentária ainda é uma doença altamente prevalente em todo o mundo, métodos para reverter e controlar esse processo têm sido estudados. Os métodos não invasivos ainda são a primeira alternativa de uso, visando paralisar a progressão da lesão cariosa ou diminuir a desmineralização. Com esse propósito, tem sido estudado o uso de novos materiais bioativos, que prometem realizar a incorporação de minerais no interior do esmalte desmineralizado. Assim, este estudo avaliou a redução da desmineralização do esmalte tratado com diferentes agentes remineralizantes, por meio da determinação do conteúdo mineral por meio de microtomografia computadorizada e análise bioquímica de esmalte bovino tratados com diferentes agentes remineralizantes. Para isso, blocos de esmalte bovino (6x3 mm) foram polidos e selecionados pela dureza média Knoop da superfície (341,18 kg/mm2 ± 10%) e randomizados em 5 grupos de acordo com o agente remineralizante: Duraphat (Controle Positivo), MI Varnish (CPP-ACP), Curodont Repair, Curodont Protect e Sem tratamento (Controle Negativo). Os blocos foram desmineralizados utilizando-se solução desmineralizadora. Então, cada bloco foi dividido em três partes iguais que foram submetidas a três condições de substrato: 1. Esmalte desmineralizado/cárie-ED (2x3mm - linha de base); 2. Esmalte desmineralizado/remineralizado- EDR (2x3mm - aplicação de agentes remineralizantes); e, 3. Esmalte desmineralizado/remineralizado/submetido à simulação de desafios cariogênicos- EDRC (2x3 mm – 8 ciclos de pH- pH *cycling*). Após o término de cada tratamento, os blocos de esmalte foram submetidos às seguintes análises: 1) determinação do conteúdo mineral (ΔZ) por análise em microtomografia computadorizada -μ-CT (n=5); 2) quantificação de CaF_2 na superfície do esmalte (n=24); e 3) Quantificação de fluorapatita (FAp - Biopsia) no interior do esmalte (n=24). Os dados foram submetidos ao teste de normalidade (Kolmogorov-Smirnov) para todas as análises. Na análise por μ-CT, foi aplicado o teste ANOVA um fator. Na análise de CaF_2 e FAp, foi utilizado ANOVA dois fatores com medidas repetidas ($p \leq 0,05$). A densidade mineral, avaliada por μ-CT, mostrou não ser influenciada pelos diferentes agentes remineralizadores no estágio de remineralização ($p = 0,123$) ou mesmo após a ciclagem de pH, neste sentido, todos os grupos de tratamento diferiram significativamente do Controle Negativo ($p=0,001$), mostrando que todos os agentes foram capazes de formar mineral no interior da

lesão. Os grupos Duraphat e MI Varnish, formaram quantidades maiores de CaF_2 e FAp ($p<0,001$) no grupo remineralizado e nos grupos de pH *cycling* em relação aos outros tratamentos. No grupo de ciclagem de pH, não houve diferença significativa entre Duraphat e MI Varnish ($p = 0,259$) na formação de CaF_2, e os outros não diferiram entre si, apresentando menor formação de CaF_2. Em relação a FAp, não houve diferença significativa entre Duraphat e MI Varnish para os blocos remineralizados ($p < 0,001$) e após ciclagem de pH ($p < 0,001$), esses tratamentos não diferiram do CP, mostrando significativamente os maiores valores de FAp. Pode-se concluir que, agentes bioativos são uma alternativa aos agentes fluoretados na redução da desmineralização do esmalte após ciclagem de pH, formando minerais no interior da lesão inicial de cárie.

Palavras-chave: esmalte dental, cárie dentária, desmineralização, biomimético, bioativo, materiais dentários.

ABSTRACT

Dental caries is still a highly prevalent disease throughout the world; methods to reverse and control this process have been studied. Noninvasive methods are still the first alternative of use, aiming to paralyze the progression of the carious lesion or to diminish the demineralization. With this purpose, the use of new bioactive materials has been researched, which promise to perform the incorporation of minerals inside the demineralized enamel. Thus, this study evaluated the reduction of enamel demineralization treated with different remineralizing agents, through the determination of mineral change (ΔZ) by computerized microtomography and biochemical analysis of bovine enamel treated with different remineralizing agents. For this, bovine enamel blocks (6x3 mm) were polished and selected by the surface Knoop hardness (341.18 kg / mm2 ± 10%) and randomized into 5 groups according to the remineralizing agent: Duraphat (Positive Control), MI Varnish (CPP-ACP), Curodont Repair, Curodont Protect and No Treatment (Negative Control). The blocks were demineralized using a demineralising solution. Then, each block was divided into three equal parts that were submitted to three substrate conditions: 1. Demineralized enamel / caries-ED (2x3mm - baseline); 2. Enamel demineralized and remineralized-EDR (2x3mm - application of remineralizing agents); and, 3. Demineralized enamel, remineralized and submitted to the simulation of cariogenic challenges-EDRC (2x3 mm - 8 cycles of pH-pH cycling). After the end of each treatment, the enamel blocks were submitted to the following analyzes: 1) determination mineral content by computerized microtomography -μ-CT analysis (n=5); 2) quantification of CaF_2 on the enamel surface (n=24); and 3) Quantification of fluorapatite (FAp - Biopsy) inside the enamel (n=24). The data were submitted to the normality test (Kolmogorov-Smirnov) for all analyzes. In the μ-CT analysis data, the one-way ANOVA test was applied. In the analysis of CaF_2 and FAp data, ANOVA two-way with repeated measures were used, considering a level of significance of 5%. The mineral density, measured by μ-CT, was not influenced by the different remineralizing agents in the remineralization stage ($p = 0.123$) or even after pH cycling, all treatment groups differed significantly from Negative Control ($p = 0.001$), showing that all agents were able to form mineral within the lesion. The Duraphat and MI Varnish groups formed larger amounts of CaF_2 and FAp ($p < 0.001$) in the remineralized group and in the pH Cycling groups relative to the other treatments. In

the pH cycling group, there was no significant difference between Duraphat and MI Varnish ($p = 0.259$) on CaF_2 formation, and the others did not differ from each other, presenting lower CaF_2 formation. In relation to FAp, there was no significant difference between Duraphat and MI Varnish for the remineralized blocks ($p < 0.001$) and after pH cycling ($p < 0.001$), these treatments did not differ from CP, showing significantly the highest values of FAp. It can be concluded that, bioactive agents are an alternative to fluoride agents in reducing enamel demineralization after pH cycling, forming minerals within the caries-like lesions.

Key words: dental enamel, dental caries, tooth demineralization, biomimetic materials, bioactive, dental materials

SUMÁRIO

1. INTRODUÇÃO

A cárie dentária tem sido reportada como um dos principais fatores de morbidade e em alguns locais, ainda apresenta alta incidência e prevalência (Kassebaum et al., 2017). Inicia-se por um desequilíbrio no sistema des-remineralização do esmalte, por meio de um processo dinâmico, com influência do biofilme cariogênico. Este metaboliza carboidratos em ácidos orgânicos diminuindo o pH do meio bucal e, consequentemente, removendo minerais que compõem a estrutura dentária. No esmalte, a lesão de cárie começa pela desmineralização subsuperficial, deixando uma superfície mineral porosa cobrindo o corpo da lesão (Brochner et al., 2010; Bertassoni et al., 2011), sendo esse processo denominado lesão inicial de cárie. Dessa forma, se a lesão inicial não for controlada, poderá progredir para uma cavitação (Nyvad & Fejerskov, 1997; Ehrlich et al., 2009).

O esmalte dental é composto por 96% de matéria inorgânica e 4% de matéria orgânica e água (Alkattan et al., 2018). O material inorgânico é composto principalmente pelo mineral hidroxiapatita (HAp), uma estrutura cristalina composta por cálcio (Ca), fosfato (P) e hidroxila. Quando há a presença do fluoreto (F), esses íons podem substituir grupos hidroxila e diminuir a solubilidade da apatita, formando um novo mineral chamado apatita fluoretada (fluorapatita) (Dowker et al., 1999). O fluoreto, quando disponível, tem o papel de equilibrar os desafios des-remineralização, reduzindo a perda mineral e retardando a progressão da lesão, porém não é capaz de evitar a doença (Cury et al., 2016).

Um dos meios de se utilizar o fluoreto é de forma tópica por dentifrícios fluoretados ou aplicações profissionais de soluções ou géis concentrados, contribuindo para agir na desmineralização (Arnold et al., 2006; Souchois & Vieira, 2012; Kantovitz et al., 2013; Gelani et al., 2014). Dentre os mais preconizados, os vernizes encontram respaldo para pacientes com pouca idade e com atividade de cárie.

A ação dos vernizes fluoretados se dá devido á formação de produtos de reatividade do tipo fluorapatita e fluoreto de cálcio na superfície do dente. Neste processo o fluoreto de cálcio é formado em maior quantidade, funcionando como depósito de fluoreto, onde esses íons são liberados quando ocorre um desafio cariogênico (ten Cate, 1997). Com essa liberação de fluoreto para o meio, este se torna livre e disponível para atuar no processo de cárie, reduzindo a desmineralização e ativando o processo de remineralização pela precipitação de apatita fluoretada, a fim de substituir os minerais perdidos (Retief et al.,1983).

Parece provável que a paralização ou redução da desmineralização seja importante em relação ao mecanismo de ação do fluoreto, porém, encontra-se consolidado que o mesmo é mais eficaz na superfície da lesão da cárie, o que não resulta numa remineralização total da lesão (ten

Cate & Arends, 1980). Na atualidade, novos materiais bioativos que prometem resultados similares ou complementares ao fluoreto estão sendo desenvolvidos. Estes materiais prometem um mecanismo de ação diferente dos materiais fluoretados. Segundo Lynch & Smith, 2012, os agentes presentes nestes materiais elevam as concentrações de cálcio no ambiente bucal, migrando esses íons para o interior da lesão de cárie. A associação de cálcio e fluoreto em agentes remineralizantes tem como proposta efetivar a consolidação mais completa das lesões, servindo como abordagens adicionais para controlar a progressão da doença (Lynch & Smith, 2012).

Nesta abordagem, ganha destaque o complexo CPP-ACP. Neste agente o fosfopeptídeo de caseína (CPP), uma proteína presente no leite, é utilizada para estabilizar os íons cálcio e fosfato em altas concentrações de nanocomplexos amorfos designados por fosfato de cálcio amorfo (ACP) (Reynolds, 1997). O CPP permite que altas concentrações dos íons cálcio e fosfato sejam estabilizados em soluções para que estejam biodisponível promovendo a remineralização (Cochrane et al., 2008). A associação CPP-ACP tem sido incorporada em dentifrícios, gomas de mascar, enxaguatórios bucais (Iijima et al., 2004) e vernizes fluoretados. A tecnologia sobre CPP-ACP se dá devido ao transporte de cálcio (e fosfato) em esmalte desmineralizado, que pode ocorrer através de pares de íons neutros, que se dissociam uma vez dentro da lesão e contribuem para a remineralização, tornando-o um agente remineralizador bioativo (Cochrane et al., 2008). A adição de CPP-ACP nos vernizes fluoretados, resultando no MI Varnish, poderá tanto potencializar os efeitos terapêuticos do flúor proporcionando a remineralização de lesões de cárie iniciais como servir como método preventivo da desmineralização (Cochrane et al., 2010; Karlinsey et al., 2010; Schemehorn et al., 2011; Cochrane et al., 2014).

Outra proposta atual de alguns novos materiais é remineralização biomimética do esmalte que foi recentemente ampliada pelo uso de arcabouços de auto-montagem de peptídeos para promover a recristalização das lesões subsuperficiais de esmalte (Kirkham et al., 2007; Benesch et al., 2008; Segman-Magidovich et al., 2008). Esses peptídeos foram projetados para se auto organizarem em função do pH, com membranas lipídicas, e já estão sendo produzidos e utilizados em aplicações nanotecnológicas (Aggeli et al., 1997a; Aggeli et al., 2001; Zhang, 2003; Rajagopal e Schneider, 2004; Reches & Gazit, 2006). Seu mecanismo de ação tem sido atribuído ao fato da projeção dos peptídeos que se reúnem espontaneamente em resposta a gatilhos ambientais para formar arcabouços biomiméticos 3D sendo capaz de nuclear a hidroxiapatita de novo (Kirkham et al., 2007). Algumas pesquisas mostraram que o tratamento com peptídeos (Curodont Repair P11-4) reduziu significativamente a desmineralização do esmalte, medida pelo conteúdo de fosfato da solução de desmineralização, após submeter as lesões artificiais de cárie a ciclagem de pH. Os autores especularam que a inibição da desmineralização pode ter sido atribuída à formação de minerais na lesão induzida pela automontagem dos peptídeos (Kirkham et al., 2007). Porém, neste

estudo a esta ação anticárie foi constatada quando comparada ao grupo sem nenhum tratamento, sendo que para que um agente apresente potencial para uso sua ação seja comparável à ação anti-cárie dos tradicionais produtos fluoretados, que são atualmente indicados para tratamento de lesões de cárie inicial.

Dessa forma, estudos envolvendo agentes remineralizadores que sejam capazes de promover a diminuição da desmineralização, incorporação de minerais na superfície do esmalte e no interior da lesão, avaliando ganho ou perda mineral através de análises bioquímicas e microscópicas, quando submetido aos desafios similares aos que ocorrem no meio ambiente bucal são de relevância para se encontrar evidências para realização de futuros estudos clínicos.

2 ARTIGO - Demineralization of the enamel caries-like lesions decreased by bioactive remineralizing agents

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Abstract

Dental caries is still a highly prevalent disease throughout the world; methods to reverse and control this process have been studied. Noninvasive methods are still the first alternative of use, aiming to paralyze the progression of the carious lesion or to diminish the demineralization. With this purpose, the use of new bioactive materials has been researched, which promise to perform the incorporation of minerals inside the demineralized enamel. Thus, this study evaluated the reduction of enamel demineralization treated with different remineralizing agents, through the determination of mineral change (ΔZ) by computerized microtomography and biochemical analysis of bovine enamel treated with different remineralizing agents. For this, bovine enamel blocks (6x3 mm) were polished and selected by the surface Knoop hardness (341.18 kg / mm2 ± 10%) and randomized into 5 groups according to the remineralizing agent: Duraphat (Positive Control), MI Varnish (CPP-ACP), Curodont Repair, Curodont Protect and No Treatment (Negative Control). The blocks were demineralized using a demineralising solution. Then, each block was divided into three equal parts that were submitted to three substrate conditions: 1. Demineralized enamel / caries-ED (2x3mm - baseline); 2. Enamel demineralized and remineralized-EDR (2x3mm - application of remineralizing agents); and, 3. Demineralized enamel, remineralized and submitted to the simulation of cariogenic challenges-EDRC (2x3 mm - 8 cycles of pH-pH cycling). After the end of each treatment, the enamel blocks were submitted to the following analyzes: 1) determination mineral content by computerized microtomography -μ-CT analysis (n=5); 2) quantification of CaF_2

on the enamel surface (n=24); and 3) Quantification of fluorapatite (FAp - Biopsy) inside the enamel (n=24). The data were submitted to the normality test (Kolmogorov-Smirnov) for all analyzes. In the μ-CT analysis data, the one-way ANOVA test was applied. In the analysis of CaF_2 and FAp data, ANOVA two-way with repeated measures were used, considering a level of significance of 5%. The mineral density, measured by μ-CT, was not influenced by the different remineralizing agents in the remineralization stage ($p = 0.123$) or even after pH cycling, all treatment groups differed significantly from Negative Control ($p = 0.001$), showing that all agents were able to form mineral within the lesion. The Duraphat and MI Varnish groups formed larger amounts of CaF_2 and FAp ($p <0.001$) in the remineralized group and in the pH Cycling groups relative to the other treatments. In the pH cycling group, there was no significant difference between Duraphat and MI Varnish ($p = 0.259$) on CaF_2 formation, and the others did not differ from each other, presenting lower CaF_2 formation. In relation to FAp, there was no significant difference between Duraphat and MI Varnish for the remineralized blocks ($p <0.001$) and after pH cycling ($p <0.001$), these treatments did not differ from CP, showing significantly the highest values of FAp. It can be concluded that, bioactive agents are an alternative to fluoride agents in reducing enamel demineralization after pH cycling, forming minerals within the caries-like lesions.

Key words: dental enamel, dental caries, tooth demineralization, biomimetic materials, bioactive, dental materials

INTRODUCTION

Fluoride is the most commonly used remineralizing agent. Some authors have proved the efficacy of fluoride to increase the resistance of tooth mineral to demineralization by acids as well as to promote remineralization of incipient lesions. The primary preventive actions of caries with fluoride are posteruptive due to topical effects that can interfere in the dynamic balance at the interface between mineral surface and oral fluid (Rošin-Grget *et al*., 2013). The action of high fluoride concentration agents occurs by the reactivity that occurs between fluoride ions and the enamel forming mainly loosely bound fluoride (CaF_2-like) and firmly bound fluoride (FAp) on the enamel surface to promote a protective effect against acidic challenges (ten Cate, 1997; Fernandez *et al*., 2014). The CaF_2-like is covered by phosphate and protein, and in the demineralization process with low pH, fluoride and calcium ions are released (ten Cate, 1997), forming fluorapatite to

regulate demineralization / remineralization process (Retief *et al*., 1983). Despite the known effect of fluoride, finding other innovative non-fluoridated agents is desirable in order to enhance the fluoride effect or to serve as an new alternative to fight against caries process.

Remineralizing agents that have a bioactive effect have been studied in the remineralization process of initial caries lesions. The progress obtained with calcium based remineralizing agents such as CPP-ACP (amorphous calcium phosphate - casein) is encouraging and scientifically accepted. This technology on CPP-ACP occurs due to the transport of calcium and phosphate in demineralized enamel, which can occur through pairs of neutral ions, which dissociate once inside the lesion and contribute to remineralization (Cochrane *et al*. 2008). The addition of CPP-ACP to fluoride varnishes, may both enhance the therapeutic effects of fluoride by providing remineralization of early caries lesions and serve as a preventive demineralization method (Cochrane *et al*., 2010; Karlinsey *et al*., 2010; Schemehorn *et al*., 2011, Cochrane *et al*., 2014).

Although not fully elucidated, it is suggested that mineralization in natural systems, as well as the synthetic hydrogel, is initiated by the formation of poorly crystalline calcium apatites, which then undergo group transitions to form stable apatites with higher crystallinity. The process is mediated by acidic proteins in the extracellular matrix, or anionic groups in the polymeric hydrogel that serves as a model for joining the inorganic cations and aligns them with the interlaced crystal of the apatite. Mineralization occurs on surfaces with repetitive patterns of negatively charged groups. In addition, the peptide hydrogel modified with the sequence of adhesion of bioactive amino acids like Arg-Gly-Asp proved to be scaffold ideal for the proliferation of odontogenic cells and regeneration of dentin and enamel (Palmer *et al*., 2008).

Another alternative, through biomimetic remineralization of enamel has recently been expanded by the use of self-assembling frameworks of peptides to promote the recrystallization of subsurface enamel lesions (Kirkham *et al*., 2007; Benesch *et al*., 2008; Segman-Magidovich *et al*., 2008). These peptides were designed to self-organize as a function of pH, with lipid membranes, and are already being produced and used in nanotechnological applications (Aggeli *et al*., 1997a; Aggeli *et al*., 2001; Zhang 2003; Rajagopal & Schneider, 2004; Reches and Gazit, 2006). Its mechanism of action seems to be due to the projection of the peptides that meet spontaneously in response to

environmental triggers to form biomimetic 3D frameworks being able to nucleate the hydroxyapatite de novo (Kirkham *et al.*, 2007). Some research has shown that treatment with peptides significantly reduced enamel demineralization, as measured by the phosphate content of the demineralization solution, after subjecting artificial caries lesions to pH cycling. The authors speculated that inhibition of demineralization may have been attributed to the formation of minerals in the injury induced by self-assembling of peptides (Kirkham *et al.*, 2007) but didn't compare with a gold standard fluoride remineralizing agent.

Given the supposed successes obtained with the related materials, it is extremely important to test *in vitro* the formation of minerals and decrease of the demineralization promoted by the exposed remineralizing agents, comparing them with the fluoride agents, in conditions that simulate a high cariogenic challenge. The hypotheses tested by this study were: 1. The amount of mineral loss (ΔZ) of caries-like lesion by micro-CT treated with remineralizing agents based on fluoride and bioactive remineralization are different; 2. The amount of loosely bound fluoride (CaF_2-like) and firmly bound fluoride (FAp) found on enamel caries-like lesion is different after application of different compositions remineralizing agents; 3. Remineralizing agents based on fluoride and bioactive are different concerning CaF_2-like and FAp in caries-like.

The aims of this study were:

1. To quantify the mineral change (ΔZ) of caries-like lesions treated with remineralizing agents by µ-CT.
2. To quantify *in vitro* formation of loosely bound fluoride *(*CaF_2-like) and firmly bound fluoride (FAp) in enamel artificial caries-like lesions after application of different compositions of remineralizing agents.
3. Measure the capacity of these remineralizing agents of inhibiting the enamel demineralization and enhance its remineralization.

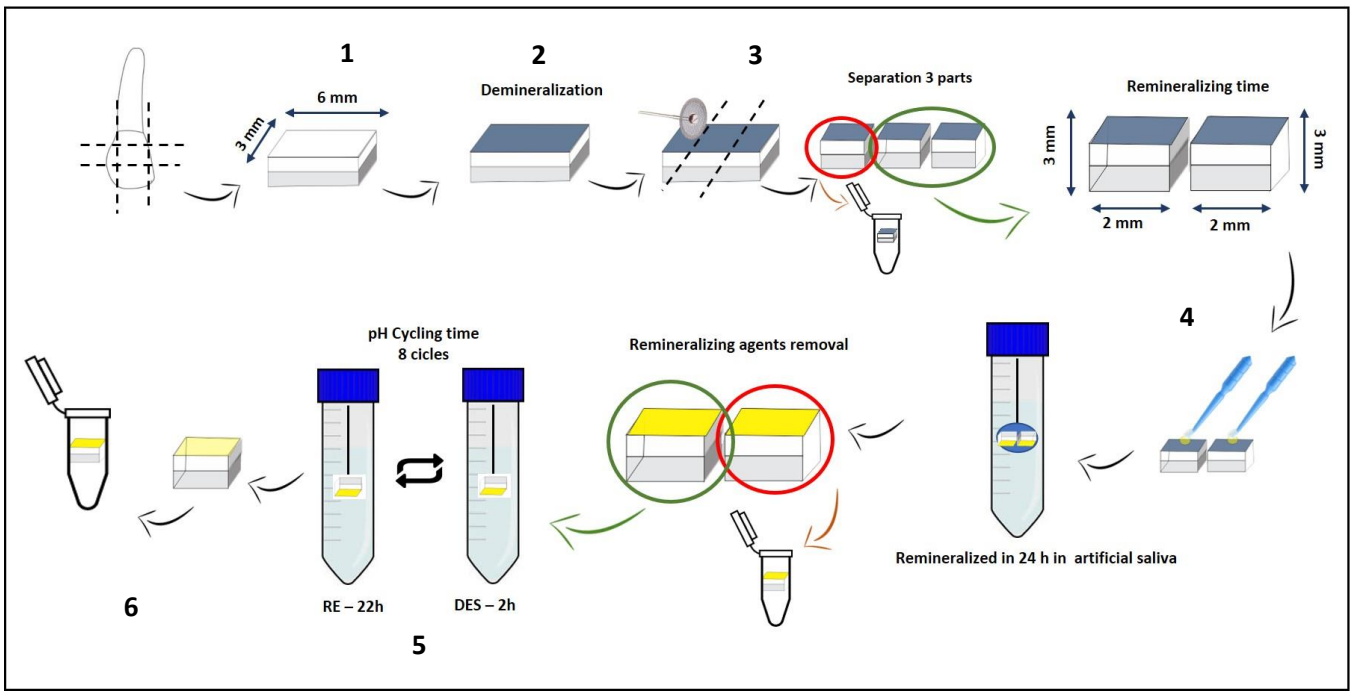
MATERIAL AND METHODS

Experimental Design

This *in vitro* experimental exploratory study was carried out in a completely randomized design to study the application of remineralizing agents on enamel surface submitted in

different condition (demineralized-ED, demineralized+remineralized-EDR, demineralized+remineralized+ cariogenic challenge-EDRC) were done in a dependent way. The specimens used were enamel bovine blocks (6 x 3 x 2 mm), which were randomly divided into 5 groups (n=29) according to the treatment: Duraphat-DU, MI Varnish-MI, Curodont Repair-CR, Curodont Protect-CP and without treatment/negative control-NC. Then, substrate conditions were accomplished using parts of the same block divided into three equal parts, which was submitted previously to demineralization condition. Briefly, each block had the enamel polished surface demineralized. Then, a third part of each block was immersed in artificial saliva. The other part was submitted to a remineralization and then divided into two equal parts. A half was kept in artificial saliva and the other one submitted to a ph cycling (Figure 1). The dependent variables of this study were: mineral concentration obtained by µ-CT, CaF_2-like concentration in enamel (µgF/cm^2); FAp formed in enamel (µg F/g of enamel) obtained by enamel biopsy.

Figure 1. Experimental design outlined in this study. 1. Enamel bovine blocks (6x3x3 mm) from bovine teeth; 2. enamel demineralization; 3. enamel blocks cutting on 3 equal parts (2x3x3 mm); 4. two out of three specimens were submitted to remineralizing agents following the protocol described on material and methods; 5. one of two specimens was submitted to pH Cycling; 6. stored in relative humidity until analysis.



Specimen preparation

One hundred forty-five blocks with 6x3x2 mm were cut from bucal surface of bovine incisors using a cutting machine (Isomet® Low Speed Saw, Buehler, IL, USA) using two water-cooled diamond discs (Extec Corp., Enfield, Conn., USA). The dentin was ground flat using silicon carbide paper of 340-grit to obtain parallelism between top and bottom surfaces of each block. Then, the enamel surface of the blocks was ground flat with water-cooled silicon carbide discs (400-, 600-, 1,200 and 2,500 grade papers; Buehler), and wet-polished with felt paper using diamond spray (6, 3, and 1 µm; Buehler, Lake Bluff, Illinois, USA). A baseline enamel surface hardness was determined on the outer enamel surface by making 3 indentations, spaced 100 µm from each other, using a Knoop hardener indenter with a 25 g load for 5 s and a microhardness tester coupled to FM-ARS 900 software (Future-Tech FM, Kawasaki, Japan). The blocks presenting hardness of 341.18 Kg/mm^2 ± 10% were randomly divided into five groups (n = 29), according to the remineralizing agents described on the table below:

Table 1 – Material composition, amount of fluoride and manufacturers of the remineralizing agents used in the study.

Material	Composition	Fluoride	Manufacture
Duraphat® 	30–60% colophonium, 10–30% ethanol, 5% sodium fluoride, white wax, shellac, mastic saccharin, raspberry essence (containing Ethyl butyrate, geraniol, resinoid iris, isoamyl acetate, essence of jasmine, vanilla and propylene glycol).	5% NaF (22.600 ppm)	COLGATE® (New York, USA)
MI Varnish™ 	30–50% polyvinyl acetate, 10–30% hydrogenated rosin, 20–30% ethanol, 1–8% sodium fluoride, 1–5% CPP-ACP, 1–5% silicone dioxide.	5% NaF (22.600 ppm)	GC CORPORATION (Tokyo, Japan)
Curodont Repair® 	self-assembling β-peptide	No fluoride containing	CREDENTIS (Windisch, Swiss)
Curodont Protect® 	self-assembling β-peptide gel	900 ppm of Sodium monofluorophosphate (MFP)	CREDENTIS (Windisch, Swiss)

The fluoride of the studied products was dosed previously to the experiment phases. Fluoride amount was similar to the dose described by the manufacturers on the package.

All surfaces of each block were covered with acid-resistant varnish (Colorama, CEIL Exp. Exp. Ltda, São Paulo, SP, Brazil), leaving the highly polished enamel surface exposed. Surface enamel was cleaned using a brush and with a mixture of pumice + deionized water to remove residues and them it was rinsed with distilled water for 30 s. Next, a prophylaxis was performed on the surface for the removal of residues.

Enamel demineralization

All enamel blocks (6 x 3 x 2 mm) were immersed individually in demineraralizing solution (0.05 mol/L acetate buffer, pH 5.0, 1.28 mmol/L Ca, 0.74 mmol/L Pi and 0.03 µg F/mL from the salts $Ca(NO_3)_2.4H_2O$, KH_2PO_4 and NaF, respectively - 2 mL/mm^2 of enamel area) for 32 h to induce caries-like lesion formation on enamel with no surface erosion (Queiroz *et al*., 2008).

After demineralization, all blocks were washed with deionized water and dryed with absorbent paper. The blocks were cut into three equal parts (2 x 3 x 2 mm) using double face diamond disc (Microdont, São Paulo, Brasil), and all of them had their sides and bases protected with dental wax. The first slab from the enamel block was stored in the refrigerator to posterior analysis.

Enamel remineralization

The other two parts of the block were submitted to remineralization, following the manufacturer instructions. Immediately after the remineralizing agent application, each block was placed in an individual container containing 37.45mL of artificial saliva (1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/ mL in 0.1 mol/L Tris buffer, pH 7.0 in the solution proportion of 3.12 mL/mm^2) for 24 h (Fernandez *et al*., 2014; Delbem *et al*., 2006).

Both Duraphat (Colgate, New York, USA) and MI Varnish (GC Corp.; Tokyo, Japan) were applied in a thin uniform layer on the enamel surface with a disposable brush and the block was storage in artificial saliva.

Curodont™ Repair (Credenits AG, Windisch, Switzerland) was applied according to the manufacturer's instructions. Product powder was dissolved in 0.05 mL of H_2O, then it was applied on the enamel surface, after 5 min the blocks were stored in artificial saliva to be in the same conditions as the varnishes groups.

Curodont™ Protect (Credenits AG, Windisch, Switzerland) was applied using a microbrush according to the manufacturer's instructions over the white spot lesion, after 1-2 minutes the blocks were stored in artificial saliva in the same conditions.

The negative control enamel blocks received no treatment, but they were stored in artificial saliva as described for experimental groups.

After 24 h, the varnishes were removed from the enamel block surfaces with acetone solution-imbibed cotton swab (acetone and deionized water 1:1) and, the blocks were washed with deionized water (Retief *et al*., 1983; Brunn & Givskov, 1991; Fernandez *et al*., 2014).

Acid challenge using pH cycling

After remineralization time, the third part of each block was submitted to acid challenge, in order to simulate a high caries activity patient, based on the dynamic model of de-remineralization cycling proposed by Queiroz et. al. (2008). The pH cycling consisted of 8 cycling days of des and remineralization. Blocks were immersed for 2 h in the demineralizing solution and for 22 h in remineralizing solution at 37°C. On the 4th cycle day, the de- and remineralizing solutions were replaced by fresh ones. After the treatments, the enamel blocks were washed with deionized water and stored in refrigerator.

The demineralizing solution (DE) was prepared from 0.05 mol/L acetate buffer pH 5.0 and containing 1.28 mM/L Ca, 0.74 mM/L and 0.03 µg F/mL from the $Ca(NO_3)^2$ $4H_2O$, KH $2PO_4$ and NaF salts, respectively. The remineralizing solution (RE) was prepared from 1.5 mM/L Ca, 0.9 mM/L Pi, 150 mM/L KCl, 0.05 µg F/mL in 0.1 M Tris/L, pH 7.0. The ratio of DE and RE solutions per exposed area was 6.25 mL/mm^2 and 3.12 mL/mm^2, respectively.

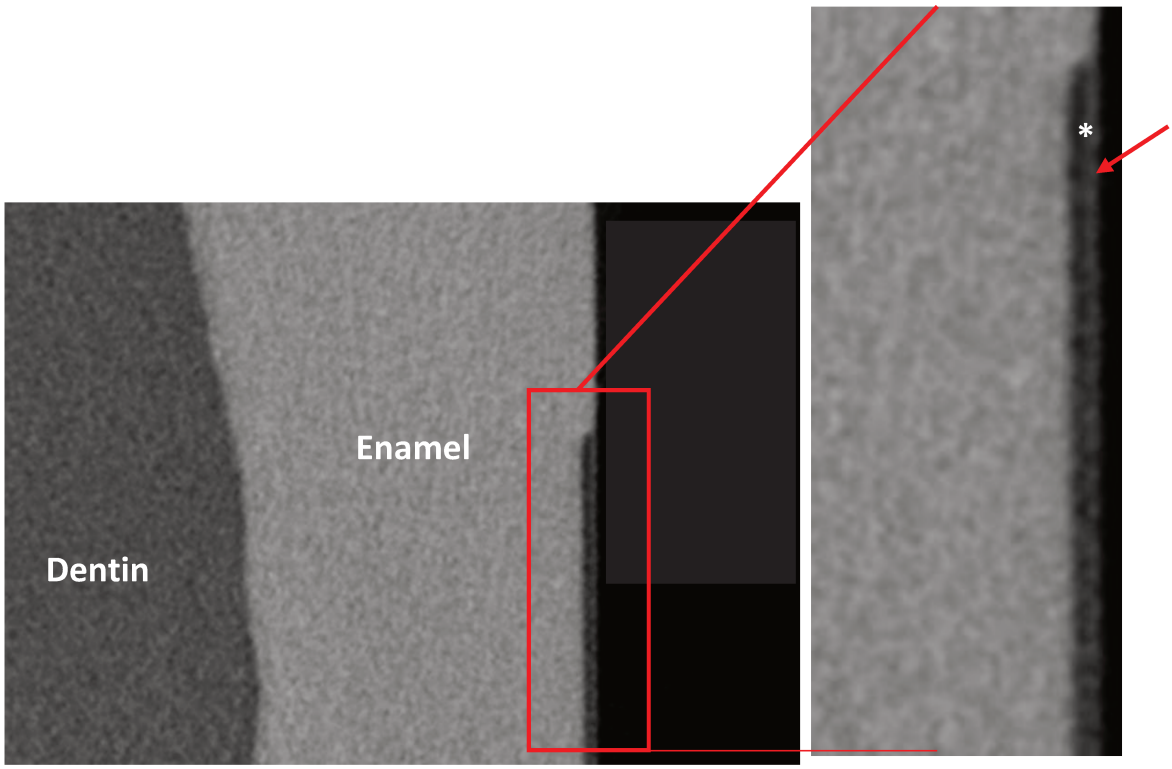
Micro-CT acquisition and reconstruction procedures

The specimens (n = 5) were scanned in a nanofocus X-ray inspection system (Phoenix Nanome|X, GE, Saint Paul, MN, USA). Blocks were wrapped in parafilm™ during the scanning procedures to avoid desiccation. Acquisition parameters included: 80 kV, 200 µA, detector size 890 X 1000 pixels, 20.30 µpixel size. Scanning time was approximately 85 min for each specimen. After acquisition, cross-sections of each specimen were reconstructed using a proprietary software (Datos|x, GE, Saint Paul, MN, USA).

The reconstructed stack was imported into an open source 3D visualization software interface (3D Viewer, FIJI) and a volume of interest (VOI) containing the enamel surface was selected. The selected VOI was projected using a median algorithm (Z project function, FIJI). Each projection was used to obtain linear density profiles starting from the enamel surface up to 250 µm deep into the enamel layer.

Profiles from each study group (demineralized, remineralized, pH cycled) were plotted and the mineral loss parameter ΔZ was calculated for remineralization and pH cycling groups in each specimen. The ΔZ values from the demineralized profiles were subtracted from values obtained from the remineralizaed and pH cycled groups to calculate the final mineral change in each specimen. Values are represented in pixel/µm.

Figure 2: µ-CT image from a specimen obtained after caries producing. Red square/biggest image shows the enamel caries-like lesion under enamel surface (subsurface demineralization) with 50 µm depth.



* identifies the demineralized enamel under enamel surface; red arrow identifies the enamel surface covering the demineralized enamel.

Determination of loosely bound fluoride CaF_2-like found on Enamel

All enamel blocks (n=24) were individually immersed in 0.2 mL of 1 M KOH (4.44 mL/cm²) for 24 h under mechanical agitation at room temperature to extract the CaF_2 ion (Caslavska *et al*., 1975). The extract was buffered with an equal volume (0.2 mL) of TISSAB II containing 1 M HCl; then, fluoride was determined using a specific ion electrode (Orion model 96-09, Orion Research Cambridge, MA, USA) coupled to an ion analyzer (Orion EA 940, Cambridge, MA, USA). The results were expressed as μg F/cm^2 of enamel area (Fernández *et al*., 2014).

Determination of Firmly bound fluoride FAp found on Enamel

Blocks from all groups were immersed in 0.25 M HCl (3.57 mL/cm²) for 15, 30 and 60 s as under constant agitation (150 rpm) to extract an enamel layer. Consequently, this extract was buffered with an equal volume (0.25 mL) of TISAB II (total ionic strength adjustment buffer), pH 5.0, modified with 20 g NaOH/L (Koo & Cury, 1998). The F concentration was determined using a specific F electrode as described for CaF_2-like. Fluoride concentration was expressed in μg F/ cm^2 of enamel (Delben *et al*., 2005)

Statistical Analysis

The Kolmogorov-Smirnov and Levene tests were applied to verify the normality of the data and homogeneity of variances. The micro-CT data showed normal distribution, then, data were submitted to One- way ANOVA.

The CaF_2 and FAp data was transformed by box cox. Then it was used Two-way ANOVA with repeated measures, the factors considered was the treatment group and the phases (demineralized enamel, remineralize enamel and pH cycled enamel). All the statistical analyses were performed using the software SPSS (SPSS 22.0; SPSS, Munich, Germany) with significance level set at 5%.

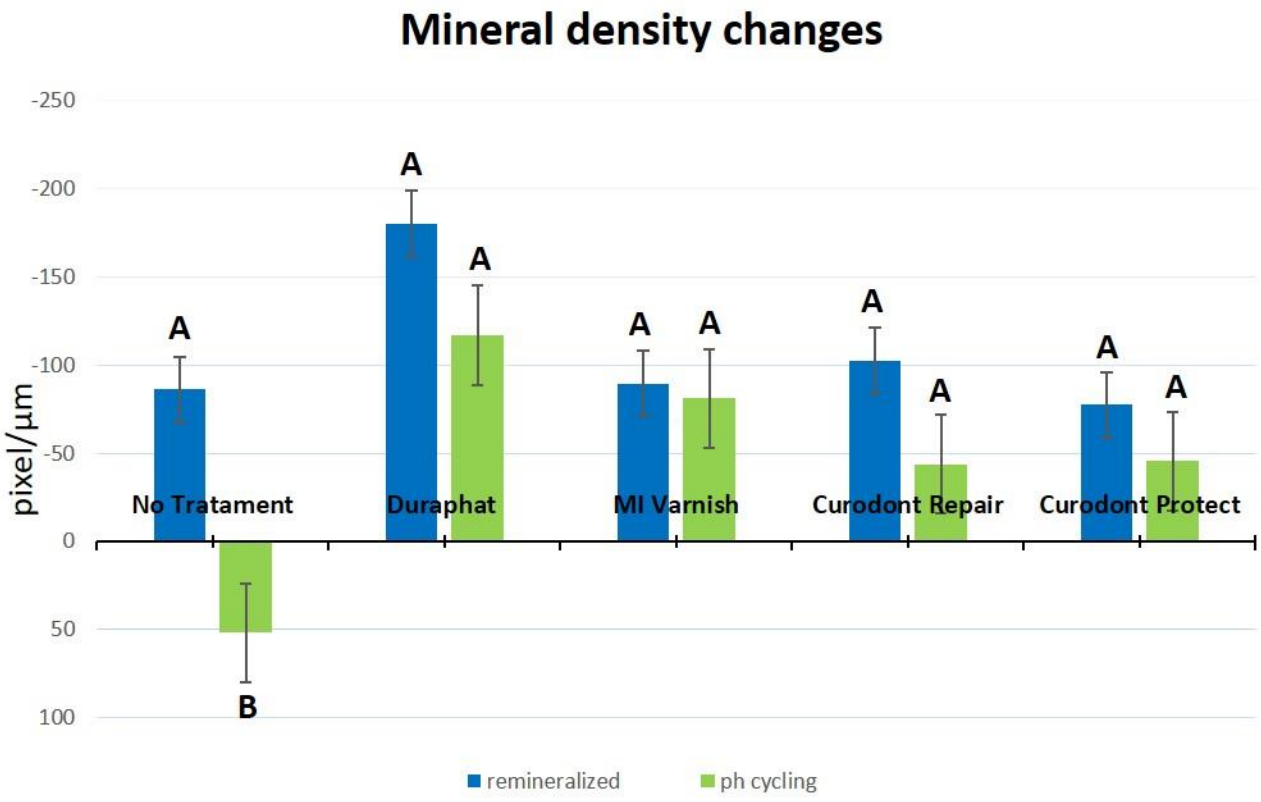
RESULTS

µ-CT analyses

For EDR group, there was no significant difference between groups in relation to mineral density (p=0.123).

For pH cycling group, every treatment group showed statistical difference in relation to the negative control group (p=0.001). The treatment groups Duraphat (p=0.001) and MI Varnish (0.006) showed a better performance in relation to Negative Control group.

Figure 3. Mineral density changes after remineralization and pH Cycling groups. Bars followed by similar capital letters means no significant statistically difference, concerning each group, Remineralized and pH Cycling.



Determination of CaF_2-like

For demineralized group (ED), as expected, the amount of CaF_2-like did not differ between the treatment groups, which reinforces the validity of the experiment, since the specimens showed similar conditions.

For remineralized group (EDR), it was detected a statistically significant difference among the groups concerning CaF_2 amount in enamel. The highest amount of CaF_2 was found when demineralized enamel was treated by Duraphat (27.8 ugF/cm^2), followed by MI Varnish (18.4 ugF/cm^2). The Curodont Repair and no treatment (negative control) group showed the lowest amount of CaF_2 in enamel; however, there was no significant difference between them concerning CaF_2. Even with low fluoride concentration (900 ppm) compared to Duraphat and MI Varnish, the Curodont Protect group showed significant higher CaF_2 than Curodont Repair and no treatment group. This showed that, even at low concentrations, the formation of CaF_2 was sufficient to differ from the use of non-fluoride products and even the non-use of any remineralizing agent.

After pH cycling, the remineralizing agents influenced the amount of CaF_2 formation present on enamel surface. The Curodont Protect and Curodont Repair treatment groups did not differ from no treatment group, showing that the low amount of fluoride (900 ppm) was not sufficiently capable of maintaining the calcium fluoride reserve in the enamel. However, the groups Duraphat (5.04 µgF/cm^2) and MI Varnish (2.98 µgF/cm^2) showed the greatest values.

Determination of FAp

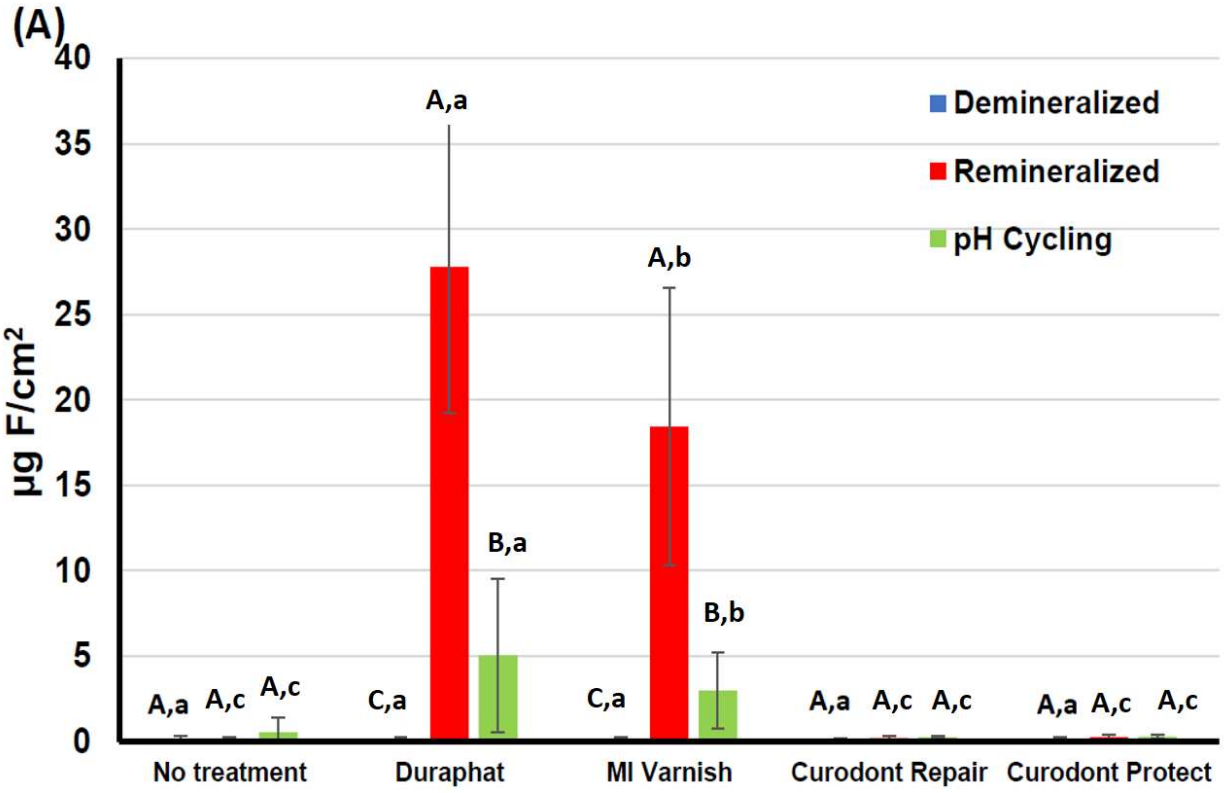
As expected on ED blocks, the specimens presented similar characteristics, showing no statistical difference among the groups on FAp amount in enamel.

For EDR, the formation of FAp was influenced by the treatments. No statistically significant difference was found between the Duraphat and MI Varnish as both groups presented a higher FAp formation in relation to the no treatment, Curodont Repair and Curodont Protect groups, which did not present statistical difference on the formation of FAp among them.

For pH cycling groups, the formation of FAp was influenced by the treatments. The Duraphat and MI Varnish groups presented higher FAp formation after pH cycling than the

other groups. No significant statistical difference was found between the Curodont Protect, Curodont Repair and no treated groups in the FAp formation after pH cycling. No statistical significant difference was detected between the Duraphat and MI varnish, concerning FAp formation after pH cycling. Although Curodont Protect presented 900 ppm of F there was no significant statistical difference between that group and Curodont Repair, which that does not present F in their composition.

Figure 4. Means and standard deviations of loosely bound fluoride (CaF_2) **(A)** and firmly bound fluoride (FAp) **(B)** found on enamel (µg F/cm^2). Unlike capital letters show difference between treatment groups within the same substrate (ED, EDR and EDRC), and unlike lower case letters show difference between substrate (ED, EDR and EDRC) within the same treatmend group ($p < 0,05$).


(A)
µg F/cm²
40
35
30
25
20
15
10
5
0
Demineralized
Remineralized
pH Cycling
A,a
A,c
A,c
C,a
A,a
B,a
C,a
A,b
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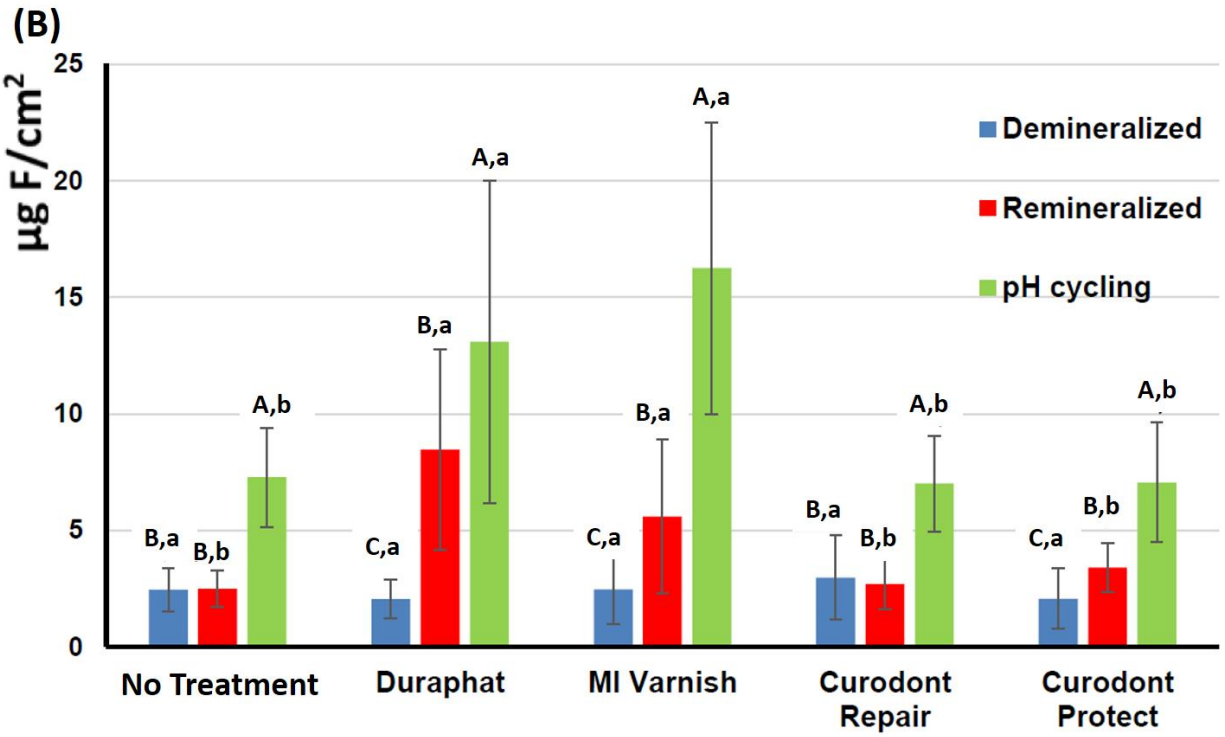
A,a
A,c
A,c
No treatment
Duraphat
MI Varnish
Curodont Repair
Curodont Protect


(B)
µg F/cm²
25
20
15
10
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0
Demineralized
Remineralized
pH cycling
B,a
B,b
A,b
C,a
B,a
A,a
C,a
B,a
A,a
B,a
B,b
A,b
C,a
B,b
A,b
No Treatment
Duraphat
MI Varnish
Curodont Repair
Curodont Protect

DISCUSSION

The present *in vitro* study compared the effect of bioactive remineralizing agents (fluoride-containing CPP-ACP and self-assembling peptides with and without fluoride) compared with fluoride-containing varnish in the reduction of demineralization of caries-like lesions under demineralizing conditions. Hypothesis tested were confirmed since different remineralizing agents based on fluoride or bioactives remineralization showed different performance concerning ΔZ, CaF_2 and FAp formation.

Duraphat was used as a positive control in this study as it is a commonly used fluoride varnish that has been tested in other studies and have been success in terms of fluoride release and caries prevention (Shen *et al*., 2002; Castillo *et al*., 2001; Marinho *et al*, 2013; Shen *et al*., 2016).

The pH-cycling protocol can be used to simulate acid challenges (des-and-remineralization) similar to that occurs in oral environment. This study designed to mimic the dynamics of mineral loss and gain involved in caries formation (Vieira *et al*., 2005; Delbem *et al*., 2006; Queiroz *et al*., 2008; Lata *et al*., 2010). The pH cycling protocol simulated in this study was based on the model described by Queiroz *et al* (2008). This validated pH cycling model that was used to simulate the potential demineralized enamel area reduces using dentifrice formulations due simulates *in vivo* high caries risk situations and also measures the net result of the inhibition of demineralization and the enhance of remineralization (Queiroz *et al*., 2008). Due these positive results and because it is a very well designed model, was the one chosen for the development of this study.prevention

The time of the blocks treated immersion in artificial saliva in this study was 24 h, in accord of Fernandez *et al* (2014), relates the reactivity of the whole varnish with enamel was stabilized only after 24 h. Therefore, following this same protocol for all treatment groups. As it has already been seen, the fluoride acquired by three layers extracted of enamel by biopsy was significantly increased, extending the contact time of the agents to the dental surfaces for 24 h (Retief *et al*., 1980).

Currently, a computadorized microtomography has been used to evaluate the mineral content of carious or remineralized lesions (Damen *et al*., 1997). The illustration can be shown with the mineral density of the sample. However, the opposite of transverse microradiography, as it does not require preparation of the samples over a long period of

time to obtain the ± 100 µm bands, is a nondestructive analysis by digital scanning of the entire sample, preventing the fields from being important for the analysis (Elliot *et al*., 1997). This article allows for several sample scans and consequent 3D reconstruction. Assuming these advantages, it was decided to use a micro-CT in this study.

The micro-CT is a non-invasive, commonly accepted, technique to evaluate the tooth mineral density (TMD). This analyze showed that the different remineralizing treatment did not influenced the mineral density after 8 days cycling pH period. This analysis calculates the density from the gray shadows of the sample scanned in a computerized microtomography. Unlike other biochemical analyzes, this analysis allowed to identify other minerals within the caries lesion that are not linked with fluoride. Every remineralizing agents presented a potential remineralizing in this analysis after pH cycling, with greater mineral density when compared with the no treatment group. Different from what happened with the FAp analysis, which quantifies only fluorapatite, the micro-CT evaluation can show a whole mineral content inside the enamel.

An *in vitro* study carried out by Kucuk *et al* (2016), found that fluoride and CPP-ACP containing-products were more effective in remineralizing the incipient caries lesions evaluated by micro-CT when compared with saliva artificial in demineralized human enamel using the similar cycling protocol of this study, with a period of 30 cycles.

This study showed that both Duraphat and MI Varnish formed higher amount of CaF_2 than the other agents, and it can be explained because the high fluoride concentration of this varnishes (22.600 ppm fluoride). While Curodont Repair (no fluoride) and Curodont Protect (900 ppm fluoride) did not differ from demineralized group, Curodont Protect has formed CaF_2 after remineralization group higher than Curodont Repair and No treatment group. The CaF_2 formation is dependent on the amount of fluoride in the composition of the product. Despite of the low concentration of fluoride containing gel self-assembling peptide, Curodont Protect, it can be observed significant amount of CaF_2 than no treatment or even to the Curodont Repair that containing similar composition, except fluoride. It is known that CaF_2 plays an important role in preventing demineralization, since it is considered a mineral reservoir, as mostly forms calcium fluoride (CaF_2) or calcium fluoride-like material, which is often referred as “loosely bound” fluoride compared with fluorapatite or “firmly bound” fluoride. It is the most likely source of fluoride ions during cariogenic challenges. As fluorapatite has lower solubility than calcium fluoride, firmly bound fluoride

is presumably superior to loosely bound fluoride in slowing mineral diffusion within dental tissues (Tenuta *et al.*, 2008). Therefore, the presence of calcium fluoride on the enamel surface, as well as onto the demineralized enamel surface is the main mechanism for prevention of further demineralization considering high caries activity patients (White & Nancollas, 1990).

It is clear that the higher fluoride amount on dental products, the higher amount of calcium fluoride is formed (Tenuta *et al.,* 2009). Soares *et al* (2017) evaluated the ability of CPP ACPF, Bioactive Glass and Curodont Protect to remineralize artificial carious lesions in enamel *in vitro*, and they found considerably greater increase in the percentage of SMH (Superficial Microhardness) in Curodont Protect group as compared to the other test groups. This could have been explained by the difference of the pH cycling models and de-re solutions (8 days versus 30 days), as well as the difference in the type of analysis (biochemical analyses CaF_2-like versus SMH) (Soares *et al.*, 2017). In another study, some authors tested Curodont Protect on enamel erosions compared with fluoride agents and concluded that the self-assembling peptide-containing fluoride showed no antierosive effect (Attin *et al.*, 2017; Ceci *et al.*, 2016). In the case of self-assembling peptide gel, it is possible that it acts as a protector of enamel surface demineralization, under other conditions and purposes, which differ from this present study that evaluated the reduction of previously demineralized enamel.

Duraphat, a fluoride-based varnish, used in this study provide higher CaF_2 formation (27.8 µg F/cm^2) than found by Hayacibara *et al* (2004) who used the same varnish (19.39 µg F/cm^2). The explanation for these different results is due to the fact that in the study conducted by the aforementioned authors, no immersion of the treated block in saliva was performed, unlike the present study, where the blocks remained immersed in artificial saliva (containing calcium and phosphate) by 24 h, which allowed a greater formation of CaF_2 by the availability of calcium ions. The authors didn't simulate caries lesion, that is the fluoride agents was applied on sound enamel, and stored at 100% humidity and 37°C for 24 h (Hayacibara *et al.*, 2004).

In relation with FAp formation, "firmly bound" fluoride, when fluoride varnishes were used, it provided the highest amount of FAp (8.4 µgF/cm^2) than the other agents tested and also as no treatment groups. No statistical difference was found between Duraphat and MI varnish groups before and after the pH cycling. However, very interesting was the

performance of MI Varnish that after remineralization showed a value below Duraphat, but after cycling, its value surged, surpassing this last one. The synergistic effect of varnish containing CPP-ACP and fluoride in reducing demineralization may be attributed to the formation of CPP-stabilized amorphous calcium fluoride phosphate (Cross *et al*., 2004), resulting in the increased incorporation of fluoride ions together with increased concentrations of bioavailable calcium and phosphate ions (Reynolds *et al*., 2008). It is known that the presence of fluoride determines the mineral variations occurring in the more superficial layers of the enamel during the cariogenic challenge, and a dose-response effect of F, reducing enamel demineralization, can be observed.

The self-assembling peptides agents did not differ from the negative control concerning calcium fluoride formation on enamel neither in the EDR nor in the pH cycling group. The primary function of the peptide is to create a *scaffold* to attract the calcium and phosphate ions into the carious lesion to rework the crystal of the hydroxyapatite dissolved by the carious process (Kirkham *et al*., 2007). Due to the product doesn't contain fluoride in your composition, it used the ion from artificial saliva to form FAp, but in a lower amount than fluoride containing agents.

Results of this study, concerning FAp, were different from those found by Delben et al. (2006), where fluorapatite formation was evaluated after application of fluoride varnish on sound enamel and after submitting the blocks to pH cycling. The formation of fluorapatite means (by acid extractions at times 15, 30 and 60 s) found by the authors were 14.06 before, and 1.8 µ F/cm^2 after cycling, while in this present study the mean values were 8.45 and 13.08 µ F/cm^2 (remineralizing and after pH cycling groups) using fluoride varnish Duraphat. The difference of the results, can be attribute firstly, to the difference between products composition, while Delben *et al* used Duraphat with 2.26% F, the present study, used 5% F. Secondly, the authors had used a sound enamel, whereas in this study demineralized enamel was used. Finally, it may also be due to the difference between pH cycling protocols where the solutions used by the authors contained 0.03 µg F/mL in the remineralizing solution and the demineralized one did not contain fluoride, whereas in this study were used 0.05 and 0.03 µg F/mL F. Then, the different concentrations can be result in different amount of FAp on enamel.

In addition, it can be assumed that a difference of the micro-CT and FAp analyzes can be due to the presence of the crystallization of hydroxyapatite and not only about

fluorapatite after this period of 8 cycles. In the FAp analysis, only the Duraphat and MI Varnish groups showed a statistically significant difference in relation to the other treatment groups, whereas in the micro-CT analysis, all treatment groups with remineralizing agents had a statistically significant difference in relation to the negative control group (no treatment). In other words, the micro CT can detect a larger formation of minerals, being in the form of hydroxyapatite or fluorapatite ridge than only the biochemical analysis of FAp. Both analysis are important, micro-CT can indentify the whole amount of mineral on enamel and the bioquimical analysis as FAp quantification, the potential preventive effect. Therefore, this study showed that instead of Duraphat and MI Varnish provided the higher amount of FAp and CaF incorporation, others mechanisms can be associated with preventing the lesion progress. Further studies have to be accomplished in order to determine the incorporation and formation of FAp and hidroxiapatite into the demineralized areas.

In vitro studies are important and valid to test the efficacy and behavior of remineralizing agents in caries lesions, however, randomized clinical trials are required to confirm in vitro findings and obtain clinical validation. Moreover, this results potentially indicate that despite of high fluoride-containing remineralizing agents, all treatments tested has potential for control the demineralization progress.

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3. CONCLUSÃO

Baseados nos resultados e nas limitações impostas a um estudo *in vitro*, pode-se concluir que apesar dos agentes remineralizadores contendo alta concentração de flúor apresentarem maior formação de CaF_2 na superfície e FAp no interior da lesão inicial de cárie, todos os grupos de tratamento estudados apresentaram formação de mineral no interior da lesão de cárie, contribuindo para a diminuição da desmineralização, mesmo após desafio cariogênico, por ciclagem de pH, observada por micro-CT.

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