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MARIA DENISE RODRIGUES DE MORAES BEZERRA

ANÁLISE DA AÇÃO DE CATEQUINAS DERIVADAS DO CHÁ VERDE EM DENTINA HUMANA EROSIVAMENTE DESMINERALIZADA: ESTUDOS *IN VITRO* E *IN SITU*

FORTALEZA
2016

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Doutor em Odontologia. Área de concentração: Clínica Odontológica

Orientador: Prof. Dr. Sérgio Lima Santiago
Co-orientadora: Profa. Dra. Gislaine Cristina Padovani

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Aprovada em: _____

BANCA EXAMINADORA

_____

Prof. Dr. Sérgio Lima Santiago (Orientador)
Universidade Federal do Ceará (UFC)

_____

Profª. Drª. Linda Wang
Universidade de São Paulo (USP)

_____

Profª. Drª. Lidiany Karla Azevêdo Rodrigues Gerage
Universidade Federal do Ceará (UFC)

_____

Profª. Drª. Vanara Florêncio Passos
Universidade de Fortaleza (UNIFOR)

_____

Profª. Drª. Paula Borges Jacques
Universidade de Fortaleza (UNIFOR)

Dedico este trabalho a Deus.
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RESUMO

O presente estudo avaliou a ação de catequinas do chá verde em dentina humana sob situação de desafio erosivo e originou três capítulos. Nos dois primeiros, blocos de dentina humana (n=10) coronária ou radicular (4x4x2mm) foram imersos em saliva (2h) para formação de película adquirida. Foram submetidos de forma cíclica (3x/dia-3 dias) ao desafio erosivo pela imersão em ácido cítrico (60s), seguido pelos tratamentos: G1-Água destilada (AD), G2-Solução de gluconato de clorexidina 0,12% (CLX), G3-Infusão de chá verde (CV). Os espécimens permaneciam em saliva artificial entre os desafios e sob agitação (37ºC). A microdureza de superfície e perfilometria dos blocos foram analisadas diariamente. O estudo *in situ* foi randomizado, cego, cruzado e 20 voluntários utilizaram dispositivo intra-oral, contendo 4 blocos de dentina humana, por três fases de 5 dias cada. Foi realizada imersão extra-oral do dispositivo em 50 mL de Coca-Cola® (4x/dia-1min), seguida por gotejamento (1mL, 4x/dia) sobre os blocos: G1-solução de fluoreto de sódio (NaF) 0,05%, G2-epigalocatequina-3-galato (EGCG) 0,1%, G3-CV. Foram realizadas análises de microdureza de superfície, perfilometria e microscopia eletrônica de varredura. Para análise da inibição enzimática, blocos de dentina humana foram congelados, moídos, submetidos à erosão por ácido cítrico (24h) e extração de proteínas solúveis. O *pool* de proteínas extraído foi utilizado como fonte de metaloproteinases (MMPs), foi tratado: G1-sem tratamento, G2-CLX 0,12%, G3-NaF 0,05%, G4-CV, G5-EGCG 0,1% e foi submetido ao ensaio colorimétrico de inibição enzimática. Foi realizada a eletroforese das enzimas extraídas em gel de acrilamida sob condições não redutoras. O gel foi encubado por 3h em tampão de renaturação modificado pelo acréscimo das soluções: G1-sem modificação, G2-CLX 0,12%, G3-NaF 0,05%, G4-CV e G5-EGCG 0,1%, e corado em solução contendo 0,025% Comassie blue e descorado com solução de ácido acético (10%). O padrão de normalidade dos dados foi analisado pelo teste

de Kolmogorov-Smirnov, seguido por Análise de variância e teste de Tukey (α=5%). Foi observada redução na perda de dureza da dentina coronária *in vitro* com o uso de CLX e CV em comparação com o controle. O tratamento com CV reduziu significativamente o desgaste e a rugosidade da dentina coronária *in vitro*. O tratamento *in vitro* com CV reduziu estatisticamente o desgaste e a rugosidade da dentina radicular quando comparado ao controle e à CLX. Não houve diferença estatisticamente significante entre os grupos em relação à perda de dureza de superfície de dentina radicular *in vitro*. Quanto ao estudo *in situ*, os tratamentos com EGCG e CV reduziram a perda de dureza da dentina significativamente. Por outro lado, não houve diferença estatística nos valores de desgaste e rugosidade. A zimografia demonstrou que a CLX 0,12%, o CV e o EGCG 0,1% apresentaram ação inibitória sobre as MMPs, enquanto que no ensaio colorimétrico o CV apresentou inibição enzimática. A solução de CV reduz *in vitro* o desgaste e a rugosidade da dentina coronária e radicular erosivamente desmineralizada. O CV e a EGCG 0,1% reduzem os danos causados pela erosão em dentina coronária *in situ* e contribuem para inativação de MMPs extraídas da dentina.

Palavras chaves:

Eletroforese. Erosão dentária. Inibidores teciduais de metaloproteinases. Metaloproteinases da matriz. Desgaste dos dentes.

ABSTRACT

The objective of this study was to evaluate and compare the action of different enzyme inhibitors on human dentin in cyclical erosion challenging situation. To this were carried out 3 projects that yielded the following chapters: In the chapter 1 and 2, coronary or root human (n=10) dentin blocks (4x4x2 mm) were submitted cyclically (3x/ day/3 days) to erosive challenge by immersion in acid [dehydrated citric acid ($C_6H_8O_7$), pH 3.75, 60 s] followed by treatments depending on the group: G1-distilled water; G2- 0.12% Chlorhexidine digluconate (CHX); G3- Green tea infusion (GT). The blocks were analyzed daily by surface profilometry and hardness. In the chapter 3, a randomized, blinded, crossover, *in situ* study in which 20 volunteers used palatal intra oral device for three phases of 5 days each, containing 4 blocks of human dentin. They were immersed in human saliva for 2 hours to acquired pellicle formation. The erosive challenge was performed by the device immersion in 50 mL of Coke™ (pH 2.6, 4x/ day /1 min, extraorally) followed by treatment (4x/ day) by dropping on blocks 1 mL of the following solutions: G1- 0.05% Sodium fluoride, G2- 0.1% Epigallocatechin-3-gallate (EGCG), G3- Green tea infusion. At each phase the volunteer used only one substance. Quantitative analyzes were performed, such as percentage loss of surface hardness, roughness and wear, as well as qualitative analysis by scanning electron microscopy. Complementing the studies to analysis of enzyme inhibition, human dentin blocks were milled, subjected to erosion by citric acid and subjected to extraction of soluble proteins. Electrophoresis was performed and then the gel was incubated for 3 h in renaturation buffer modified according to the groups G1: without modification, G2- 0.12% chlorhexidine digluconate, G3- 0.05% NaF, G4- Green tea and G5- 0.1% EGCG. Gels were stained in a solution containing 0.025% Coomassie blue and destained with acetic acid 10% solution to verify the possible inhibitory action of the substances evaluated on the collagenolytic

enzymes. Then were subjected to colorimetric enzyme inhibition test, in which the absorbance was measured. After extraction of dentin proteins, protein quantification by Bradford's method was performed, which showed 0.15 µg soluble proteins. The results were analyzed using the Kolmogorov-Smirnov test to evaluate the normal range of results, followed by analysis of variance (ANOVA) and Tukey test, using a significance level of 5%. Results: A significant reduction in the dentin hardness loss in coronary dentin *in vitro* with the use of CHX and GT in comparison to the control ($p < 0.05$). Treatment with GT significantly reduced wear and roughness of dentin *in vitro* ($p < 0.05$). In relation to *in vitro* study on root dentin, the treatment with GT reduced the wear and roughness ($p < 0.05$). There was no statistically significant difference between the groups regarding the loss of surface hardness of root dentin *in vitro* ($p > 0.05$). As for the *in situ* study, treatment with EGCG and GT reduced the loss of dentin hardness significantly ($p < 0.05$). On the other hand, there was no significant difference in relation to wear and roughness values ($p > 0.05$). For zymography analysis, 0.12% CHX, green tea and 0.1% EGCG showed inhibitory action on the extracted metalloproteinases dentin and, the colorimetric assay green tea has enzimatic inhibition similar to standard inhibitor. Conclusion: Green tea solution reduces *in vitro* wear and roughness of coronary and root dentin erosively demineralized. The 0.1% EGCG and green tea reduce erosion damage on coronal dentin *in situ* and contribute to inactivate MMPs extracted dentin.

Keywords:

Electrophoresis. Dental erosion. Tissue inhibitors of metalloproteinases. Matrix metalloproteinases. Tooth wear.

LISTA DE ABREVIATURAS

MOD	**Matriz orgânica desmineralizada**
MMPs	**Metaloproteinases da matriz**
EGCG	**Epigalocatequina-3-galato**
DW	**Distiled water; água destilada**
CHX	**Chlorhexidine; Clorexidina**
GT	**Green Tea; chá verde**
Ca2+	**Íon Cálcio**
Zn2+	**Íon Zinco**
Fe2+	**Íon Férro**
MMP-2	**Metaloproteinases da matriz-2**
MMP-9	**Metaloproteinases da matriz-9**
Ra	**Averagen roughness; Média de rugosidade**
SEM	**Scanning electron microscopy; Microscopia eletrônica de varredura**
IBGE	**Instituto Brasileiro de Geografia e Estatística**

SUMÁRIO

INTRODUÇÃO GERAL

A erosão dentária é uma patologia com alta prevalência em adolescentes e adultos (36% - 61%), causada pela ação de ácidos de origem não bacteriana, os quais promovem a dissolução dos tecidos dentais mineralizados, podendo causar dor e complicações endodônticas (VERED *et al*., 2014; IMFELD, 1996; GANSS, 2014). Apresenta-se em superfícies lisas com cavidades pouco profundas, ocorrendo coronal à junção cemento-esmalte, e na superfície oclusal observa-se aspecto de chanfrado distinto e cúspides arredondadas (GANSS, LUSSI, 2014; (MAGALHÃES *et al*., 2009a).

Sabe-se que o hábito de dieta ácida, doenças gastroesofágicas, radioterapia da cabeça e pescoço, e uso crônico de medicamentos são alguns dos fatores que elevam o risco do indivíduo de erosão dos dentes (LIESHOUT; BOTS, 2014). A idade, composição salivar e abrasividade da língua, bem como natureza, composição e frequência de ingestão de ácidos são fatores que influenciam na suscetibilidade e no grau da erosão (HOOPER *et al*., 2015). No Brasil, segundo a pesquisa nacional de saúde, realizada em 2013 pelo Instituto Brasileiro de Geografia e Estatística (IBGE), 23,4% da população consomem refrigerantes ou sucos artificiais pelo menos 5 dias na semana (PNS, 2013). Esse consumo de bebidas carbonatadas é um fator de risco para erosão dental. Estudos nacionais apontam para uma grande variabilidade nos resultados sobre prevalência, assim como nos estudos internacionais, apresentando uma variabilidade entre 3,4% até 58% em crianças e adolescentes (FARIAS *et al*., 2013). A alta frequência de ingestão de comidas e bebidas ácidas indica uma forte associação com desgaste dentário e exposição dentinária (WEI *et al*., 2016).

Por se tratar de uma condição multifatorial, a ocorrência de erosão dentária depende de fatores socioeconômicos e comportamentais (hábitos dietéticos), químicos (fontes ácidas intrínsecas e extrínsecas) e biológicos (presença de película adquirida e fluxo salivar), sendo a interação entre esses aspectos condição fundamental para se determinar o início e a severidade da doença (LUSSI; CARVALHO, 2014; YOUNG; TENUTA, 2011). Porém, orientações comportamentais são difíceis de serem controladas para redução de hábidos de dieta ácida (MAGALHÃES *et al*., 2009a; (COMAR et al., 2015). Portanto, diferentes abordagens podem ser utilizadas para minimizar os efeitos deletérios da doença.

A realização de uma completa anamnese, avaliando os fatores de risco individual, a fonte dos ácidos (exógena ou endógena) responsável pela perda mineral e o substrato (esmalte

ou dentina) é crucial para o diagnóstico e o esclarecimento dessa patologia. Os fatores químicos e biológicos, por serem passíveis de avaliação e terem grau de complexidade menor que os outros critérios, podem ser utilizados para o direcionamento do tratamento preventivo ou terapêutico (GANSS; LUSSI, 2014).

O desgaste causado pelas lesões de erosão pode comprometer os dentes do indivíduo por toda a vida, além de estar relacionado à hipersensibilidade dentinária. O substrato dentinário é exposto quando ocorre recessão gengival e desmineralização dos cristais do esmalte, deixando as fibras colágenas desprotegidas e vulneráveis à degradação pelas enzimas colagenolíticas (KATO, 2012). Assim, na erosão, a dentina tem sua superfície atacada, tanto por desmineralização quanto por degradação proteolítica por meio de proteases endógenas (metaloproteinases da matriz -MMPs e cisteino-catepsina). As MMPs são enzimas zinco e cálcio dependentes que se encontram inativadas na saliva e dentina e, quando associadas ao pH ácido, são ativadas e iniciam a ação proteolítica, desempenhando um papel fundamental na progressão da cárie e erosão dentária (VISSE R, 2003); CARRILHO, 2012).

Estudos demonstram que a manutenção da matriz orgânica desmineralizada (MOD) protege o tecido subjacente de sofrer mais erosão e perda de mineral (BUZALAF; KATO; HANNAS, 2012). Assim, preservar a MOD após desafios erosivos dificulta a difusão dos íons ácidos através do tecido dentinário e parece ser uma estratégia para reduzir a taxa de desgaste da dentina (GANSS; KLIMEK; STARCK, 2004; HARA *et al*., 2005; KATO *et al*., 2012).

Substâncias inibidoras de MMPs estão sendo utilizadas como tratamento preventivo de algumas doenças sistêmicas e bucais (DEMEULE *et al.*, 2000; KATO *et al*., 2014). Dentre elas, a solução de digluconato de clorexidina, o fluoreto de sódio (KATO *et al*., 2009; MAGALHÃES *et al*., 2009b; KATO *et al*., 2010, KATO *et al*., 2012;) e as catequinas isoladas do chá verde [Epigalocatequina-3-galato - EGCG (0,0065%)] (GENDRON *et al*., 1999; DEMEULE *et al*., 2000, VIDAL *et al*., 2014, de MORAES *et al*., 2016) foram utilizadas em estudos prévios para proteção da dentina contra erosão.

Soluções enxaguatórias de fluoreto de sódio são amplamente utilizadas em produtos de higiene oral. Porém não apresentam substantividade, pois a camada de CaF_2 é dissolvida em baixo pH e apresentam ação parcialmente reversível na inibição de MMP-2 e -9, não sendo eficaz na proteção da dentina contra a erosão (GANSS; SCHLUETER; KLIMEK, 2007; KATO *et al*., 2014; (ALTINCI et al., 2016). Entretanto uma estratégia que tem sido

utilizada em pesquisas na área da odontologia é a abordagem biomimética em que agentes bioativos são utilizados para aumentar ou reforçar o tecido pela alteração de propriedades biomecânicas e bioquímicas (BEDRAN-RUSSO *et al*., 2014).

O chá verde é proveniente da planta *camélia sinens*is e é um potente antioxidante e anticarcinogênico. Possui em sua composição a epigalocatequina-3-galato (EGCG), que é um polifenol responsável por aproximadamente 59% do total das catequinas da folha do chá verde (STEINMANN *et al*., 2013). Esse polifenol vem sendo avaliado como um promissor auxiliar no controle do processo de desgaste dentinário por erosão, apresentando ação inibitória relevante sobre as MMPs (DEMEULE *et al*., 2000) e ação protetora do desgaste de dentina erosivamente desmineralizada (MAGALHÃES *et al*., 2009b; (SILVEIRA et al., 2014); (KATO et al., 2010); (DE MORAES et al., 2016). Ademais, acredita-se ser um potente agente biomodificador, promovendo ligações cruzadas com o colágeno preservando-o, melhorando as propriedades mecânicas e, dessa forma, mantendo a camada de matriz orgânica que foi desmineralizada (BEDRAN-RUSSO et al., 2014). Notavelmente, a EGCG e várias preparações de chá verde estão disponíveis como remédios de prateleiras em muitos países e são acessíveis para a população (STEINMANN *et al*., 2013).

Uso de substâncias naturais, como os polifenóis provenientes de plantas, tem tido ênfase por serem fontes renováveis, sustentáveis, de baixa toxicidade e apresentarem bioatividade, biocompatibilidade e aplicabilidade na Odontologia. Portanto, a interação desses compostos com o colágeno da dentina resulta em ligações de alta estabilidade, dificultando a degradação da dentina e aumentando a proteção contra erosão (BEDRAN-RUSSO *et al*., 2014).

Assim, torna-se fundamental buscar estratégias como alternativas para a efetiva proteção dos tecidos dentários mineralizados contra o desgaste causado pela erosão. Este estudo teve como objetivo avaliar e comparar, *in vitro* e *in situ,* algumas substâncias inibidoras enzimáticas na manutenção da proteção da perda tecidual em dentina humana desmineralizada em situação de desafio erosivo cíclico. As hipóteses do trabalho são: as substâncias testadas serão eficazes na proteção da superfície dentinária e apresentarão ação inibitória sobre as MMPs extraídas da dentina.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo deste trabalho, *in vitro e in situ*, foi avaliar a ação do chá verde sobre dentina humana erosivamente desmineralizada.

2.2 Objetivos Específicos

Capítulo 1: Analisar *in vitro* a ação da solução de clorexidina e da infusão de chá verde na proteção da perda tecidual da dentina coronária sob desafio erosivo cíclico.

Capítulo 2: Analisar *in vitro* a ação da solução de clorexidina e da infusão de chá verde na proteção da perda tecidual da dentina radicular sob desafio erosivo cíclico.

Capítulo 3: Analisar *in situ* a ação da infusão de chá verde, da solução de Epigalocatequina-3-galato e de fluoreto de sódio na proteção da perda tecidual da dentina sob desafio erosivo cíclico, bem como verificar suas ações e da solução de clorexidina sobre as metaloproteinases da dentina humana erosivamente desmineralizada.

3 CAPÍTULOS

REGIMENTO INTERNO

Esta tese está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará (Anexo A), que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou coautoria do candidato. Por se tratar de pesquisa envolvendo partes de animais, o presente trabalho foi submetido ao Comitê de Ética em Pesquisa (CEP). Assim sendo, esta tese de doutorado é composta por três capítulos contendo artigos científicos publicados ou em fase de redação conforme descritos abaixo:

Capítulo 1: Effect of green tea as a protective measure against dental erosion in coronary dentin. **Brazilian Oral Research**.v.30, p.1-6. 2016.

Capítulo 2: Protective effect of matrix metalloproteinases inhibitors on radicular dentin erosion: *in vitro* study. Este artigo será submetido à publicação no periódico **Clinical Oral Investigations**.

Capítulo 3: Protective effect of green tea catechins on wear of human dentin erosion: an *in situ* study. Este artigo será submetido à publicação no periódico **Journal of Dentistry**.

CAPÍTULO 1

3.1 Capítulo 1

EFFECT OF GREEN TEA AS A PROTECTIVE MEASURE AGAINST DENTAL EROSION IN CORONARY DENTIN

SHORT TITLE: EFFECT OF GREEN TEA ON CORONARY DENTIN

Maria Denise Rodrigues de Moraes, University of Fortaleza, Fortaleza, Ceará, Brazil. Post-graduation Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil. (85) 988965991; denisermoraes@gmail.com

Jéssica Rodrigues Mendes Carneiro, Post-Graduate Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil. (85) 999108631; jessicarmendesc@gmail.com

Vanara Florêncio Passos, University of Fortaleza, Fortaleza, Ceará, Brazil. (85) 999882039; vanarapassos@hotmail.com

Sérgio Lima Santiago, Post-Graduate Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil. (85) 988842704; sergiosantiago@ufc.br

Full address of the author to whom correspondence should be sent:

Dr. Sérgio Lima Santiago

Rua Monsenhor Furtado s/nº.

CEP 60.430-355 Fortaleza, CE Brazil

Phone- +55-85-88242704 Fax- +55-85-33668232 E-mail: sergiosantiago@ufc.br

EFFECT OF GREEN TEA AS A PROTECTIVE MEASURE AGAINST DENTAL EROSION IN CORONARY DENTIN

Abstract

The aim of this study was to evaluate the effect of green tea as a protective measure on eroded dentin. Disks of human coronary dentin were selected based on surface hardness and randomly assigned to 3 groups (n=10): DW - distilled water, CHX - 0.2% chlorhexidine digluconate, and GT - green tea. The disks were allowed to acquire pellicle for 2 hours and were then subjected to 3 cycles per day of demineralization ($C_6H_8O_7$ 0.05 M, pH 3.75, 60 s), treatment (DW or CHX or GT, 5 min) and remineralization (artificial saliva, 60 min) over a period of 3 days. Changes in the dentin were determined by loss of surface hardness (%SHL) and mechanical profilometry analysis at the end of each day. Data were analyzed by two-way ANOVA followed by Tukey's test for %SHL and profilometry ($p<0.05$). Significant reductions in dentin hardness loss were observed only for the CHX group when compared to the DW group ($p<0.05$). However, there was no significant difference between the CHX and GT groups ($p> 0.05$). A significant difference was observed between DW and GT treatments for wear and roughness measurements ($p<0.05$). The green tea extract solution was able to reduce the wear and roughness caused by dentin erosion under the conditions of this study.

Keywords: tooth erosion, dentin, hardness, matrix metalloproteinase inhibitors, camellia sinensis.

Introduction

Erosive demineralization of the tooth crown is characterized by initial softening of the enamel surface. This process is followed by continuous layer-by-layer dissolution of the enamel crystals, leading to a permanent loss of tooth volume with a softened layer at the surface. The dentin becomes increasingly exposed in advanced stages and exposes the organic matrix to breakdown by host-derived enzymes, such as matrix metalloproteinases (MMPs) present in dentin and saliva.[1-3] MMPs act in the chemical degradation of the organic matrix of dentin and play an important role in the progression of dentin erosion.[4-6]

Protective measures to reduce dental erosion, such as laser therapy and topical fluoride, have been investigated. Most of these treatments are based on the action of fluoride, which is available in dentifrices, solutions, or varnishes, although the role of fluoride in the protection of dental erosion is still controversial.[7-10]

In recent years, some *in situ* studies have shown that commercial green tea and a rinse containing green tea extract were able to reduce erosive and erosive/abrasive dentin wear.[11,12] These products are rich in polyphenols and have been reported to be inhibitors of the activity of different metalloproteinases.[11,13] It has been suggested that the use of synthetic inhibitors of MMPs could be used to control the loss of the dentin matrix.[5,14] However, MMPs are secreted as inactive precursors (pro-forms), requiring activation by a low pH to degrade extracellular matrix components.[1]

Therefore, the demineralization process is increased by the action of acids on the organic matrix of dentin and the activation of the MMPs.[12] To minimize this process, green tea has a significant inhibitory effect on MMPs and dentin demineralization.[15-17] The reduced daily

exposure to acid may be minimized by the use of metalloproteinase inhibitors, which protect the organic matrix as a barrier to ion diffusion.

Other studies have attempted to evaluate the protective effect of green tea against wear promoted by erosion; however, there are still doubts concerning the maintenance of inhibitory action in the MMP after successive cyclical acidic exposures. It might be interesting to investigate the effectiveness and substantivity of these substances on the surface of the eroded dentin in cycles of erosive challenge. The absence of studies that simulate the erosion of dentin through short cyclic acid challenges, both daily and intermittent, reinforces the need for further studies.

Thus, the objective of this *in vitro* study was to assess the protective effect of green tea on dentin demineralization in three days of cyclic erosion by assessing the percentage of surface hardness loss (%SHL), roughness and wear.

Materials and methods

Experimental Design

This *in vitro* study was approved by the local research and ethics committee (protocol # 175/2010). This is a blinded experimental study design with three groups (n = 10), with two factors: time (one, two and three days of the experimental cycle) and treatments (distilled water (DW; G1- control), 0.2% chlorhexidine (CHX; G2) and green tea (GT; G3; EGCG concentration 0.0014%)). The treatments were applied by immersion under agitation for five minutes, and the specimens were randomly assigned to the defined treatments. The dependent variables were the percentage of surface hardness loss (%SHL), as quantitatively evaluated by differences in the mean values of surface hardness after treatment, and dentinal wear and roughness, as assessed by the contact profilometer Hommel Tester T1000 (Jenoptik, Schwenningen, Germany).

Specimen Preparation

Coronary dentin specimens were prepared from human third molars that had been stored in 0.01% (w/v) thymol solution at 4°C. Dentin disks were obtained using a longitudinal coupled double-sided diamond disk in a IsoMet slow speed saw (Buehler, Lake Bluff, USA). The dimensions of each disk depended on the diameter of the tooth. Sequentially, the specimens were ground in a water-cooled mechanical grinder (Arotec S.A., Cotia, Brazil) using 400-, 600-, 800- and 1200-grit aluminum oxide abrasives and polished with felt paper and 1 μm diamond spray (Extec Corp., Enfield, USA). The surface hardness values were determined using a Knoop diamond FM100 (Future-Tech Corp., Kawasaki, Japan), making five indentations with a load of 10 gf /5 s, 100 μm apart from each other, at the center of the specimens. Thirty dentin disks presenting a mean hardness of 55.81 ± 6.20 Knoop hardness number (KHN) were selected and randomly assigned using a computer-generated randomization list into three experimental groups (Microsoft Excel 2007).

Nail varnish was applied on half of the surface of each specimen to serve as the reference area for profilometry analysis. The exposed area was subjected to the acid challenge.

Pellicle Formation

On each experimental day, five volunteers without erosion, salivary dysfunction or active carious lesions donated fresh saliva samples. The secretion of saliva was stimulated by chewing on paraffin wax for five minutes. Saliva from the first minute of chewing was swallowed, and the rest was collected and deposited into a 50 mL centrifuge tubule. The saliva samples were centrifuged for 10 min at 2000 rpm in a pre-cooled centrifuge (4°C) NT-815 (Novatecnica, Piracicaba, Brazil). The clear fluid above the sediments was pooled and used for pellicle formation.[18] Prior to erosive challenge, each group of dentin disks was independently immersed in clarified saliva and incubated under agitation at 100 rpm in an

oscillating table (TE143, Tecnal, Piracicaba, Brazil) at 37°C (Olidef CZ, Ribeirão Preto, Brazil) for two hours before each experimental day to simulate the environment of the oral cavity.

Experimental procedure

The study used cyclic procedures repeated over a three-day period, including pellicle formation, erosion, treatments with test solutions (DW, CHX, GT) and remineralization with freshly prepared artificial saliva (1.5 mM Ca; 0.9 mM PO_4; 150 mM KCl and 0.1 M Tris buffer, pH 7.0), considering the influence of variables such as stirring, temperature and exposure time (Fig 1).[19] The 0.2% chlorhexidine digluconate solution was provided by a pharmacy, and the green tea (Dr Oetker, Jd. do Lago, Brazil) was freshly prepared according to the manufacturer's instructions at the beginning of each experimental day. The concentration of epigallocatechin gallate (EGCG) in this green tea was 0.0014%, as assessed using a spectrophotometer, and the pH was 5.45.

Erosion Cycling Model

After pellicle formation, each disk was submitted to a citric acid solution for 60 seconds. The acid challenge was performed using 0.05 M dehydrated citric acid, pH 3.75 (Dinâmica®, Diadema, Brazil). Each disk was then rinsed with distilled water and treated for 5 minutes with the treatment solution (DW, CHX, GT) and then immersed in artificial saliva for 1 hour, performed under agitation at 100 rpm and 37°C (Fig 1).

This cycle was repeated three times a day for three days. At the end of each experimental day, the hardness, wear and surface roughness analysis of each specimen were measured.[20]

Percentage of Surface Hardness Loss Assessment

Immediately after each experimental day, the slabs were placed in the hardness machine, and five new indentations were made using a Knoop diamond under a 10 g load for five seconds (SHafter), with the indentations spaced 100 μm apart from the previous measurements

(SHbefore). The percentage of SH loss (% SHL) was then calculated for each day, according to the following equation: %SHL = [(SHbefore – SHafter)x100/ SHbefore].

Measurement of Dentin Surface Loss

Measurements of dentin surface loss were performed using the stylus profilometer, after each experimental day. The difference between the heights of the surfaces of the reference and the treated areas was evaluated. Before analysis, the nail varnish was carefully removed, exposing the untreated reference areas. On each sample, at intervals of 100 μm, five profile traces (1.5 mm in length) were recorded, and the levels of dentin wear were determined in relation to the reference surfaces. For each sample, the mean values obtained from the five traces were calculated.[21]

Measurement of Surface Roughness

Surface roughness was described by the arithmetic mean of the absolute ordinate values Ra (average roughness as per ISO 4287) of 5 measurements made in each disk.[22,23] In profilometry, the surface of a specimen was scanned using a stylus with a diamond to generate a two- or three-dimensional profile using a contact measuring device.[24]

Statistical Analysis

Statistical procedures were performed with the Statistical Package for Social Sciences (SPSS 17.0 for Windows, SPSS Inc., Chicago, USA). A Kolmogorov-Smirnov test was applied to all groups to test for the normal distribution of errors. Because the values were normally distributed across all groups, two-way ANOVA and Tukey's post hoc tests were used for comparative purposes. The level of significance was set at 5%.

Results

Table 1 presents the means of dentin hardness loss, wear and roughness values found for all the treatments evaluated in the three-day experiment.

In relation to dentin hardness loss, dental wear and roughness, two-way ANOVA revealed a significant difference among the treatments tested [($p<0.001$; $F=3.3$), ($p<0.001$; $F=9.7$), and ($p<0.001$; $F=8.3$), respectively], as well as the duration of demineralization represented by the number of experimental days [($p<0.001$; $F=69.7$), ($p<0.001$; $F=11.4$), and ($p<0.001$; $F=49.0$), respectively]. Furthermore, the interaction between the factors was significant for the loss of dentin hardness ($p=0.009$; $F=3.6$). However, the interaction between the factors was not significant for dental wear ($p=0.745$; $F=0.4$) and for roughness ($p=0.782$; $F=0.4$). Significant differences in dentin hardness loss were observed only for the CHX group when compared to the DW group ($p<0.05$). However, there was no significant difference between CHX and GT ($p>0.05$).

For dentin hardness loss and dental wear, it was observed that with an increase in the number of experimental days, all specimens displayed statistically significant surface softening from day 1 to day 2, which stabilized at day 3, showing that citric acid was effective in eroding the dentin surface. The green tea was more effective than DW in protecting the human dentin against wear caused by the erosion of citric acid ($p<0.001$). Additionally, there was no significant difference between CHX and GT ($p<0.05$).

The chlorhexidine behaved similarly to the control ($p=0.053$) in protecting the human dentin against surface roughness caused by the erosion of citric acid. On the other hand, the green tea was more effective than the control ($p<0.001$).

Discussion

The present *in vitro* de-remineralization cycling model investigated green tea with respect to its capacity to protect human dentin from erosion. This study confirmed the expected surface softening and dentin tissue loss due to the action of citric acid, even after pellicle formation for two hours before each experimental day. The use of an *in vitro* multiple-exposure acid

model allowed for a better understanding of the erosive challenges faced by the dentition while performing a controlled investigation and reducing the experimental time and cost.[25]

The results of this study disagree with the results of Mirkarimi and Toomarian (2012)[26] because an increase in surface hardness values after immersion in green tea solution was not observed in the current study. However, the erosive challenge used in their study was shorter and lower than that used in the present study. The dentin disks subjected to this erosive cycle presented higher percentages of change in hardness, which may have influenced the indentations of the diamond when measuring hardness. Furthermore, the hardness analysis is a sensitive method for detecting changes in the mineral density of artificial eroded/abraded lesions in an enamel substrate but not in dentin;[27] therefore, another analysis, profilometry, was added to the current study.

Under great acid challenge, profilometric analysis is widely used to measure the loss of enamel and dentin surfaces.[27,28] The results of the present study corroborate those of Magalhães et al. (2009)[12] and Kato et al. (2009)[11] because the green tea was able to reduce dentin wear in the current study. A possible mechanism of action for the reduction in dentin loss might be the inhibition of MMPs by chlorhexidine and green tea extract solution.[12] Another study that involved cyclic acid challenge also used profilometry to measure the wear of dentin; Barbosa and colleagues (2011)[29] concluded that the supplementation of soft drinks with green tea extract might be a viable alternative to reduce the erosive potential against dentin.

In this study, a cycling model was used, employing citric acid three times a day over a period of 3 days. On the 3rd day of the erosive challenge, the surface roughness and dentin wear were worse than in the first day, showing that the acid used in this study was effective in eroding the dentin surface. It is also probable that the low pH of the acid induced the activation of dentin-derived MMPs.[11]

The 1 μm of wear protection observed in this *in vitro* study correlates with the erosive cycle, which involved short periods of acid exposure and remineralization. Magalhães and colleagues performed a more aggressive challenge (4 times/day, Coca Cola, 5 min) than in this study (3 times/day, citric acid, 1 min)[12] and observed a similar outcome. Additionally, in that study, green tea extract with approximately 30 ± 3% of catechin was used, while the present study used a commercial product, which limited the concentration of catechin (0.0014%).

One possible mechanism of action of green tea on the reduction of dentin erosion could be the inhibition of MMPs. If it is true, the component responsible for this effect may be polyphenols. Green tea polyphenols, especially epigallocatechin-3-gallate (EGCG), were found to have potent and distinct inhibitory activity against MMPs in cell culture tests.[11] The green tea polyphenols and their major constituent, EGCG, have been shown to inhibit metalloproteinases in other areas of medicine and dentistry.[30,31] It can be speculated that the small concentration of EGCG in the tea used in the present study can justify the unsatisfactory results in protecting the loss of dentin hardness. However, it must be acknowledged that the protocol employed in the present study does not suggest that the effect of green tea on the reduction of the wear and roughness of dentin specimens is due to its inhibitory action on MMP activity, as we did not test this directly.

Because the erosive/remineralization cycle was performed under *in vitro* conditions, it is clear that the results are not completely transferable to an *in vivo* situations, the natural protective effects of the oral cavity are lacking. However, the present study utilized variables such as the erosive solution, stirring method and temperature in order to replicate the oral environment. Thus, the use of green tea might be a viable alternative as a protective measure against erosive wear in short acid exposures. However, further studies are necessary to explore the inhibitory action of green tea solution on MMPs present in erosively demineralized dentin.

Conclusion

According to the conditions of the present study, the use of green tea extract is a promising protective measure in reducing dentin erosion, as it has a protective effect on the dentin roughness and wear caused by erosion.

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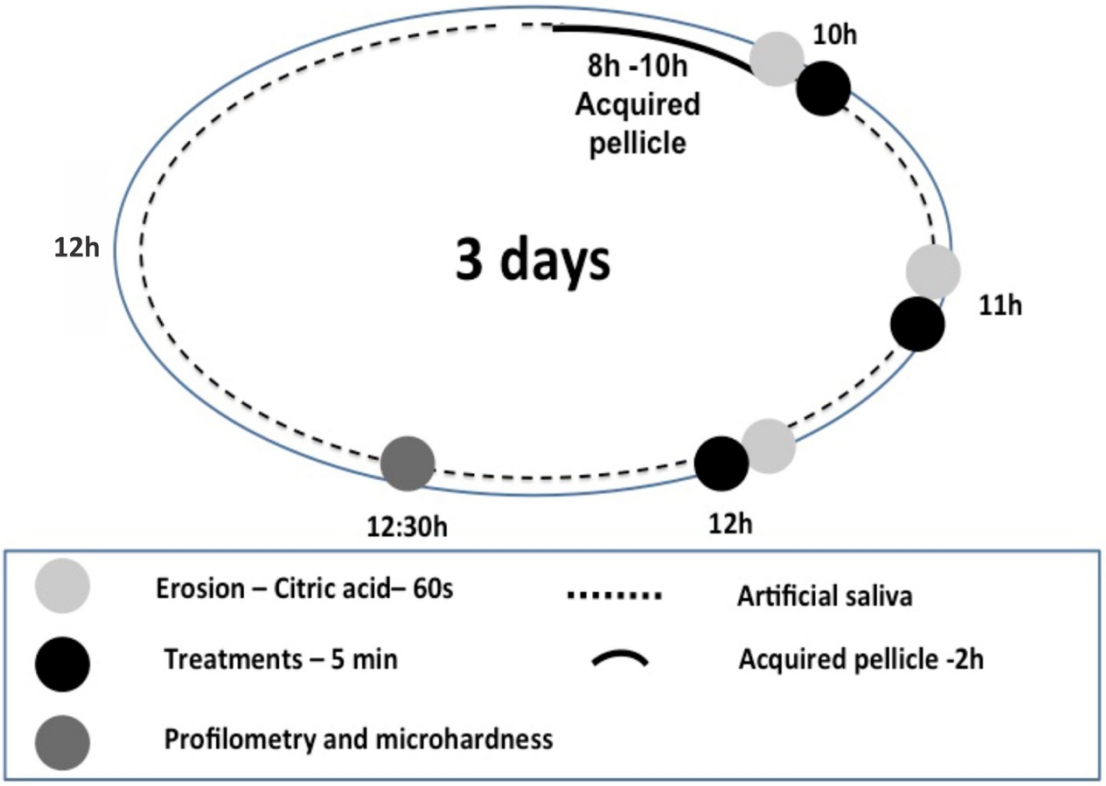


Figure 1. Erosion cycle model and treatments

Table 1. Means and standard deviations of the loss of dentin hardness, wear and roughness values for all the treatments evaluated.

Group	***Analyses***		
	Dentin Hardness Loss (%)	***Wear (µm)***	***Roughness (Ra)***
DW	55.67 (9.13) a	2.07 (0.77) a	0.25 (0.04) a
CHX	61.83 (7.59) b	1.78 (0.78) ab	0.22 (0.03) ab
GT	58.78 (9.48) ab	1.25 (0.48) b	0.20 (0.04) b

* Different lowercase letters indicating significant differences between groups in columns.

CAPÍTULO 2

3.2 Capítulo 2

PROTECTIVE EFFECT OF MATRIX METALLOPROTEINASES INHIBITORS ON RADICULAR DENTIN EROSION: AN *IN VITRO* STUDY

Maria Denise Rodrigues de-Moraes, University of Fortaleza, Fortaleza, Ceará, Brazil. Graduate Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceara, Fortaleza, Ceara, Brazil. (85) 988965991; denisermoraes@gmail.com

Marcelo Victor Sidou Lemos, Graduate Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceara, Fortaleza, Ceara, Brazil. (85)996119893

Vanara Florêncio Passos, University of Fortaleza, Fortaleza, Ceara, Brazil. (85) 999882039; vanarapassos@hotmail.com

Sérgio Lima Santiago, Department of Operative Dentistry, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceara, Fortaleza, Ceara, Brazil. (85) 988842704; sergiosantiago@ufc.br

Address for correspondence:
Sérgio Lima Santiago
Rua Monsenhor Furtado, s/n – Rodolfo Teófilo
CEP 60430-355 – Fortaleza – CE – Brasil
E-mail: sergiosantiago@ufc.br

PROTECTIVE EFFECT OF GREEN TEA INFUSION ON RADICULAR DENTIN EROSION: AN *IN VITRO* STUDY

Abstract

Objective This study aims to evaluate the effect of green tea infusion in protecting tissue loss caused by erosion on human root dentin.

Materials and methods Root dentin blocks were immersed in human saliva (2h) and subsequently submitted to three-day-cycles of erosive challenge. The specimens (n=10) were selected based on their surface hardness and submitted to erosive challenge-3 times/day (citric acid 0.05 M, 1 min) and treatments (5 min): DW-Distilled water; CHX-0.12% Chlorhexidine digluconate, and GT-Green tea infusion, with immersion in artificial saliva between (60 min) and at the end of the cycles (overnight). At the end of each experimental day, measurements were performed to verify surface wear, roughness and % of surface hardness loss (%SHL). Scanning Electron microscopy (SEM) were qualitatively analyzed. Data were analyzed by two-way repeated-measures ANOVA and Tukey test (α=0.05).

Results ANOVA two way repeated-measures revealed in the results of the wear difference between the groups and the time of experiment ($p<0.05$). Tukey's test showed less wear to GT than the DW ($p<0.05$). For the roughness, Tukey's test showed the lowest mean value was for the treatment with GT ($p<0.05$). In %SHL, it revealed significant differences for the time and not for the groups. Tukey's test showed on the third day the %SHL was higher.

Conclusion The green tea infusion has shown to be promising as a protective agent of root dentin in cyclic erosive challenge *in vitro*.

Clinical Relevance Root dentin treatment with green tea infusion can be considered effective against erosive tooth wear.

Keywords *Camellia Sinensis* Citric acid Tooth erosion Tooth wear Green tea

Introduction

Non-carious cervical lesions are associated with gingival recession exposing root dentin [1]. It is difficult to diagnose these lesions in early stages, and in advanced stages the dentine becomes more and more exposed [2]. In such cases, the deleterious effect of erosion seems to be sharper due to a more rapid and extensive exposure of the dentin, because the cementum layer overlying the surface of the root is thin and can be easily removed [3].

During demineralization, collagen fibrils present in the organic matrix are exposed[4]. The exposed collagen fibrils are subject to degradation due to the action of a family of collagenolytic enzymes known as matrix metalloproteinases (MMPs) [5,6]. MMPs are endopeptidases, zinc and calcium dependent which contribute to the organization and mineralization on the dentin matrix. Such enzymes are deposited in human dentin during formation of the teeth and remain inactive, but when in acidic environments, as in caries and erosion lesions, are activated [6,7]. Although, few studies evaluated the role of MMPs in dental erosion, it can be speculated that such MMPs may be directly related to the degradation of exposed collagen [4,8,9].

It was demonstrated that MMPs inhibitors reduce the loss of dentin substrate when it is exposed to an erosive challenge [4,9-11]. Study has shown that chlorhexidine (CHX) has MMP-2, -8 and -9 activity inhibiting potential. The effect of CHX to inhibit MMPs is attributed due to a mechanism of zinc chelation, since the MMPs -2, -9 inhibition may be avoided by adding calcium chloride to the CHX connections [12].

Besides CHX, fluorides and ferrous sulfate ($FeSO_4$) have also been related as potential anti-erosion agents [13], in relation to fluorides, its effects are mainly attributed to the formation of a protective layer of calcium fluoride on dental tissue. This fact would make the teeth more stable and less susceptible to demineralization, making a lower pH level necessary to cause such effect [14-16]. In relation to ferrous sulfate, its action is attributed to the replacement by cations $Fe^{2+}$of the essential ions in the MMPs structure ($Ca^{2+}$and Zn^{2+}), causing reversible inactivity [17].

The investigation of organic anti-erosion agents has gained increased importance. Green tea polyphenols (*Camellia sinensis*) have been identified as promising agents in the strategy to preserve collagen fibrils exposed to collagenolytic degradation [9,10,18,19]. The flavonoid epigallocatechin-3-gallate (EGCG) is the main polyphenol found in green tea, and presents ability to inhibit the expression and activity of MMP-2 and MMP-9 [20-22]. Green tea extract has shown the ability to reduce the dentin erosion progression [9,10,17,18]. EGCG

seems to interact with such enzymes via hydrogen bonds, which are responsible for the change of its secondary structure and consequently inhibition [22].

The green tea extract is often used daily in diets and seems particularly attractive, since this tea contains EGCG and others catechins that control dentin degradation. However, there are still doubts concerning the action of treatment solutions to dentin erosion after cyclical exposures of acids. Since the concentration and distribution of MMPs in the dentin varies along different dentin depths [23], it seems valuable to evaluate whether using green tea as a protease inhibitor would also protect loss of the root eroded dentin.

Therefore, the objective of this *in vitro* study was to evaluate the effect of green tea extract and chlorhexidine in protecting tissue loss of demineralized dentin by *in vitro* cyclic erosive protocol. The hypothesis is that the green tea infusion and 0.12% chlorhexidine digluconate has a protective effect against wear of the demineralized dentin by short erosive protocol of three-days.

Materials and methods

Experimental design

This is an *in vitro*, randomized and blind study with two factors under investigations: time (one, two and three days of the experimental cycle) and treatments DW: distilled water (control), CHX: 0.12% chlorhexidine digluconate and GT: green tea infusion [GT- EGCG (concentration 0.0014%)]. The specimens were randomly assigned into three groups (n=10) in accordance with the treatment.

The protocol was approved by the local committee (#003438/2016). The response variable used was the percentage of surface hardness loss (%SHL), evaluated by Knoop microhardness (FM100 Future-Tech Corp., Kawasaki, Kanagawa, Japan), and dentin wear and roughness, assessed by the contact profilometer Hommel Tester T1000 (Jenoptik, Schwenningen, Germany). The dentin surface was qualitatively evaluated by Scanning Electron microscopy (SEM).

Specimens preparation

Ninety recently extracted non-carious human third molars, which had been stored in a thymol solution at 0.01% (w/v) at 4 °C, were used in this study. The crowns were sectioned

from the roots with a diamond saw. Ninety dentin blocks (4x4x2 mm) from cervical third of the roots were obtained using a longitudinal coupled double-sided diamond disk in a IsoMet slow speed saw (Buehler, Lake Bluff, USA). Dentin blocks were mounted on acrylic appliances and grounded in a water-cooled mechanical grinder (Arotec S.A., Cotia, Brazil) using 400-, 600-, 800- and 1200-grit aluminum oxide abrasives, polished with felt paper and 1 μm diamond spray (Extec Corp., Enfield, USA) [18,24]. The initial values of surface Knoop hardness were determined using a microhardness tester (FM100 Future-Tech Corp., Kawasaki, Kanagawa, Japan), which were five indentations with a load of 10 gf for 5 s, distancing 100 micrometres from each other, taking as reference the center of the specimen. Thirty root dentin blocks with an average of Knoop hardness (KH) of 61.96 ± 6.19 were selected and randomly distributed in three experimental groups using a computer-generated randomization list (Microsoft Excel 2007, EUA). Subsequently, a thin coating layer of acid-resistant was applied on their polished surface, leaving the half area (2x2 mm) not protected by the varnish and subjected to acid challenge [18].

Erosive Cycling

On each experimental day, five volunteers without erosion, salivary dysfunction or active carious lesions donated fresh saliva samples. The secretion of saliva was stimulated by chewing on paraffin wax for five minutes. Saliva from the first minute of chewing was swallowed, and the rest was collected and deposited into a 50 mL centrifuge tubule. The saliva samples were centrifuged for 10 min at 2000 rpm in a pre-cooled centrifuge (4°C) NT-815 (Novatecnica, Piracicaba, Brazil). The clear fluid above the sediments was pooled and used for pellicle formation [18,25]. The specimens from each group were immersed in clarified saliva and incubated for two hours under stirring (oscillatory table TE143, Piracicaba, SP, Brazil), at 37°C (Olidef CZ, Ribeirão Preto, SP, Brazil) in order to simulate the formation of the acquired pellicle [25].

After the formation of the acquired pellicle, each block was immersed in a citric acid solution at 0.05M (dehydrated citric acid, pH 3.75; Dinâmica®, Diadema, SP, Brazil) for 60 s. They were then washed with distilled water and subjected to respective treatments for 5 min, and subsequently immersed in artificial saliva for one hour, with all steps performed under stirring at 100 rpm and kept at 37°C. This cycle was repeated for three days and three times daily. The cycle time was defined by previous pilot study. At the end of each experimental day, hardness, wear and surface roughness of each specimen were measured

[26]. After each experimental day analyses, the blocks were immersed in artificial saliva under stirring and stored at 37 ° C overnight.

The cyclic erosive challenge comprised the following steps: formation of the acquired pellicle, erosion, treatments and remineralization with artificial saliva, considering the influence of variables such as stirring, temperature and time of exposure [27] (Fig.1). The treatments were: 0.12% chlorhexidine digluconate (pH 5.5) prepared in a local compounding pharmacy; the green tea (Dr Oetker, Jd. Do Lago, SP, Brazil) was prepared before each cycle (200 mL), being 0.0014% the concentration of epigallocatechin-3-gallate (EGCG), obtained after analysis in spectrophotometer, and pH of 5.45.

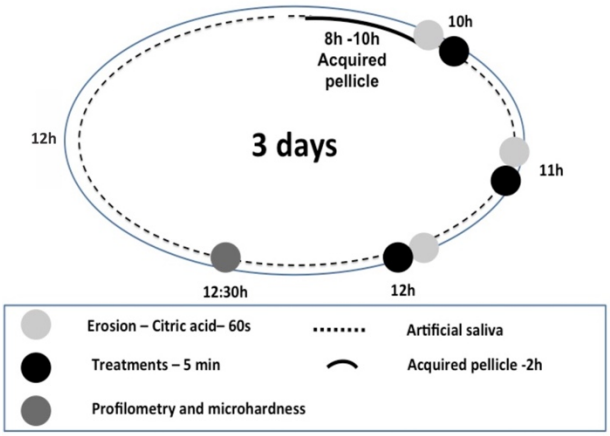


Fig. 1 Erosive cycle [18]

Surface Hardness Loss Percentage

Immediately after each experimental day, the blocks were analyzed in a microdurometer, where five new indentations were made using the Knoop tip load of 10 gf /5 s (SH-after), being such indentations carried to 100 μm of the previous measurements. The percentage of SH loss (% SHL) was then calculated for each day, according to the following equation: %SHL = [(SHbefore – SHafter) x100/ SHbefore] [18].

Measurement of wear and roughness

Before analysis, the nail varnish was carefully removed with acetone exposing the untreated reference areas. These measurements were performed as described in De-Moraes et al., (2016) [18]. Measurements of dentin surface loss were performed using the stylus profilometer (HommelTesterT1000 Hommelwerke GmbH, Alemanha), after each

experimental day. The diamond stylus moved from the reference to the exposed area (1.5 mm). The difference between the heights of the surfaces of the reference and the treated areas was evaluated. On each sample, at intervals of 100 μm, five profile traces (1.5 mm in length) were recorded, and the levels of dentin wear were determined in relation to the reference surfaces. For each sample, the mean values obtained from the five traces were calculated [28]. Surface roughness was described by the arithmetic mean of the absolute ordinate values Ra (average roughness as per ISO 4287) of 5 measurements made in each block [29].

Scanning Electron microscopy – SEM

Representative samples, three of each group, were selected and prepared for analysis. The surfaces of the specimens were visualized. The samples were fixed on stubs, stored at room temperature for 24 hours in a desiccator and sprayed with gold. The samples were analyzed by scanning electron microscopy Quanta FEG 450 (FEI Company, Oregon, USA). The acceleration voltage was 25 kV and the magnifications used were 500, 2000 and 4000 x [17].

Statistical Analysis

The data were calculated and analyzed statistically. Statistical procedures were performed with the Statistical Package for the Social Sciences (SPSS 17.0) for Windows. A Kolmogorov-Smirnov test was applied for the normal distribution of errors. Because the values were normally distributed across all groups, the data were analyzed by two-way ANOVA and the Tukey's *post hoc* test was used to evaluate the influence of treatment and number of experimental days. One-way ANOVA was applied for the means of experimental days. The level of significance was set at 5%.

Results

Two-way repeated-measures ANOVA revealed in the results of the wear significant difference between the groups ($p=0.019$) and the time of experiment ($p=0.017$). Tukey's test showed significant difference between the first to third day of experiment ($p=0.013$) and, in relation to the mean of three-day treatment, GT showed the less wear than the control

(p=0.018) and did not differ significantly from CHX (p=0.11) (Table 1).

Table 1. Mean and standard deviation of wear after each experimental day (μm).

Wear	DW	CHX	GT
Day 1	0.72 (0.23) Aa	0.67 (0.20) Aa	0.49 (0.17) Aa
Day 2	0.78 (0.15) Ab	0.71 (0.23) Aa	0.73 (0.27) Aa
Day 3	0.90 (0.26) Aa	0.87 (0.26) Aa	0.66 (0.32)Aa
Mean of treatments	0.80 (0.09) A	0.75 (0.10) AB	0.63 (0.12) B

* Similar lowercase letters together in the same column represent the absence of statistically significant differences (p> 0.05). Similar capital letters in the same line represent the absence of statistically significant differences (p> 0.05). DW: Distilled water, CHX: Chlorhexidine digluconate, GT: Green tea.

Table 2 shows mean and standard deviation of roughness values. Two-way repeated-measures ANOVA showed in the results of the roughness significantly difference for the groups (p=0.003) and not for the time (p=0.271). The lowest mean value was observed for the treatment with GT, and it was statistically significant between DW (p=0.009) and CHX (p=0.006).

Table 2. Mean and standard deviation of roughness values after each experimental day (μm).

Roughness	DW	CHX	GT
Day 1	0.05 (0.06) Aa	0.06 (0.05) Aa	0.05 (0.05) Aa
Day 2	0.06 (0.06) Aa	0.04 (0.03) Aa	0.02 (0.04) Aab
Day 3	0.05 (0.05) Aa	0.06 (0.05) Aa	0.02 (0.04) Bb
Mean of treatments	0.05 (0.01) A	0.05 (0.01) A	0.01(0.03) B

* Similar lowercase letters together in the same column represent the absence of statistically significant differences (p> 0.05). Similar capital letters together in the same line represent the absence of statistically significant differences (p> 0.05). DW: Distilled water, CHX: Chlorhexidine digluconate, GT: Green tea.

For % surface hardness loss (%SHL), Two-way repeated-measures ANOVA demonstrated significant differences for the time (p=0.010) and not for the groups (p=0.129). Tukey's test showed significant difference between day 1 and day 3 (p=0.008). On the third

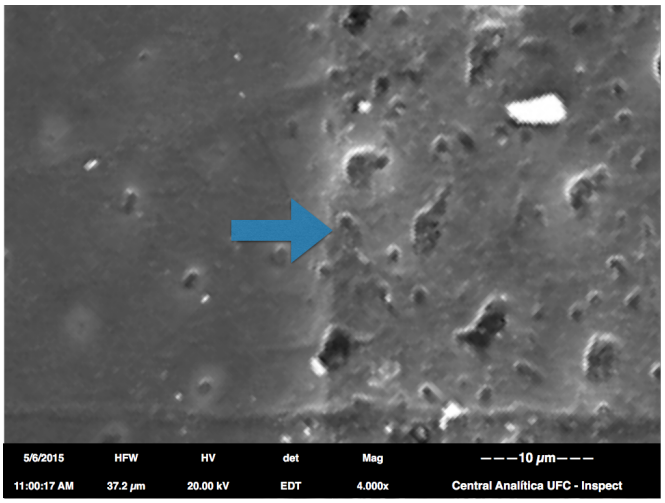
experimental day the %SHL was higher, showing the effectiveness of the erosive cycle challenge (Table 3).

Table 3. Mean and standard deviation of values of surface hardness loss (SHL) after each experimental day, in %.

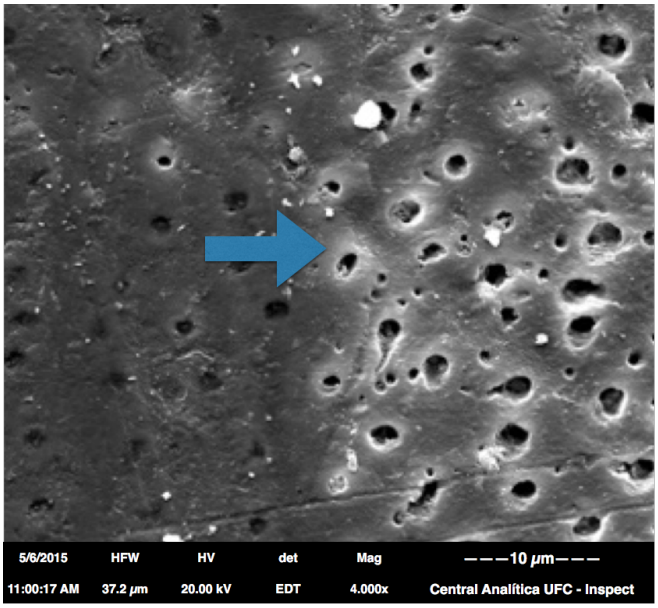
%SHL	DW	CHX	GT
Day 1	35.00 (8.68) Aa	31.12 (12.10) Aa	37.08 (5.92) Aa
Day 2	41.55 (15.36) Aa	32.92 (13.63) Aa	35.83 (10.76) Aa
Day 3	47.02 (9.31) Ab	42.21 (10.21) Bb	45.24 (14.00) ABb
Mean of treatments	41.19 (6.01) A	35.42 (5.94) A	39.38 (5.11) A

* Similar lowercase letters together in the same column represent the absence of statistically significant differences ($p > 0.05$). Similar capital letters in the same line represent the absence of statistically significant differences ($p > 0.05$). DW: Distilled water, CHX: Chlorhexidine digluconate, GT: Green tea.

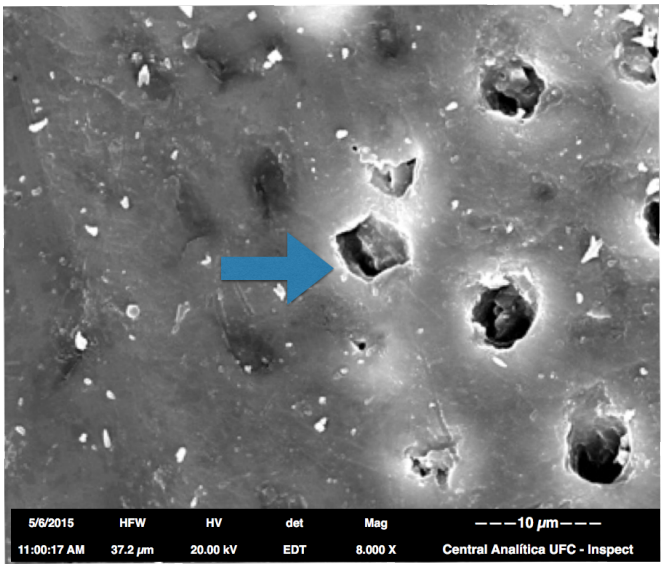
The scanning electron microscopy images were performed in order to verify the behavior of the root dentin surface subjected to cyclic erosive challenge process and treated with inhibitors. Figure 2 shows the interface between the reference area and eroded area (arrow). In the control group (DW), the interface is more perceptible whereas in the groups that received treatment with CHX and GT it was less visible. The tublules were partially oblitered when the root dentin was treated with green tea.



a) DW: Specimem treated with DW. The interface is more perceptible.



b) CHX: Specimem treated with CHX. The interface between reference and treated ared is less visible.



c) GT: Specimem treated with GT. The interface is less visible and the tubules in the treated area were partially oblitered.

Fig. 2 Scanning electron microscopy (SEM) of interface between reference area and erosion/treated area. DW-Distilled water (Control) (4.000x), CHX-0.12% Chlorhexidine digluconate (4.000x) e GT-Green tea (8.000x).

Discussion

Dental erosion is presented as a public health problem since there was an increased incidence in society. The development and progression of erosive lesions is modified by behavioral and biological factors such as acidic food (extrinsic origin) or gastro-esophageal problems (intrinsic origin) [30,31]. The constant search for low cost and highly efficient products that can prevent the loss of tooth structure and are easily available has gained importance in the context of current dentistry, since the etiologic factors of erosive lesions are difficult to control [32].

Previous study showed that treatment with green tea significantly reduced the loss of dentin by approximately 40% after erosive challenges [11]. Magalhães and colleagues (2009) [9] tested a green tea infusion compared with chlorhexidine and meridol. When compared to meridol, green tea infusion and chlorhexidine solution promoted less wear. The results of this study corroborate with that data in relation to treatment with green tea infusion, since GT treatment caused less wear than the control, reducing wear approximately 21%. Although erosion employed in this study was *in vitro*, a cyclic erosive challenge was used which allowed simulate acid frequent exposures occurring in *in situ* and *in vivo* studies. Moreover,

the human saliva was used for the acquired pellicle formation prior to the beginning of the erosion cycle, making possible the comparison between the *in vitro* and *in situ* methodology [31].

Kato and colleagues (2010) [17] have opted for testing EGCG and chlorhexidine in gel formulations, including a gel without active principle as a placebo and a control with 1.23% of sodium fluoride. The group with MMPs inhibitors have acted preventing erosion. Data obtained in the present study agree with these results, since the group treated with green tea has presented statically significant reduction in the wear and roughness in relation to the control. Moreover, in that investigation, the EGCG was tested isolated, while in the present study the green tea infusion was used. The components of the tea did favorable outcomes since the EGCG, even in a lower concentration (0.0014%), may favor a synergistic effect with the other catechins.

In the present investigation the group treated with green tea presented better results in relation to roughness analyzing by profilometry, not being observed differences between the control group and chlorhexidine. To the interpretation of these results it is desirable an understanding of the complex structure of dentin. The lack of a positive outcome for CHX group, maybe explained why there is no consensus in literature about the method used to measure the roughness after the dentin erosion challenge. Specially for the measurement of low roughness values in dentin, the atomic force and focus-variation 3D microscopy are better than stylus profilometer [33,34].

Analyzing the results of the surface hardness loss it can be observed, statistically, significant difference when increased the number of erosion cycles (day 1 to day 3), showing the effectiveness of the short erosive protocol of three-days conducted in the present study. Mirkarimi and colleagues (2012) [10] showed reduction of Knoop hardness measurements of dentin subjected to erosive challenge and treated with green tea infusion solution for one minute. However it has not corroborated with the results of the present study. The cyclical erosive challenge used may be the possible reason for the increase in the percentage of hardness loss at the end of the three experimental days.

The dentin erosion prevention by the tested substances may occur due to the potential of catechins derived from green tea inhibit endopeptidases that degrade demineralized organic matrix layer [35], thereby reducing the degradation of collagen fibers. In previous study, the GT was effective in reducing the wear of coronary dentin [18], in a similary erosive challenge cycle. However, there was a limitation of the methods used in this study, since does not prove the inhibition capacity, like in zymography studies which was tested by our group in another

study. Through scanning electron microscopy analysis could be observed that the solutions tested reduced the gap visualized in the interface (reference area/treated area).

Even though the experiment has been performed in laboratory conditions, it is important to analyze the side effects of treatments in repeated applications by cyclic erosive protocol [36]. Isbrücker and colleagues (2006) [37] evaluated the toxicity of a highly concentrated solution of EGCG (90%) in Wistar rat's diet during 13 weeks and this was not toxic at doses up to 500 mg/kg /day. The ingestion of 8-16 cups of green tea per day for healthy subjects, with similar EGCG concentration used in this experiment, for a period of four weeks, has shown to present transient side effects which is considered safe. On the other hand, the chlorhexidine, when applied by repeated periods, may cause reversible adverse effects such as mucosal irritation, dental staining and taste alteration [38].

Although erosive tooth wear has multifactorial causes, like a behavioral approach, the search for supporting treatments such as the use of natural substances with anti-erosion action is an important strategy to prevent tooth wear. Especially, when there is a substance that gathers various qualities such as accessible cost to the population, easily found in the market, low toxicity risk and side effects [37]. In this context, green tea infusion solutions represent a promising alternative to control wear on teeth damaged by erosion, since it brings several qualities described above and also has an inhibitory activity against the MMPs.

Conclusion

Within the limitations of this study, treatment with green tea infusion has shown to be a promising protective agent of root dentin, from the third day of treatment, in cyclic erosive challenge *in vitro*.

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CAPÍTULO 3

3.3 Capítulo 3

EFFECT OF GREEN TEA CATECHINS ON PROTECTIVE OF WEAR ON ERODED HUMAN DENTIN: AN *IN SITU* STUDY

GREEN TEA CATECHINS ON ERODED DENTIN

Maria Denise Rodrigues de-Moraes[1,4]; Vanara Florêncio Passos[1]; Gislaine Cristina Padovani[2]; Lady Clarissa Brito da Rocha-Bezerra[3]; Ilka Maria Vasconcelos[3]; Sérgio Lima Santiago[4]

1. University of Fortaleza, Ceara, Brazil; Graduate Program in Dentistry, Faculty of Pharmacy, Nursing and Dentistry, Ceara, Brazil
2. Department of Physic, Federal University of Ceara, Ceara, Brazil
3. Department of Biochemistry, Federal University of Ceara, Ceara, Brazil
4. Department of Operative Dentistry, Faculty of Pharmacy, Nursing and Dentistry, Ceara, Brazil

EFFECT OF GREEN TEA CATECHINS ON PROTECTIVE ERODED HUMAN DENTIN: AN *IN SITU* STUDY
GREEN TEA CATECHINS ON ERODED DENTIN

Abstract

Objectives: This study aims to evaluate the protective effect of green tea and Epigallocatechin-3-gallate on eroded dentin and endogenous proteases.

Methods: In the *in situ* experiment, twenty healthy volunteers (aged 20-33) participated in a single-center, double-blind and, three-treatment crossover study. Subjects have worn upper removable appliances containing four human dentin blocks. The erosive challenge (coke-1 min) was performed extraorally 4 times/day/5 days. Blocks were treated (1 drop-1 min) as follows: Control-No treatment, NaF-0.05% Sodium fluoride, EGCG-0.1% Epigallocatechin-3-gallate and GT-Green tea infusion. The specimens were subjected to Knoop hardness, stylus profilometry and scanning electron microscopy analyses. Matrix metalloproteinases (MMPs) were extracted from powder demineralized human coronary dentin (citric acid, pH 2.3) and were performed colorimetric assay and electrophoresis in gelatin. The gels were exposed to buffers containing G1-No treatment, G2-0.05% NaF, G3-0.12% Chlorhexidine digluconate (CHX), G4-GT, G5-0.1% EGCG and the activity was analyzed using zymography.

Results: Kolmogorov-Smirnov, analysis of variance (ANOVA) and Tukey's test in *in situ* study showed significant differences in dentin hardness loss for EGCG and GT ($p<0.05$), but not for roughness and wear ($p>0.05$). In Zymography analysis the 0.12% CHX, GT and 0.1% EGCG treatments inhibited the action of MMPs and in Colorimetric assay only the green tea inhibited the action of MMPs.

Conclusion: Green tea and EGCG may reduce the dentin damage caused by erosion and could contribute to inactivate endogenous dentin MMPs.

Clinical significance: The use of EGCG or green tea solutions immediately after acid intake can prevent the progression of dentin erosive lesions.

keywords: tooth erosion, tooth wear, collagen cross-linkers, matrix metalloproteinases inhibitors, epigallocatechin gallate, camellia sinensis.

1. Introduction

Dentin erosion is defined as an irreversible chemical loss from the hard dental tissue without the involvement of bacteria, with significant association with dentin hypersensitivity [1-4]. It is clinically characterized as shallow cavities on smooth surfaces, occurring the coronal from the enamel-cementum junction and, distinctive beveled and rounded cusps on the occlusal surface [5,6]. The mineral loss of peritubular dentin promotes a widening of dentinal tubules and an exhibition of collagen fibers, which become susceptible to a proteolytic degradation of matrix metalloproteinases (MMPs) [4]. Data regarding the prevalence of erosive tooth wear varies widely in different parts of the world, between 23.4% to 71.1%, with high prevalence in adults and adolescents [3,7]. The incidence of dental erosive wear increases with the age and acid dietary intake [1,8].

Preventive strategies should be placed on early lesions, however, in advanced stages, it was suggested to maintain demineralized organic matrix (DOM) in order to prevent ion diffusion and reducing acid damage on dentin. The preservation of DOM degradation by MMPs and other collagen-degrading proteases could be done by MMPs inhibitors, such as sodium fluoride, chlorhexidine digluconate solutions, and green tea catechins, like as epigallocatechin-3-gallate [6,9-16].

Plant-derived polyphenols have presented biocompatibility and bioactivity. The bioactivity means that these polyphenols interact with dentin collagen making an effective crosslinking which reduce dentin biodegradation [17]. This strategy can be used to protect dentin against erosion. Green tea and its main component, epigallocatechin-3-gallate, showed good results *in vitro* as a promising aid in controlling dentinal wear erosion process [10,14,16,18].

However, clinical studies about erosive tooth wear are laborious to be performed because of the difficulty in distinguishing it from other non-carious cervical lesions types and due to the dental erosive wear scoring systems have not been standardized yet [8]. Some *in vitro* studies have been published on methods to reduce or prevent erosion. Unfortunately, extrapolation of *in vitro* results is further complicated due to the different experimental conditions, divergent conclusions, and even using human saliva did not adequately reflect *in situ* conditions [19,20]. Thus, *in situ* studies could be a useful tool to investigate the protective effect on dentin lesion, since the natural protective effects of the oral cavity mimics an *in vivo* situation.

Specifically, the aim of this study was to verify that rinse with green tea polyphenols solutions protect eroded dentin surface loss and inhibit the endogenous proteases. The tested hypotheses were that 1) there is difference between the tested substances in the protection of

the dentin tissue loss, and 2) substances exhibit inhibitory action on the extracted MMPs dentin.

2. Materials and methods

2.1. Study design

This was a single center, randomized, double-blinded (blinded to the subject and to person responsible for performing the contact profilometry and hardness measurements), cross-over *in situ* study for erosive induction by acidic solution exposure involving three phases of 5 days each. The factor under investigation was treatment solutions (four levels): absence (control), Sodium Fluoride (0.05% NaF), Epigallocatechin-3-gallate solution - EGCG (0.1%), and the Green tea infusion. The dependent variables were quantitatively evaluated by profilometry and percentage of surface hardness loss (%SHL) and qualitatively evaluated by scanning electron microscopy (SEM), zymography, and colorimetric assay.

2.2. Sample preparation

Three hundred fresh extracted, undamaged human teeth from patients with informed consent under a protocol #003438/2016 approved by local committee, which were stored at 4ºC in 0.01% thymol solution until use.

The coronal dentin samples (4x4x2 mm) were obtained from extracted human third molars and the preparation procedures were conducted as previously described [16]. The baseline surface Knoop hardness (Microhardness Tester FM 100, Future Tech, Fujisaki, Kawasaki-City, Japan) was performed and 240 dentin blocks have showed a mean hardness of 68 ±10% Knoop hardness number (KHN). They were selected and randomly assigned; using a computer-generated randomization list (Microsoft Office 2007, EUA), into four experimental groups as folowing: G1 (Control) - no treatment, G2 - 0.05% NaF, G3 - 0.1% EGCG, G4 - Green tea infusion. The solutions were prepared immediately before the start of the in situ phase, except the green tea that was prepared daily.

2.3. *In situ* study

At a significance level of 0.05 and a power of 0.80, the sample size suggested was of 15 individuals. A total of twenty volunteers, undergraduate dentistry students, ranging from 20 to 32 years, were recruited to compensate possible dropout during the experimental period [21]. They agreed to participate and signed the informed consent form prior to enrollment in the study. Visual oral examinations were carried out by an experienced dentist. Inclusion criteria included good oral health; the absence of active caries (unless repaired prior to the study); no evidence of reflux and dental erosion; no periodontal disease that could compromise the study or the subject's health; absence of any oral appliance; subjects taking antibiotic or any medication that could interfere significantly with saliva flow; and showing abnormal salivary parameters, stimulated and unstimulated saliva rates (>1 and 0.50 mL/min respectively), and salivary pH≥6.8 [22].

The blocks were individually packaged and identified for sterilization by ethylene oxide before the *in situ* phase. Prior to the experiment, adhesive unplasticised polyvinyl chloride (UPVC) tapes were then placed on their polished surface, leaving half (2x2mm) exposed to subsequent testing [22]. Each 4 samples were fixed with wax into the recesses of the individual acrylic palatal appliances [6]. The position of the samples in the rows and the order of treatments in the phases were randomly determined for each volunteer (Microsoft Office 2007, EUA).

In each experimental phase, twenty volunteers used acrylic mucus-supported upper removable appliances. The devices were built in acrylic resin using the palate as a retentive area. A computer-generated randomization list (Microsoft Office 2007, EUA) was used to assign the patients to the phases and the positions of specimens on the device. The devices were prepared with four cavities (5x5x3 mm), in which four dentin blocks were randomly positioned and fixed with wax. The specimens were carefully positioned to allow the position 1mm below the device surface, avoiding contact with the tongue. Three treatment solutions were compared to the ability to protect human dentin surface of erosive lesions. In each phase the participants used only one of the treatments, characterizing a cross-over design, in which all volunteers were randomly subjected to all treatments (Figure 1). At each phase, the dentin samples and the treatments were changed [21].

Subjects were required to wear their appliance 12 h prior to the study to allow mineral equilibrium with saliva and also the formation and maturation of the salivary pellicle. They

have used day and night during three phases of 5 days each, and were instructed to avoid eating, drinking, or carrying out oral hygiene procedures using intra-oral devices. In order to prevent any damage of the volunteers, the erosive challenge of specimens was performed extraorally [20]. The volunteers immersed the appliance in a cup containing 50 mL Coke (pH 2.6, 0.32 ppm F, Coca-Cola Company, Brazil) at room temperature, for 1 min, 4 times a day (8h, 12h, 16h, 20h). Immediately after erosion, the appliances were rinsed for 60 s in water, the excess was removed with absorbent paper, and the volunteers dripped 1 mL onto blocks (4x/day) of the test solutions at room temperature: G2-0.05% NaF (Dinâmica, Diadema, SP, Brazil), G3-0.1% EGCG (Sigma-Aldrich, St Louis, MO, USA), G4-Green tea extract (Dr Oetker, SP, Brazil) or no treatment (G1-control) and, then reinserted into the mouth [6,23] (Figure 1). The pH of the substances was checked before the beginning of the experiment.

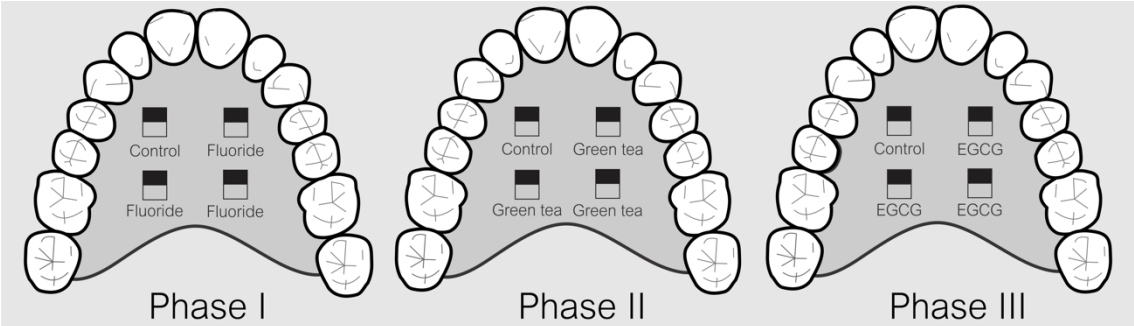


Figure 1. Schematic drawing illustrating experimental design for the clinical phase.

The volunteers received non-fluoridated toothpaste (Bitufo, Tupeva, São Paulo, Brazil), toothbrushes and were required to carefully brush the appliances extra-orally and not over the specimens. Over a two-day lead-in period the volunteers were instructed to standardize oral hygiene with the toothpaste provided (modified Bass brushing technique) and not used any mouthwashes before the start of each phase. A 2-day washout period between each phase was carried out in order to have no interference of the product used in the previous phase.

2.4. Measurement of dentin surface hardness loss, wear and roughness

Immediately after five-experimental days, the specimens were removed from the device and stored in moisture and refrigerator until the time of the analyses. Measurements of dentin surface loss, wear and roughness were performed after the experimental cyclic. The adhesive tape stuck on the half block was carefully removed, exposing the reference untreated area.

For Knoop hardness, five new indentations, 100 μm distant from each other and 500 μm far from the interface, were made on the same equipment (Microhardness Tester FM 100, Future Tech, Fujisaki, Kawasaki-City, Japan) and the same settings (under load of 10g/5s) used in baseline [24]. The percentage of SH loss (% SHL) was calculated, according to the following equation: %SHL = [(SHbefore – SHafter)x100/ SHbefore].

The dentin surface wear and roughness measures were performed with a stylus profilometry (Hommel Tester T1000, Hommelwerke GmbH, Germany). At intervals of 100 mm, three profile traces (1.5 mm in length) were recorded on each specimen. The dentin wear and roughness levels were determined in relation to reference surfaces. For each sample, the mean values were obtained [6,24,25].

2.5. Scanning Electron Microscopy - SEM

Representative samples of each group were selected and prepared for analysis. The surfaces of the specimens were visualized, as well as the transverse section of the dentin. The samples were fixed on stubs, stored at room temperature for 24 hours in a desiccator and sprayed with gold. The samples were analyzed by scanning electron microscopy Quanta FEG 450 (FEI Company, Oregon, USA). The acceleration voltage was 25 kV and the magnifications used were 500, 2000 and 4000 X [10].

2.6. Zymography and Colorimetric Assay

2.6.1. Sample obtainment and processing

Thirty-five higid human third molars had been obtained within two months after extraction. Dentin blocks with average dimensions of 4x4x3 mm were prepared as previously described [16] and stored at 4ºC, 100% humidity. Dentin blocks were frozen in liquid nitrogen and triturated using a Retsch mill (MM400, Retsch GmbH, Haa, Germany) at 30Hz for 15 min to obtain a fine powder, which was stored in at -20°C until used [26].

Green tea (Dr Oetker, Sao Paulo, Brazil) was obtained commercially and prepared by infusion of dehydrated leaves with 200 mL distilled water for 3 min prior to immediate use.

2.6.2. MMPs extraction

Mineralized dentin powder was brought in contact with 0.87 M citric acid (1:10, m/v), pH 2.3, for 24 h under moderate agitation in an orbital shaker (AP22, Phoenix Luferco, Araraquara, Sao Paulo, Brazil), at 4ºC. The suspension was then centrifuged at 10,000 x *g* (Hettich Rotina 380R-Tuttlingen, Germany) at 4ºC for 10 min, and the supernatant was discarded. The pellet was resuspended in distilled water, centrifuged at the same above conditions; this procedure was repeated three times. For extraction of metalloproteases from dentin, the pellet was resuspended in 10 mL extraction buffer (0.05 M Tris-HCl buffer, containing 0.005 M $CaCl_2$, 0.1 M NaCl, 0.1% [v/v] Triton X-100, 0.0001 M $ZnCl_2$, 0.02% [m/v] NaN_3, pH 7.5), and subjected to moderate agitation in an orbital shaker for 24 h at 4°C. The extract was centrifuged at 10.000 x *g*, at 4 ºC for 30 min [27]. The supernatant, rich in MMPs from dentin, was collected and dialyzed through a 12-14 kDa membrane (Sigma-Aldrich Co., St. Louis, MO, EUA) against distilled water at 4ºC for 24 h, lyophilized and stored at -20 ºC until use. The quantification of soluble protein was determined following the method described by Bradford (1976) [28], using bovine serum albumin (BSA) as standard protein.

2.6.3. Colorimetric assay

For evaluation of inhibitory activity of the MMP inhibitors tested in this study, it was performed a colorimetric assay using a SensoLyte Generic MMP Assay kit (#1022, AnaSpec, Freemont, CA, USA) in a 96-well microplate according to manufacturer's recommendations, with minor modifications. A pool of proteases extracted from dentin as previously described was used as source of MMPs. Fourty microliters of the enzyme solution were mixed with MMP inhibitors (50 µL of 0.05% [m/v] NaF or 0.12% [v/v] chlorexidine digluconate; or 20 µL of green tea or 0.1% [m/v] EGCG) and final volume was brought to 100 uL with assay buffer (component C). The plate was incubated at 37ºC for 10 min and then reaction was started after addition of 50 uL of 0.2 mM substrate (component A). Plate was let to stand at the same termperature and absorbances were measured at 412 nm every 10 min for 2 h using a microplate reader (EpochTM, BioTek, Winooski, VT, USA). The assay, performed in triplicate, was accompanied by a control group without inhibitor solution and another with a standard inhibitor (10 uL of 20 uM MMP inhibitor - component D).

2.6.4. Zymography

Visualization of in-gel profile of dentin MMPs in the absence and presence of inhibitors was performed according to Kato et al (2011). Initially, samples of MMP protein extract (40 μL) were applied to a 12.5% (m/v) polyacrylamide gel (8.5 x 8.0 cm) prepared in 0.025 M Tris-HCl buffer, pH 8.9, containing 1% SDS [29] and 0.1% (m/v) gelatin in a vertical system. Electrophoresis was performed at 20 mA constant current. After electrophoretic run, the gel was incubated in renaturation buffer twice (0.05 M Tris HCl buffer, containing 2.5% [v/v] Triton X-100 and 0.02% [m/v] NaN_3, pH 7.5) for 30 min at 37ºC and washed with distilled water. Gel was cut in 2 cm strips and then gels were incubated for 3 h at 37ºC in activation buffer (0.05 M Tris-HCl buffer, containing 0.005 M $CaCl_2$, 0.0001 M $ZnCl_2$ and 0.02% [m/v] NaN_3, pH 7.5), in the absence (control group – G1) or presence (experimental groups) of 0.05% (m/v) NaF – G2; 0.12% (v/v) chlorexidine digluconate – G3; green tea (used in preparation of activation buffer instead of distilled water) – G4 or 0.1% (m/v) EGCG - G5. Staining was done with a solution containing 0.025% (m/v) Coomassie Brilliant Blue G-250 in 10% (v/v) acetic acid, overnight. Excess dye was removed from the gel with a solution of 10% (v/v) acetic acid.

2.7. Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS 17.0 for Windows, SPSS Inc., Chicago, USA). A Kolmogorov-Smirnov test was applied to all groups to test for the normal distribution of errors. Because the values were normally distributed across all groups, one-way ANOVA and Tukey's post hoc tests were used for comparative purposes. The level of significance was set at 5%.

3. Results

The treatment compliance was satisfactory, the protocol used was not modified and all volunteers completed the study.

3.1. Dentin surface hardness loss, wear and roughness

Table 1 presents the mean and standard deviation of percentage dentin hardness loss, wear and roughness values found for all the treatments evaluated in the five-day experiment. In relation to dentin hardness loss (%SHL), no significant differences in dentin hardness loss

were observed for control and G2 (p=0.99), but it was different from G3 (p=0.007) and G4 (p=0.02). The groups G3 and G4 presented no significant differences (p=0.98).

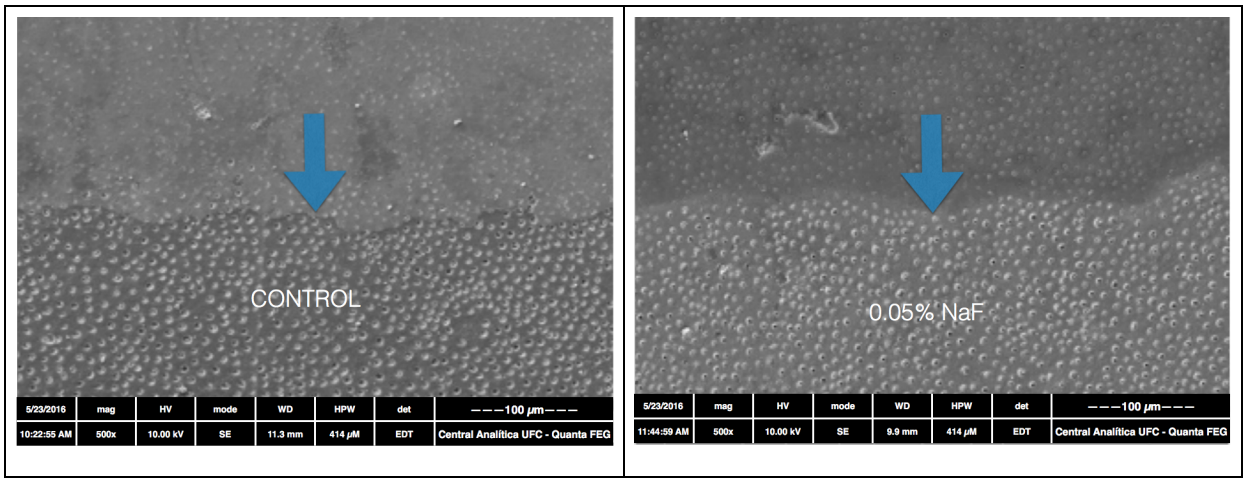
When the treatments were compared no significant differences were found among the treatments for roughness (p>0.05) and wear (p>0.05).

Table 1 Mean and standard deviation of dentin hardness loss, wear and roughness values found for all the treatments evaluated in the five-day experiment. Different letters indicating significant differences between groups in columns, p<0.05.

Group	Analyses		
	Dentin Hardness Loss (%SLH)	Wear	Roughness
G1- Control	37.39 (5.5) A	0.64 (0.1) A	0.19 (0.4) A
G2- 0.05% NaF	36.72 (9.5) A	0.65 (0.2) A	0.17 (0.6) A
G3- 0.1 % EGCG	45.59 (6.9) B	0.66 (0.1) A	0.17 (0.6) A
G4- Green tea	44.63 (8.5) B	0.58 (0.2) A	0.16 (0.8) A

3.2. Scanning electron microscopy (SEM)

The effectiveness of the erosive cyclic challenge of this study may be observed in figure 2. In these images a visible interface and the difference of the dentinal tubules between the reference and eroded/treated area were observed.



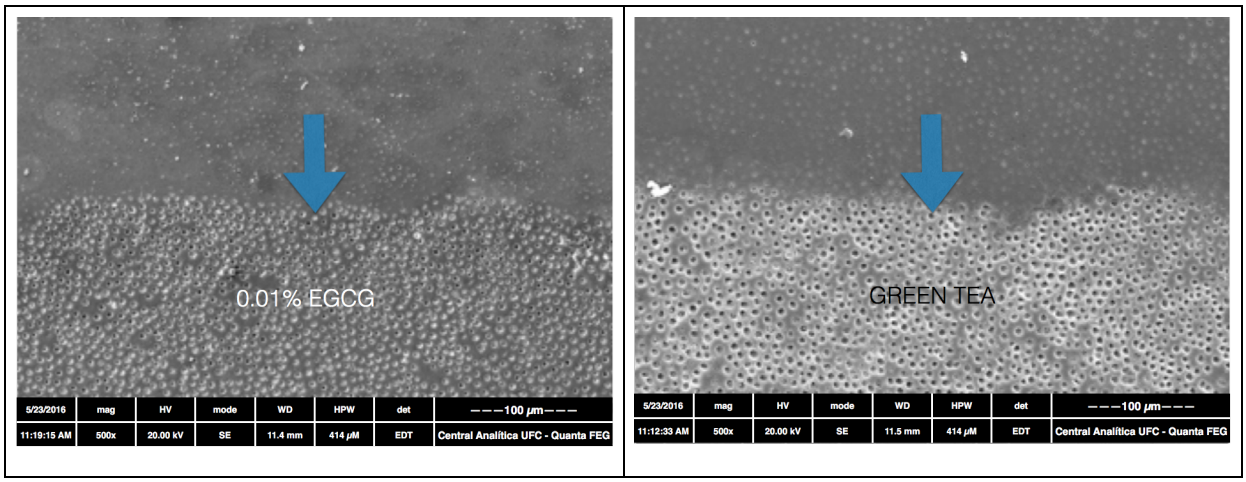


Fig. 2. Scanning electron microscopy (SEM) (magnification 500x) representative of interface between reference and eroded area without treatment (G1-Control) or treated with G2-NaF 0.05%; G3-EGCG 0.1%; G4-Green tea solution.

Figure 3 shows that in the groups G2, G3 or G4 the tubules are partially blocked, and, the G3 and G4 treatments preserve the peritubular dentin, while in the G1 and G2 groups the peritubular dentin was not preserved.

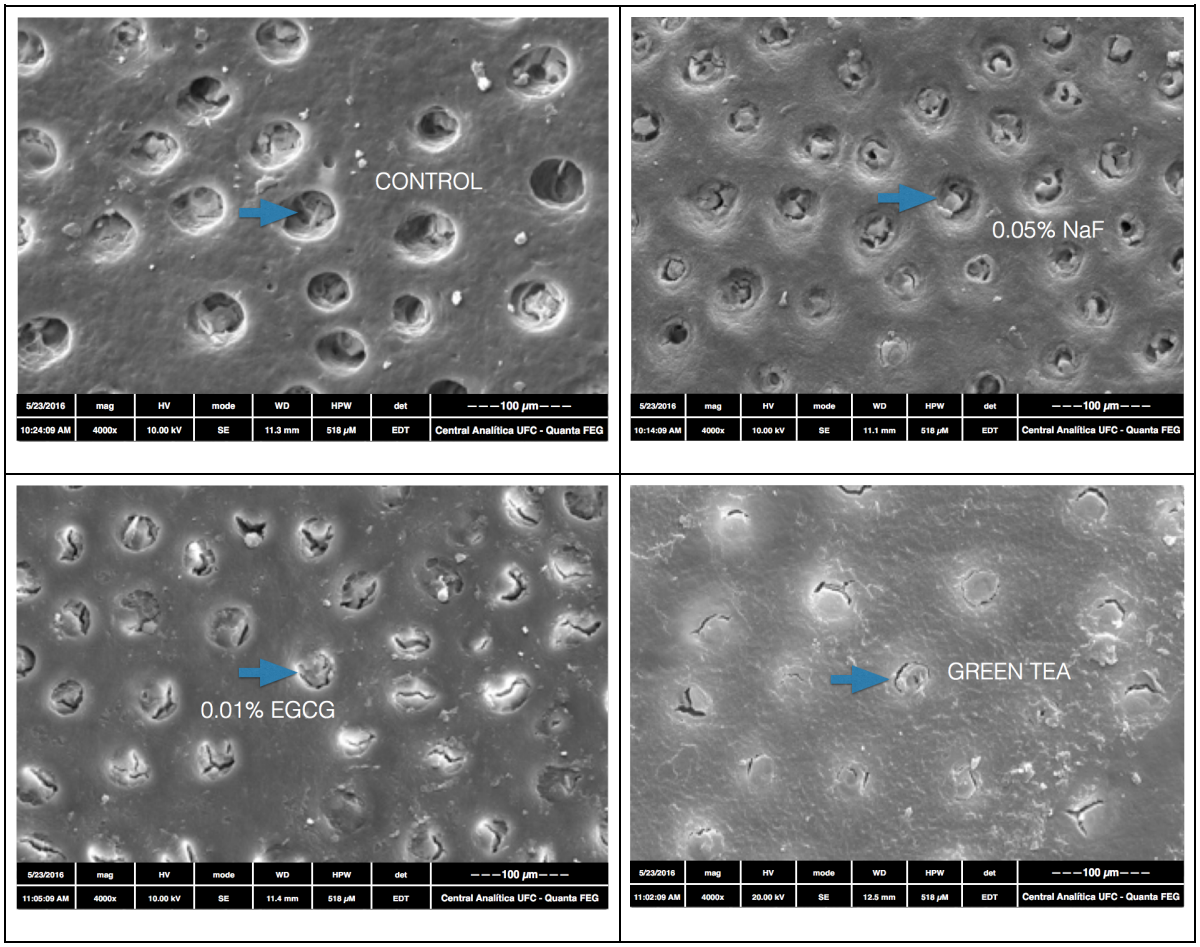


Fig. 3. Scanning electron microscopy (SEM) (magnification 4000x) of eroded dentin without treatment-G1 or treated with G2-NaF 0.05%; G3-EGCG 0.1%; G4-Green tea solution.

The Figure 4 shows that the eroded surface untreated presents a more obvious gap compared to reference area than the treated groups. Although this fact has not been statistically proven in roughness and wear analyses, it's clearly visible the interface in G1 and G2 groups, while the G3 and G4 showing the protector potential on dentin erosion.

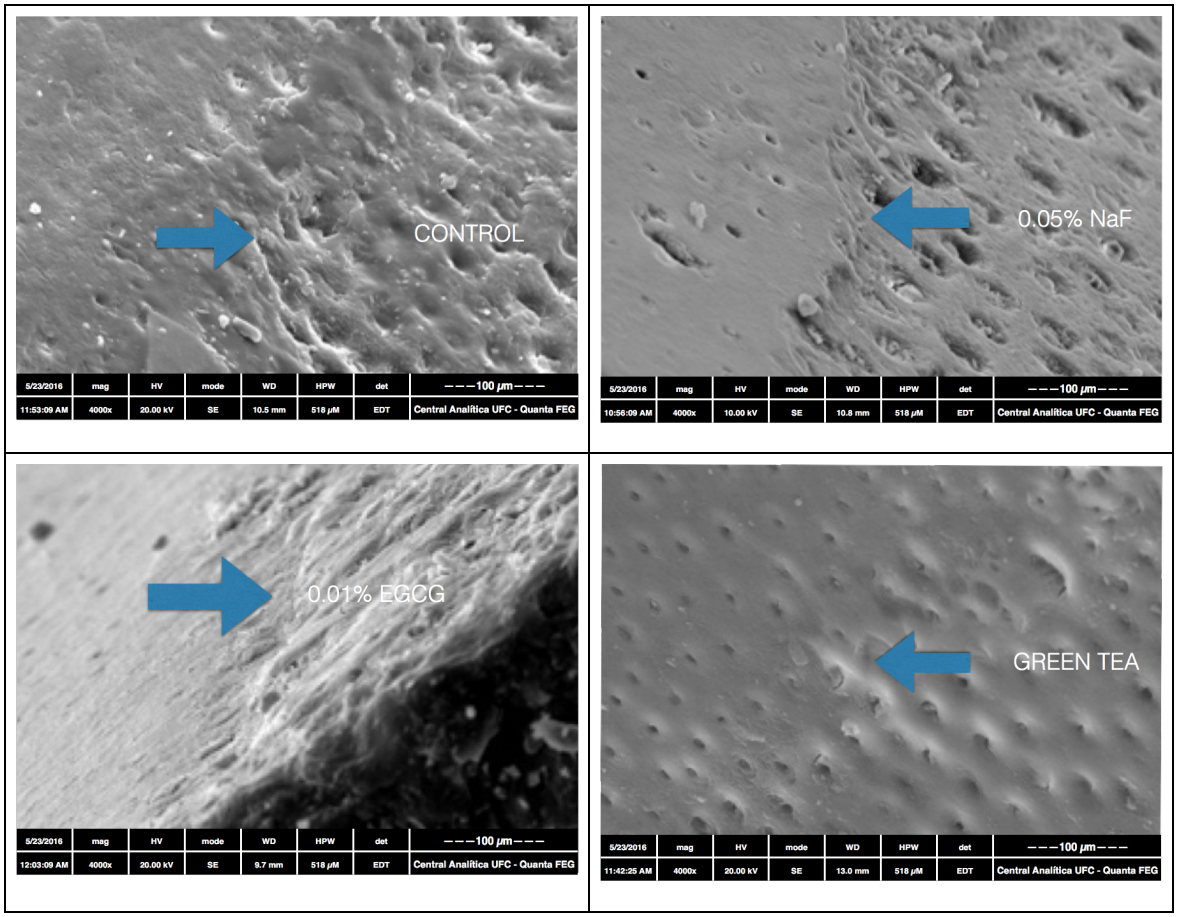


Fig. 4. Scanning electron microscopy (SEM) (magnification 4000x) representative images of the interface between reference and eroded area without treatment-G1 or treated with G2-NaF 0.05%; G3-EGCG 0.1%; G4-Green tea solution.

3.3. Colorimetric assay

Figure 5 shows the results of the colorimetric assay. The green tea extract demonstrated the best inhibitory activity against MMPs, being similar to standard inhibitor. After 60 min of reaction, it presented inhibitory activity similar to the standard inhibitor. The EGCG showed a little inhibitory activity until 60 min and then stabilized, but it was better than chlorhexidine

and sodium fluoride. The treatment with 0.12% CHX and 0.05% NaF did not show inhibitory activity, since they have similar behavior to the negative control.

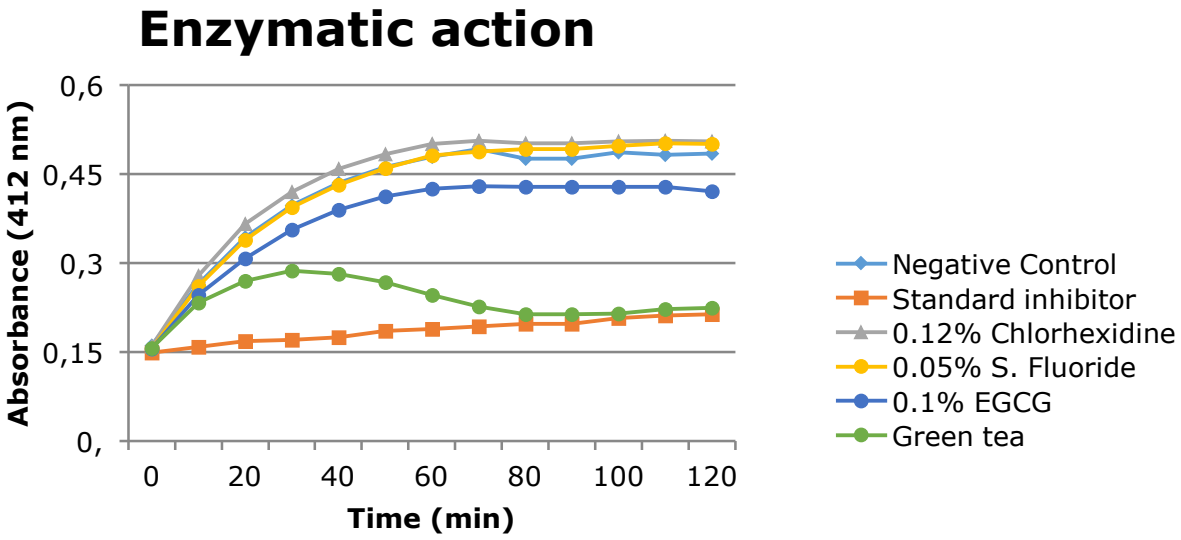


Fig. 5. Absorbance x time. Residual enzymatic activity of MMPs treated with the inhibitors.

3.4. Zymography

Figure 6 shows the results of gelatin zymography. The treatment with 0.12% chlorhexidine digluconate, green tea and 0.1% EGCG were able to inhibit proteolytic activity of MMPs, revealed by absence of clear protein bands in dark blue background. However, activity of dentin MMPs was maintained in the presence of 0.05% NaF, since protein bands of proteolytic activity were visualized, in a similar way to control (in absence of inhibitor).

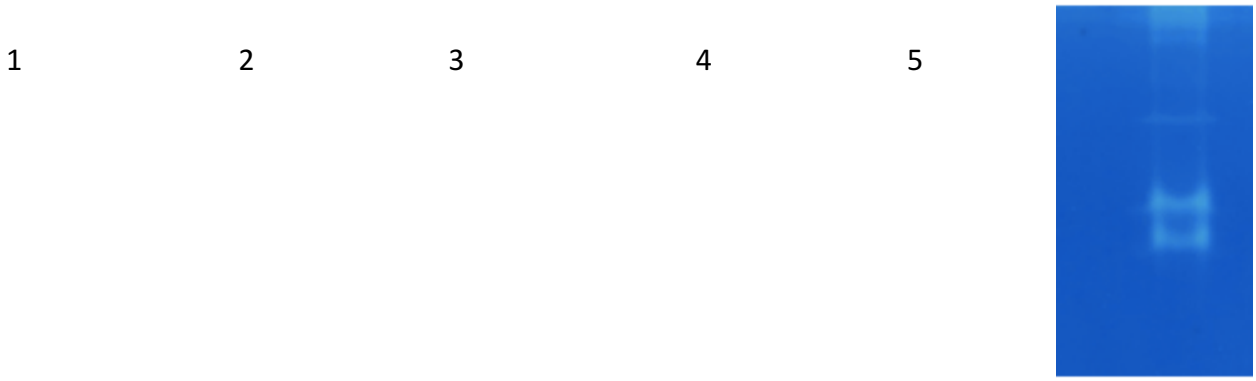


Fig. 6. Gelatin zymography of MMPs from dentin extract after treatments. 1 (no treatment), 2 (0.05% NaF), 3 (0.12% chlorhexidine digluconate), 4 (green tea), 5 (0.1% EGCG), respectively.

4. Discussion

The current experimental, *in situ* model, was designed to simulate clinical conditions as closely as possible. The main conditions related to dental erosion were reproduced with dentin human substrates, natural formation of salivary pellicle and the presence of physiological secreted saliva [10,19,30]. The cyclic acidic challenge followed by treatments suggested a positive effect on the reduction in dentin erosion when exposed by the action of the soft drink.

The results of this study showed that the green tea infusion and 0.1% epigallocatechin-3-gallate solution were important to control the dentin hardness loss when cyclic erosive challenge was used. Previous results strongly suggest that the preventive effects of the protease inhibitors against dentin erosion are due to their ability to reduce the degradation of the demineralized organic matrix [11]. It can be speculate that these substances present a potential inhibition of MMPs thereby reducing proteolytic degradation of dentin, demonstrated by the results of zymography and colorimetric assay. Additionally, polyphenols derived from plants, like green tea polyphenols, biomodify the collagen matrix improving mechanical properties and resist enzymatic degradation [15,17, 31]. Thus, the present results demonstrated that green tea infusion and 0.1% EGCG may emerge as a powerful tool in preventing the progression of dentin erosive lesions.

Scanning electron microscopy (SEM) examination allows providing information and contributes positively to visualized dental wear lesions features [32]. The green tea and EGCG show in the present SEM analysis important evidences in prevention of erosive dentin wear, as previously demonstrated [6,14]. This analysis confirms the quantitative results of this study showing that the best results for protecting the dentin against wear in erosive cyclic *in situ* challenge was due to the use of green tea and EGCG. As shown in SEM analysis (Figure 4), rinsing with 0.1% EGCG and green tea infusion solution led to lesser wear when compared to the control and NaF solution, however the difference between these solutions was not significantly demonstrated by profilometry analysis. This outcome may be explained due to the fact that before the start of the *in situ* study, the specimens were subjected to 12h-acquisition of a salivary pellicle and the salivary fluid prevents direct contact with acids. On the other hand, the size of the diamond tip may difficult the profilometer to identify subtle peaks and valleys [24,33].

A NaF mouthrinse was applied because this fluoride compound is widely used in oral

hygiene products. Additionally, in this study was carried out the most appropriate analyses used in studies about erosion [34]. The present zymography and colorimeter assay results do not corroborate with a zymography study that clearly shown inhibition by fluoride of salivary MMPs [12]. A possible explanation for this outcome, in the present study, was because it used a different source of MMPs, derived from dentin. Furthermore, when fluoride was used the results of wear and roughness did not show statistically significant difference in relation with the control, and SEM analysis shows no preserved peritubular dentin (Figure 3). Even though fluoride solutions are widely tested to prevent dental erosion, NaF was not effective on MMP-mediated collagen degradation [35]. To our knowledge, studies have never been conducted comparing surface quantitative and qualitative analyses with the tests that clearly show the inhibitory effect as zymographic and colorimetric assays, like as realized in present study.

In relation to the effect of the chlorhexidine, the result was different from others study that evaluate dentine loss by surface measurements [36]. The results of MMPs inhibitory effect of CHX in the zymographic outcome were consistent with results obtained previously, since inhibited MMPs [10]. Nevertheless, it should be emphasized that the chlorhexidine was incubated with MMPs extracted from demineralized dentin, favoring to proof of inhibitory effect on MMPs.

The mechanism of the direct effect of tested substances on collagen degradation, by assaying Hydroxyproline or NMR spectroscopy, was not investigated in this study. However, within the limitations of the present study, it was conducted to verify and confirm the inhibitors effect on MMPs extracted from dentin. The results of zymography assays demonstrated that the 0.1% EGCG and green tea infusion solution were effective to inhibit the MMPs activities, as was shown previously [14]. The colorimetric assay confirms these results since the green tea infusion demonstrated the best inhibitory action against MMPs, being similar to standard inhibitor. Additionally, 0.1% EGCG showed better inhibitors action than chlorhexidine and NaF, partially corroborating the results of Kato and colleagues (2012) [11]. This result can be justified due to the characteristic of bioactivity that have been reported for polyphenols from plants [17]. This is an approach to improve the mechanical properties of collagen and prevent enzymatic degradation [31].

These results suggest that the green tea infusion solution and epigallocatechin-3-gallate may reduce dentin damages caused by erosion and, could have inhibitory action on human coronary dentin MMPs. The results provide new insights for dentin erosion permanent

prevention by green tea catechins repeated applications. The study encourages investigation of the *in vivo* effects of these natural agents on demineralized dentin through erosion.

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4 CONCLUSÕES GERAIS

1. De acordo com os resultados do estudo *in vitro* em dentina coronária e radicular, o uso do chá verde é promissor na redução dos danos dentinários causados por desafio erosivo cíclico *in vitro*.

2. O chá verde e a Epigalocatequina-3-galato foram capazes de reduzir os danos causados pela erosão dentinária *in situ*.

3. O Chá verde e a Epigalocatequina-3-galato possuem efeito inibidor sobre as MMPs extraídas da dentina humana coronária.

4. O uso contínuo das catequinas derivadas do chá verde parece ser uma estratégia para prevenção da erosão dentinária. Contudo, necessita-se de mais estudos para confirmar o efeito desse produto natural em dentina erosivamente desmineralizada em situações clínicas.

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APÊNDICE A - TERMO DE DOAÇÃO DE DENTES

Eu, _____, RG _____, residente a _____, sob o meu consentimento estou doando ____ dentes, extraídos por razões independentes da pesquisa, para a realização da pesquisa intitulada "ANÁLISE DA AÇÃO DE CATEQUINAS DERIVADAS DO CHÁ VERDE EM DENTINA HUMANA EROSIVAMENTE DESMINERALIZADA: ESTUDOS *IN VITRO* E *IN SITU*", que será realizada na Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará pela pesquisadora: Maria Denise Rodrigues de Moraes.

Fortaleza,____de_____de20__.

_____

Assinatura do voluntário

Documento (RG):

Telefones:

_____

Assinatura do Responsável pelo estudo

Universidade Federal do Ceará / Departamento de Odontologia Restauradora

Rua Cap. Francisco Pedro s/n. Rodolfo Teófilo. CEP. 60430-170

Fone: 33668410/33668426

ATENÇÃO: A SUA PARTICIPAÇÃO EM QUALQUER TIPO DE PESQUISA É VOLUNTÁRIA. EM CASO DE DÚVIDAS, REALIZAR CONTATO COM O COMITÊ DE ÉTICA EM PESQUISA DA UFC.

Telefone do Comitê de Ética: (85)33668344.

APÊNDICE B - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título da Pesquisa- ANÁLISE DA AÇÃO DE CATEQUINAS DERIVADAS DO CHÁ VERDE EM DENTINA HUMANA EROSIVAMENTE DESMINERALIZADA: ESTUDOS *IN VITRO* E *IN SITU*

Objetivo da Pesquisa- O objetivo deste trabalho, *in vitro e in situ*, será avaliar a ação de catequinas derivadas do chá verde sobre dentina humana erosivamente desmineralizada.

Justificativa

Você é aluno da pós-graduação em odontologia e está sendo convidado a contribuir com a realização de uma pesquisa. Leia atentamente as informações abaixo e faça qualquer pergunta que desejar, para que todos os questionamentos sejam esclarecidos.

Convidamos você a participar da pesquisa intitulada:

ANÁLISE DA AÇÃO DE CATEQUINAS DERIVADAS DO CHÁ VERDE EM DENTINA HUMANA EROSIVAMENTE DESMINERALIZADA: ESTUDOS *IN VITRO* E *IN SITU*.

A população tem recebido maiores informações sobre a doença cárie e tem-se observado a manutenção da dentição natural de inúmeros pacientes por mais tempo na cavidade bucal. Tal fato enfatiza a necessidade de se aperfeiçoar métodos preventivos já existentes, com a introdução de técnicas inovadoras que possam agir como coadjuvantes na proteção e controle da erosão dental.

Desta forma, novos materiais são frequentemente lançados no mercado odontológico com o apelo de conseguirem controlar, ainda que de uma forma localizada, o aparecimento de novas lesões de erosão.

Entretanto, devido a razões éticas e às vantagens de um melhor controle experimental das variáveis, além de maior custo efetividade, parece desejável a utilização de modelos *in situ* para testar materiais e técnicas e sua capacidade de interferir no desgaste dental promovido pela erosão antes mesmo da realização de extensos e dispendiosos estudos clínicos. No entanto, ainda há na literatura científica uma escassez de estudos *in situ* que testem substâncias com efeito protetor contra erosão.

Procedimentos

Será realizado um estudo do tipo cruzado que compreenderá 3 tratamentos, sendo três fases de 5 dias, durante a qual você utilizará um dispositivo intra-oral palatino contendo blocos de dentina humana previamente esterilizados. Os 4 tratamentos serão:

Grupo 1: Sem tratamento-controle

Grupo 2: Solução de fluoreto de sódio 0,05%

Grupo 3: Solução de EGCG 0,1%

Grupo 4: Extrato de chá verde

Em um período anterior ao início das fases do experimento (2 dias), você deverá fazer uso do dentifrício pré-determinado a fim de padronizar as concentrações de flúor na saliva.

Instruções:

a) Fase clínica: Durante os cinco dias de tratamento, será realizado um procedimento erosivo quatro vezes ao dia (8:00, 12;00; 16:00; 20:00 h) utilizando bebida ácida (fornecida pelo pesquisador) em temperatura ambiente, para isso os dispositivos serão imersos em 50 mL por 60 segundos.

A seguir, o voluntário deve remover o dispositivo e lavar em água corrente com pouca pressão e gotejar os produtos fornecidos pelo pesquisador em cada quadradinho do dispositivo. Em seguida, todos os blocos contidos no dispositivo deverão receber uma gota (**uma gota sobre cada bloco**) com a solução específica designada, 4 vezes ao dia respeitando os horários pré-determinados pelo pesquisador (**8:00, 12:00, 16:00, 20:00 horas**).

b) utilizar o dispositivo intra-oral palatino diariamente, inclusive para dormir;

c) remover o dispositivo intra-oral somente durante as refeições ou ingestão de qualquer bebida ácida, durante este período o mesmo deve ser conservado no estojo fornecido e envolto com gase molhada em água com o objetivo de manter umidade;

d) fazer uso do dentifrício padronizado três vezes ao dia durante a escovação. Durante a escovação, o dispositivo deverá ser removido e os voluntários deverão limpar seus dispositivos cuidadosamente, sem escovar por cima dos blocos para evitar realizar abrasão sobre os blocos. O tempo de escovação do dispositivo e dos dentes não deve exceder a 3 minutos.

e) fazer uso de água fluoretada de abastecimento de Fortaleza (0,7 ppm F).

Desconfortos e Riscos

Vocês poderão apresentar discreta halitose apenas durante o período experimental, o que poderá ser resolvido com adequada higiene dental. Mesmo com remotas possibilidades, caso esta halitose persista após o período experimental, será realizada uma profilaxia dentária, bem como lhe será fornecido enxaguatório bucal com clorexidina até que o problema seja resolvido. O dispositivo intra-oral pode causar um leve desconforto na fonética, que é, entretanto, semelhante ao desconforto causado por um aparelho ortodôntico móvel. Durante todo o período da pesquisa, acompanhamentos semanais serão realizados, para verificar as condições do aparelho e da sua saúde bucal. Cabe ressaltar que não haverá consumo direto da substância, pois a mesma será gotejada sobre os blocos.

O benefício que vocês terão será um auxílio indireto, contribuindo para a realização deste projeto e o conhecimento que vocês adquirirão sobre a prevenção contra erosão. Este conhecimento poderá ser utilizado futuramente em prol da população alto risco de erosão dentária.

Forma de acompanhamento e assistência

Os pesquisadores envolvidos na pesquisa estarão à disposição de vocês para ajuste no aparelho intra-oral a fim de minimizar qualquer desconforto.

Garantia de esclarecimento

Você tem garantia de que receberá resposta ou esclarecimento de qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados à pesquisa. Também os pesquisadores supracitados assumem o compromisso de proporcionar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando. Qualquer dúvida ou problema com o dispositivo intra-oral, por favor, comunicar-nos com a maior brevidade possível.

Tel: 3366-8410 (Clínica de Dentística)

Formas de ressarcimento

Vocês serão ressarcidos de despesas com o transporte-alimentação para a retirada das amostras contidas nos dispositivos.

Formas de indenização

Não há danos previsíveis decorrentes desta pesquisa.

Garantia de sigilo

Os pesquisadores asseguram a sua privacidade quanto aos dados confidenciais envolvidos na pesquisa.

Liberdade para se recusar em participar da pesquisa

O participante poderá recusar a continuar participando da pesquisa e também poderá retirar o seu consentimento, sem que isso lhe traga qualquer prejuízo. Tendo compreendido perfeitamente tudo o que me foi informado sobre a minha participação no mencionado estudo e estando consciente dos meus direitos, das minhas responsabilidades, dos riscos e dos benefícios que a minha participação implicam, concordo em dele participar e para isso DOU O MEU CONSENTIMENTO SEM QUE PARA TAL EU TENHA SIDO FORÇADO OU OBRIGADO.

ATENÇÃO: A sua participação em qualquer tipo de pesquisa é voluntária. Em caso de dúvidas, realizar contato com o **Comitê de ética em pesquisa da UFC**.

ATENÇÃO: Se você tiver alguma consideração ou dúvida, sobre a sua participação na pesquisa, entre em contato com o Comitê de Ética em Pesquisa da UFC. Rua Coronel Nunes de Melo, 1000 - Rodolfo Teófilo, fone: 3366-8344. (Horário: 08:00-12:00 horas)

O abaixo assinado _____,___anos, RG:_____, declara que é de livre e espontânea vontade que está como participante de uma pesquisa. Eu declaro que li cuidadosamente este Termo de Consentimento Livre e Esclarecido e que, após sua leitura, tive a oportunidade de fazer perguntas sobre o seu conteúdo, como também sobre a pesquisa, e recebi explicações que responderam por completo minhas dúvidas. E declaro, ainda, estar recebendo uma via assinada deste termo.

_____

Assinatura do voluntário

_____

Assinatura do Profissional que aplicou o TCLE

_____

Assinatura do Responsável pelo estudo

Universidade Federal do Ceará - Departamento de Odontologia Restauradora

Rua Cap. Francisco Pedro s/n. Rodolfo Teófilo. CEP. 60430-170. Fone: 33668410/33668426

APÊNDICE C - INSTRUÇÕES AOS VOLUNTÁRIOS

1. Todos os materiais utilizados não acarretarão em custo ao voluntário.

2. Durante o experimento, os voluntários deverão escovar seus dentes com o dentifrício fornecido pelo pesquisador.

3. A pesquisa será composta por 3 etapas, com duração de 5 dias cada uma e com um intervalo de 2 dias entre as mesmas.

4. Os voluntários utilizarão um dispositivo intra oral que possui 4 cavidades contendo cada uma delas um bloco de dentina humana previamente esterilizada para a pesquisa. O dispositivo deverá ser instalado um dia antes do início da fase experimental (Domingo), à noite, após a última higiene, idealmente às 19h00min.

5. Os voluntários deverão utilizar o dispositivo e só o removerão para as principais refeições (café da manhã, almoço e jantar), ocasião em que o dispositivo ficará envolvido em gaze encharcada por água de abastecimento.

6. Durante o uso do dispositivo, nenhum tipo de alimento ou bebida poderá ser ingerido, exceto água.

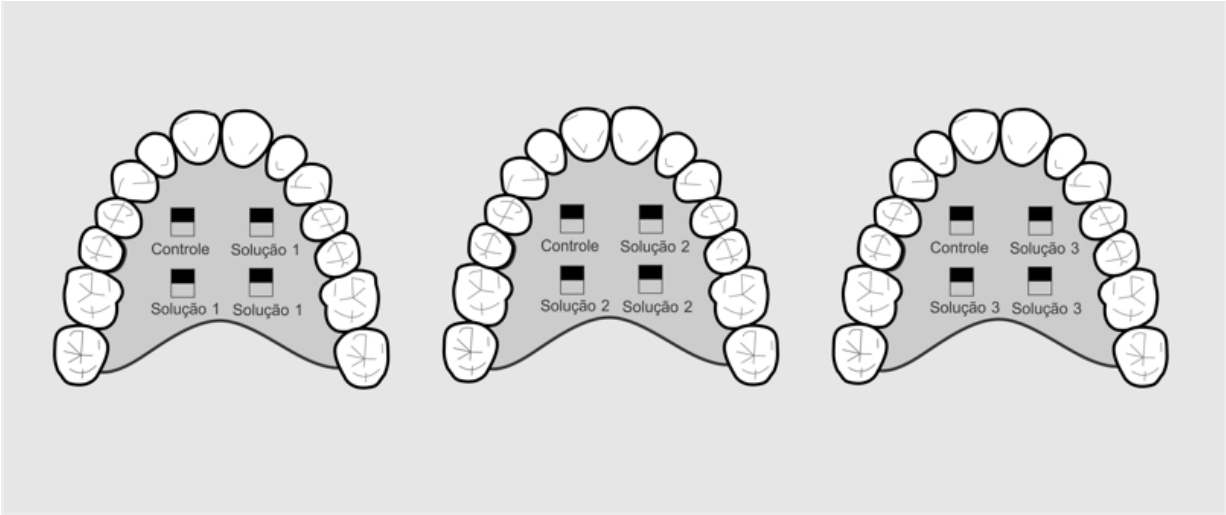
7. Evite que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para a refeição (máximo 1 hora).

8. Realize sua higiene bucal três vezes ao dia, utilizando o dentifrício dado pelo autor.

9. Durante os cinco dias de tratamento, o procedimento erosivo deverá ser realizado quatro vezes ao dia (8:00, 12;00; 16:00; 20:00 h) utilizando bebida ácida (fornecida pelo pesquisador) em temperatura ambiente, para isso os dispositivos serão imersos em 50 mL por 60 segundos.

10. A seguir, o voluntário deve remover o dispositivo e lavar em água corrente com pouca pressão e gotejar os produtos fornecidos pelo pesquisador em cada quadradinho do dispositivo. Em seguida aguardar em torno de 2 minutos antes de inserir na boca.

11. Para cada quadradinho colorido do dispositivo, terá um frasco da mesma cor para indicar qual substância deve ser gotejada em cada quadradinho (1, 2 ou 3 – conforme a figura exemplifica). As substâncias devem ser gotejadas quatro vezes ao dia (8:00, 12;00; 16:00; 20:00 h).



13. Os voluntários deverão retornar à clínica, logo acabe o período de experimento, para a troca dos dispositivos.

14. Quando qualquer material estiver acabando, entrar em contato com o pesquisador, para que este seja reposto.

15. Qualquer dúvida, entrar em contato com o pesquisador responsável do trabalho, no telefone: 988965991 - Denise.

Obs.: Favor observar todos os dias se os quadradinhos coloridos encontram-se na mesma posição e, caso desloquem de lugar, entrar imediatamente em contato com o pesquisador.

ANEXO 1 - ARTIGO 46 DO REGIMENTO INTERNO DO PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA DA UNIVERSIDADE FEDERAL DO CEARÁ

UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE FARMÁCIA, ODONTOLOGIA E ENFERMAGEM

CAPÍTULO VI

DOS EXAMES E DA DEFESA DE DISSERTAÇÃO E TESE

Art. 46 – As dissertações e as teses apresentadas ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará poderão ser produzidas em formato alternativo ou tradicional. O formatoalternativo estabelece: a critério do orientador e com a aprovação da Coordenação do Programa, que os capítulos poderão conter cópias de artigos e/ou relatórios de patentes de autoria ou coautoria do candidato, publicados ou submetidos para publicação em revistas científicas, escritos no idioma exigido pelo veículo de divulgação.

§1º - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

§2º - A dissertação e a tese em formatos tradicionais ou formatos alternativos deverão seguir as normas preconizadas pelo Guia para Normalização de Trabalhos Acadêmicos da Biblioteca Universitária disponível no sítio http://www.biblioteca.ufc.br. As partes específicas do formato alternativo deverão ser feitas em concordância com o *Manual de Normalização para Defesa de dissertação de Mestrado e tese de Doutorado no formato Alternativo do PPGO,* disponível no sítio http://www.ppgo.ufc.br.

§3º - As dissertações defendidas no formato alternativo deverão constar de, no mínimo, 01(um) capítulo, enquanto que as teses no mesmo formato deverão constar de, no mínimo, 02 (dois) capítulos.

§4º - Admite-se que a dissertação ou a tese sejam escritas e/ou defendidas em língua estrangeira seguindo as diretrizes definidas no regimento interno do Pro

ANEXO 2 – PARECER CONSUBSTANCIADO DO CEP

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ANÁLISE DA AÇÃO DE BIOMODIFICADORES EM DENTINA HUMANA EROSIVAMENTE DESMINERALIZADA

Pesquisador: MARIA DENISE RODRIGUES DE MORAES

Área Temática:

Versão: 1

CAAE: 52627316.8.0000.5054

Instituição Proponente: Departamento de Odontologia Restauradora

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.403.825

Apresentação do Projeto:

Projeto de doutorado da discente Maria Denise Rodrigues de Moraes sob orientação do Prof. Sérgio Santiago, pautado na avaliação da ação de diferentes inibidores enzimáticos sobre a dentina humana em situação de desafio erosivo cíclico. O trabalho constará de uma fase in vitro, na qual blocos de dentina humana serão submetidos de forma cíclica ao desafio erosivo pela imersão em ácido cítrico desidratado, seguido por quatro diferentes tratamentos (Solução de gluconato de clorexidina 0,12%; Extrato de chá verde; Epigalocatequina 3-galato 0,5% e Solução de fluoreto de sódio 0,05%. No estudo in situ, 20 voluntários utilizarão dispositivo intra-oral palatino, por três fases de 5 dias cada, contendo 4 blocos de dentina humana. O desafio erosivo será realizado por meio da imersão do dispositivo em ácido cítrico, seguido por tratamento com as mesmas substâncias utilizadas na fase in vitro. Os blocos de dentina e o dispositivo intra-oral serão submetidos aos testes de microdureza, perfilometria, zimografia e análise por MEV. Os resultados serão analisados por meio dos testes de Kolmogorov- Smirnov para avaliar o padrão de normalidade dos resultados, seguido de testes estatísticos apropriados, utilizando o nível de significância de 5%.

Objetivo da Pesquisa:

Objetivo Geral

Endereço: Rua Cel. Nunes de Melo, 1000
Bairro: Rodolfo Teófilo **CEP:** 60.430-275
UF: CE **Município:** FORTALEZA
Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

Avaliar a ação de biomodificadores sobre dentina humana erosivamente desmineralizada, in vitro e in situ.

Objetivos Específicos
Verificar a ação da solução de clorexidina, do chá verde, da Epigalocatequina-3-galato e do fluoreto de sódio sobre as metaloproteinases da dentina humana erosivamente desmineralizada.
Analisar in vitro a ação da solução de clorexidina, do chá verde, da Epigalocatequina-3-galato e do fluoreto de sódio na proteção da perda tecidual da dentina sob desafio erosivo cíclico.
Analisar in situ a ação do chá verde, da Epigalocatequina-3-galato e do fluoreto de sódio na proteção da perda tecidual da dentina sob desafio erosivo cíclico.

Avaliação dos Riscos e Benefícios:
A pesquisa é de baixo risco pois os voluntários podem apresentar dificuldade transitória na fonética e halitose na fase de uso do dispositivo intra-oral palatino.
Quanto aos benefícios destaca-se a contribuição para a descoberta e aprimoramento de materiais e técnicas para proteção de erosão dentinária, presente em cerca de 31-60% da população.

Comentários e Considerações sobre a Pesquisa:
Trata-se de uma pesquisa em duas fases onde o estudo in vitro é do tipo cego no desenho experimental e o in situ é cruzado e duplo cego no qual os voluntários utilizarão dispositivos intra-orais palatinos, por três fases de 5 dias. Todas as orientações para reduzir o aparecimento de halitose e dificuldades fonéticas estão contempladas.

Considerações sobre os Termos de apresentação obrigatória:
A pesquisadora apresentou ao comitê: projeto, folha de rosto devidamente preenchida e assinada, orçamento, cronograma, declaração de concordância, autorização do Laboratório da Pósgraduação em Odontologia, TCLE, currículo lattes da pesquisadora principal, carta de encaminhamento e termo de doação de dentes.

Recomendações:
Recomenda-se que os alunos da pósgraduação que concordarem em participar da pesquisa não estejam em atividades de aula com o orientador do projeto com o intuito de se evitar o "sentimento de coação" e não se sentirem obrigados a integrar o experimento.

Conclusões ou Pendências e Lista de Inadequações:
Não há pendências éticas nem documentais, s.m.j. deste colegiado.

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

Continuação do Parecer: 1.403.825

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_648866.pdf	21/01/2016 00:13:34		Aceito
Folha de Rosto	Folha_de_rosto_20_01.pdf	21/01/2016 00:12:11	MARIA DENISE RODRIGUES DE MORAES	Aceito
Orçamento	DECLARACAO_DE_CUSTEIO.docx	20/01/2016 23:53:25	MARIA DENISE RODRIGUES DE MORAES	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_20_01_2016.docx	20/01/2016 23:52:45	MARIA DENISE RODRIGUES DE MORAES	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TERMO_DE_CONSENTIMENTO_LIVRE_E_ESCLARECIDO.docx	20/01/2016 23:52:23	MARIA DENISE RODRIGUES DE MORAES	Aceito
Outros	DECLARACAO_DE_VINCULO.pdf	16/01/2016 15:14:11	MARIA DENISE RODRIGUES DE MORAES	Aceito
Outros	Carta_de_encaminhamento_ao_COMEPE.docx	16/01/2016 15:12:30	MARIA DENISE RODRIGUES DE MORAES	Aceito
Declaração de Pesquisadores	DECLARACAO_DE_PESQUISADORES.docx	16/01/2016 14:51:16	MARIA DENISE RODRIGUES DE MORAES	Aceito
Declaração de Instituição e Infraestrutura	Carta_de_Autorizacao.docx	16/01/2016 14:49:34	MARIA DENISE RODRIGUES DE MORAES	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Endereço: Rua Cel. Nunes de Melo, 1000
Bairro: Rodolfo Teófilo **CEP:** 60.430-275
UF: CE **Município:** FORTALEZA
Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

FORTALEZA, 04 de Fevereiro de 2016

Assinado por:
FERNANDO ANTONIO FROTA BEZERRA
(Coordenador)

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