UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

TAMIRES DE LUCCAS BUENO

Effect *in vitro* and *in situ* of proanthocyanidin-based dentifrices at the demineralized organic matrix submitted to erosion and dental abrasion

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Efeito *in vitro* e *in situ* de dentifrício à base de proantocianidina na matriz orgânica desmineralizada submetida à erosão e abrasão dentária

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

Orientador: Prof. Dr. Heitor Marques Honório

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RESUMO

Efeito *in vitro* e *in situ* de dentifrício à base de proantocianidina na matriz orgânica desmineralizada submetida à erosão e abrasão dentária

Este estudo teve como objetivo avaliar "*in vitro* e *in situ*" o efeito de dentifrício experimental à base de Proantocianidina na matriz orgânica desmineralizada, após ciclos de erosão e abrasão dentária. O estudo in vitro foi realizado com 50 espécimes obtidos de dentes bovinos, limpos e cortados em blocos de dentina 4x4x2 mm, polidos com tiras de lixa e com feltro. Os espécimes forma divididos em 5 grupos (N=10), de forma randomizada: G1- placebo (controle negativo); G2 – clorexidina a 0, 012% (1° controle positivo); G3 - NaF 1110 ppm (2° Controle Positivo); G4- proantocianidina 10% (1° grupo teste); G5- proantocianidina + flúor (2° grupo teste). Foram realizadas 3 ciclagens erosiva, 3x/dia por 5 min cada uma, por 5 dias. Logo após a segunda e terceira ciclagem, a escovação dos espécimes foram realizadas em uma máquina de escovação com uma carga 150g por 30s. O estudo in situ foi duplo-cego cruzado, realizado em 5 fases de 5 dias cada, com 10 voluntários saudáveis, que usaram 5 dispositivos palatais (1 para cada fase) com 4 amostras de dentina. Assim como realizado no in vitro, os blocos de dentina foram divididos de forma randomizada nos 5 grupos. Os participantes submeteram os dispositivos palatais em ciclagem erosiva, conforme o descrito para o estudo *in vitro*. No entanto, a escovação foi realizada pelos próprios participantes com escova elétrica por 30s. Tanto *in vitro*, quanto *in situ* a análise de microdureza foi realizada para seleção dos espécimes, já a perfilometria foi conduzida nos tempos iniciais e finais, sendo efetuadas cinco medidas para cada período. No estudo *in vitro* os dados foram analisados pelo teste de Kruskal-Wallis, Análise de Variância a um critério (ANOVA), seguido do teste de Tukey (p <0,05). Sendo os resultados obtidos: G1- 1,68 (1,52-2,08); G2- 1,25 (1,05-1,44); G3- 0,87 (0,74-1,03); G4- 0,48 (0,40-0,60); G5- 0,58 (0,55-0,62). O grupo G4 e G5 não houve diferença significativa, apresentando menor perda de dentina quando comparados aos demais grupos e G1 apresentou o maior degaste. Para o estudo *in situ*, os dados foram analisados pela ANOVA um critério, seguida do teste de LSD Fisher (p <0,05): G1- 1,76 (± 0,55); G2 - 1,19 (± 0,42); G3-1,29 (± 0,34); G4- 0,93 (± 0,38); G5 - 0,82 (± 0,34). Não apresentou diferença significativa entre os grupos G4 e G5, mas apresentou menor perda de dentina quando comparado a todos os outros grupos. Os dentifrícios de PA e sua combinação com dentifrícios fluoretados podem ser uma alternativa promissora para pacientes que sofrem com a erosão dentinária, evitando a perda mineral.

Palavras-Chaves: Dentina. Erosão. Abrasão. Metaloproteinases da Matrix. Dentifrícios.

ABSTRACT

Effect *in vitro* and *in situ* of proanthocyanidin-based dentifrices at the demineralized organic matrix submitted to erosion and dental abrasion

The objective of this study was to evaluate, in vitro and in situ, the effect of experimental dentifrice based on Proanthocyanidin in the organic demineralized matrix after erosive and abrasive challenges. The in vitro portion of the study was performed using 50 dentin specimens obtained from bovine teeth (4x4x2 mm), randomly divided in five groups after microhardness test (ranging from:41) (N=10 per group): G1- placebo (negative control); G2- 0.012% chlorhexidine (1st positive control); G3- NaF 1110 ppm fluoride (2nd positive control); G4- 10% purified proanthocyanidin (1st test); G5- fluoride + proanthocyanidin (2nd test). Erosive challenge was performed using an acid beverage (Coca-cola®), 3x per day during 5min each, for 5 days. The abrasion challenge was performed after the first and third erosive cycles using a mechanical brushing simulator with 150g of load for 30s in each specimen covered with the respective dentifrice slurry. In between the erosive/abrasive challenges, specimens were stored artificial saliva. The in situ portion of the study followed a crossover, double-blinded, design, and was performed in 5 phases of 5 days each, with 10 healthy volunteers who wore 5 palatal devices (1 for each phase) with 4 dentin specimens each. As for the in vitro portion, dentin blocks were randomly divided into 5 groups and submitted to the same erosive challenges. For abrasive challenge, brushing was performed by the volunteers using an electric brush for 30s. For both in vitro and in situ portions, the profilometry was conducted at the initial and final time points, with five measurements for each period. In vitro data were analyzed using Kruskal-Wallis test, one-way ANOVA, following by Tukey test ($p < 0.05$), and showed G4 and G5 with the lowest wear with median and Q1-Q3 of 0.48 (0.40-0.60) and 0.58 (0.55-0.62), respectively, followed by G3- 0.87 (0.74-1.03), G2- 1.25 (1.05-1.44), and G1- 1.68 (1.52-2.08). In situ data were analyzed by one-way ANOVA and Fisher's LSD test ($p < 0.05$), and showed similar trend of results: G4 and G5 showing the lowest wear 0.93 (± 0.38) and 0.82 (± 0.34) respectively, followed by G2 – 1.19 (± 0.42) and G3-1.29 (± 0.34), and G1- 1.76 (± 0.55) showing the highest wear. PA dentifrices and its combination with fluoride dentifrice can be an alternative to prevent mineral losses during erosive and abrasive challenges.

Keywords: Dentin. Erosion. Abrasion. Matrix metalloproteinases. Dentifrices.

LIST OF ILLUSTRATIONS

PAPER 1

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LIST OF ABBREVIATIONS AND ACRONYMS

g	Grams
h	Hours
min	Minutes
ml	Milliliters
mm	Milimeters
μm	Micrometers
CHX	Chlorhexidine
NaF	Sodium fluoride
1_{st}	First
2_{nd}	Second
G1	Placebo dentifrice group
G2	0.012% chlorhexidine dentifrice group
G3	NaF 1110 ppm fluoride dentifrice group
G4	10% purified proanthocyanidin dentifrice group
G5	Proanthocyanidin dentifrice + Fluoride group
s	seconds
DOM	Demineralized organic matrix
pH	Hydrogenic potential
MMP	Metalloproteinases
°C	Celsius degree
Al_2O_3	Aluminium oxide
#	Number
Ca^{2+}	Calcium
Zn^{2+}	Zinc
PA	Proanthocyanidin
CAPES	Brazilian Federal Agency for Support and Evaluation of Graduate Education
CNPq	National Council for Scientific and Technological Development

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1 Introduction

1 INTRODUCTION

In the last years, the prevalence of non-carious lesions has been increasing as a result of the increase in life expectance and maintenance of teeth in the oral cavity. Such lesions are characterized for mineral losses not caused by bacteria or bacterial subproducts (SHELLIS; FEATHERSTONE; LUSSI, 2014) and can present different classifications according with its etiology and clinical characteristics: abfraction, attrition, abrasion, and erosion.

The abfraction consists in the mineral loss characterized by occlusal overloads and stress concentration at cervical areas (ECCLES, 1982; GRIPPO, 1991). Attrition is a pathology caused by the contact between teeth (EISENBURGER; ADDY, 2002). Tooth abrasion occurs through mechanical forces during tooth brushing (which is related to brush bristle type, forces applied by patient, and dentifrices' abrasiveness) (ECCLES, 1982; RIOS *et al.*, 2006; RIOS *et al.*, 2007; MAGALHAES *et al.*, 2009; WEST; JOINER, 2014). Dental erosion consist in loss of mineral content as a result of intrinsic or extrinsic acids. Extrinsic acids are related with dietary habits such as frequent consumption of acidic beverages; intrinsic acids are produced by the patient's body, and tooth mineral losses can happen as a result of regurgitation and reflux disorders (i.e. anorexia and bulimia) (IMFELD, 1996; MEURMAN; TEN CATE, 1996; CARVALHO *et al.*, 2014; SHELLIS; FEATHERSTONE; LUSSI, 2014).

Tooth wear will start in enamel and, if the causes are not removed/controlled, the lesions will tend to progress into dentin, where its progression might be faster as result of the different tooth structures' composition (SHELLIS *et al.*, 2014). The dental enamel has a higher mineral composition (vol.85%), represented by crystals of hydroxyapatite arranged in prisms and it can suffer dissolution by acid substances, that can presented different concentrations of pH, making them fragile, soft and susceptible to wear, depending on the amount of acid challenge (HONÓRIO *et al.*, 2008; RIOS *et al.*, 2008; HONÓRIO *et al.*, 2010; SHELLIS *et al.*, 2011; GANSS; LUSSI, 2014). Dentin is a more complex structure, composed by inorganic components/apatite (47%), organic components (33%), and water (20%); and start to suffer dissolution at higher pH (5.5). The organic part consists of type I collagen (90%) and dentin phosphoproteins, proteoglycans and glycosaminoglycans (10% - non-collagen component) (SILVERSTONE; HICKS, 1985; PASHLEY *et al.*, 2004; MAGALHÃES *et al.*, 2009).

Mineral loss starts in enamel and tend to progress into dentin, when such lesions usually will start to be accompanied by dentin hypersensitivity (WEST *et al.*, 2013; Wara-

Aswapati *et al.*, 2005). Dental erosion occurs centripetally in dentin, expanding in the peritubular area and continuing towards the intertubular area (GANSS *et al.*, 2009; BUZALAF *et al.*, 2012). In addition to the acid-induced mineral losses, the collagenous organic matrix is also degraded by enzymes (i.e. metalloproteinases/MMP 2, MMP 8, MMP 9, and cathepsins K) (BUZALAF *et al.*, 2014; TJADERHANE *et al.*, 2015; TERSARIOL *et al.*, 2010).

The MMPs are activated in lower pH (during ingestion or contact with acids substances) however, these enzymes only degrade the collagen present at demineralized organic matrix (DOM) after the salivary neutralization (TJADERHANE *et al.*, 2015; ZARELLA *et al.*, 2015). Consider an important barrier in diffusion of acids, the DOM minimize the mineral loss preventing the demineralization of subjacent dentin (BUZALAF *et al.*, 2012; BUZALAF *et al.*, 2015).

Clinically, it is almost impossible to determine a single etiologic factor for non-carious lesions, being association between erosion and abrasion the most frequent causes, modulated by frequency, duration, and type/intensity of force. Brushing consists in the mainly abrasive challenge and it is also influenced by abrasives present in dentifrices, frequency, duration, and applied force, as well as toothbrush's characteristics (i.e. hardness) (DYER *et al.*, 2000; MAGALHÃES *et al.*, 2014).

It is noteworthy that roots/cement can also be susceptible to those lesions as a result of periodontal disease and/or gingival trauma, leaving the cervical area exposed to the chemical and/or physical challenges (MAGALHÃE *et al.*, 2012). Scientifics evidences proved that extrinsic factors, as well as higher frequency of acids food by patients are associate with erosive tooth wear (ECCLES; JENKINS, 1974; NUNN, 1996; ZERO, 1996; CARVALHO; MESTRINHO, 2014).

In view of the above, it is important to establish an intervention capable of avoiding collagen degradation and stabilize erosive tooth wear (Brackett *et al.*, 2015), as well as minimize dentin sensitivity, contributing for prevention of necessity of more costly and time-consuming treatments (SERRA *et al.*, 2009).

Studies have been focusing in prevent enzymes-driven degradation of dentin collagen matrix using the inhibitory effects of fluorides and chlorhexidine, as an approach to decrease lesion's progression (BUZALAF *et al.*, 2014). Nonetheless, fluoride is not capable of complete inhibit MMPs and its association with other inhibitors may be more efficient (KATO *et al.*, 2014).

Other MMPs inhibitors often includes natural products derived from fruits, vegetables, seeds, and nuts, such as the proanthocyanidins (PA) which presents with flavan 3 – ol subunit linked with C4-C6 (C8) (HAN *et al.*, 2003; KENNEDY; TAYLOR, 2003). Scientific evidences show that PA decrease collagen degradation and water sorption, as well as increase mechanical proprieties of dentin matrix (CASTELLAN *et al.*, 2010; CASTELLAN *et al.*, 2011). In addition, PA presents affinity with proteins rich in proline (as collagen), be able in synthetize of the collagen (HAN *et al.*, 2003), and also decrease endogeans proteases, inactivating and protecting areas of cleavage inside of collagen (Bedran-Russo *et al.*, 2014). The main mechanism of action consists in stabilization of dentin matrix through remineralization (forming calcium- PA complexes), resisting bacterial or enzymatic-driven degradation (BEDRAN-RUSSO *et al.*, 2011; LIU *et al.*, 2013; LIU; WANG, 2013; BEDRAN-RUSSO *et al.*, 2014; AYDIN *et al.*, 2019).

Based on the necessity of preventive treatments and on the promising properties of PA, the present thesis had as objectives to evaluate the effect of incorporating PA on dentifrices (associated or not with fluorides) and its action on dentin submitted to erosive and abrasive challenges. The primary purpose of paper 1 was to evaluate, in vitro, the behavior of different dentifrices in DOM after erosive and abrasive challenges. Paper 2 shared the same goal, but using an *in situ* evaluation.

2. Articles

2.1- Article 1

Effect of Proanthocyanidin-based dentifrices on dentin-wear – *In vitro* study

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Abstract

Objective: The use MMPs inhibitors is important for maintaining the DOM and minimize the dentin loss. Proanthocyanidin is an erosion preventive agent due to its properties. The purpose of this in vitro study was to evaluate the effect of proanthocyanidin-based dentifrices on dentin's wear resistance exposed to erosive and abrasive challenges.

Methods: Dentin specimens were divided in five groups (N=10) based on different dentifrices: G1- placebo (negative control); G2- 0.012% chlorhexidine (1st positive control); G3- NaF 1110 ppm fluoride (2nd positive control); G4- 10% purified proanthocyanidin (1st test); G5- fluoride + 10% proanthocyanidin (2nd test). Erosive challenges were performed through immersion in acid beverage (Coca-cola®), during 5 minutes, 3 times per day, for 5 days. Abrasive challenges were performed after the first and third erosive cycles, for each specimen, using a mechanical tooth-brushing simulator during 30s, with 150g load, covered with the respective dentifrices' slurry. The response variable was depth of dentin loss (in µm) measured by profilometry.

Results: Data were analyzed by Kruskal-Wallis and one-way Analysis of Variance (ANOVA) followed by Tukey's test ($p<0.001$). Data showed G4 and G5 presented median and Q1 and Q3 with the lowest wear 0.48 (0.40-0.60) and 0.58 (0.55-0.62), respectively, followed by G3- 0.87 (0.74-1.03) and G2- 1.25 (1.05-1.44), and G1- 1.68 (1.52-2.08). The Proanthocyanidin-based toothpaste showed the best results and could be an interesting approach for prevention of mineral losses due to erosive and abrasive challenges.

Keywords: dentin, erosion, abrasion, cross-liking agent, metalloproteinases, toothpastes.

1 Introduction

The erosive tooth wear results from acids of intrinsic and/or extrinsic origins, as well as possible associations with abrasion, but without bacterial involvement (non-carious lesion) [1]. The dental wear starts in enamel and, without the removal of the causes, reach the dentin tissue where the loss of tissue occurs faster.

In addition to the acid-induced mineral losses in dentin, the collagenous that support demineralized organic matrix (DOM) is also degraded by enzymes as MMP 2, MMP 8, MMP 9, that are activated by reduction in pH, but only degrade after the salivary neutralization and by cathepsin K) [2-4]. The preservation of the DOM is important for maintained the dental structure serving as mechanical barrier and avoiding the progression of erosive tooth wear in dentin [5-7].

MMPs inhibitors have been studied focusing on the maintenance of DOM and reduction of the erosive tooth wear. Natural products derived from grape seeds, nuts, cocoa seeds, green tea, are renewable sources of polyphenols (a.k.a Proanthocyanidin/PA)[8, 9], capable of inhibiting MMPs and act as cross-link agents promoting biomodification of dentin collagen in cleavage sites, which results in improved tensile strength and elastics properties, as well as in low cytotoxicity [10-13].

Maintenance of DOM not only serves as a barrier against acids, but also can resist to brushing forces, that may exceed the threshold of 4 N [14]. Dentifrices are the most used vehicle for oral hygiene. Although fluorides allow remineralization of the DOM and has some inhibitory effects on MMPs [14, 15, 16], the use of PA (associated or not with fluorides) could be an effective approach to reduce mineral loss in dentin [17]. The aim of the present study was to evaluate the effects of PA dentifrices in the wear of dentin subjected to erosive and abrasive challenges. The null hypothesis tested was that there would be no difference in the dentin mineral wear using a PA-containing dentifrice.

2. Materials and Methods

2.1. Experimental design

Fifty bovine dentin blocks (4×4×2mm) were randomly divided into 5 groups (N=10 for each group): G1- placebo dentifrice; G2- 0.012% chlorhexidine dentifrice; G3- NaF 1110 ppm fluoride dentifrice; G4- 10% purified PA dentifrice; G5- fluoride+PA dentifrice. The response variable was depth of dentin loss (μm) measured by profilometry.

2.2. Specimens Preparation and analysis

Fifty dentin blocks were prepared from bovine teeth roots using 2 diamond discs (Extec Corp, Enfield, CT, USA) interposed by a 4mm spacer, adapted on an ISOMET low-speed saw machine (Buehler Ltd, Lake Bluff, IL, USA) (Figure.1). Each specimen was ground flat with silicon carbide discs (320, 600, and 1200 grit) (Buehler, Lake Bluff, IL, EUA), and polished with felt discs impregnated with diamond solution (Buehler, Lake Bluff, IL, EUA) using a polishing machine (APL 4, Arotec, Cotia, SP, Brazil) (Fig.1). Between each disc, specimens were cleaned in ultrasonic T7 Thornton (Unique Ind. e Com. de produtos Eletrônicos Ltda., São Paulo, SP, Brasil) with deionized water for 2 minutes. Specimens were stored in 0.1% buffered thymol solution (pH 7.0) at 4°C until used.

In order to standardize the sample, Knop microhardness test (25g for 10s) (HMV-2000; Shimadzu Corporation, Tokyo, Japan) was used to assess the specimens, until 50 were obtained with values ranging from 41 (Fig.1). Each sample received 2 surface reference marks 1mm from each margin using a #11 scalpel blade, followed by profilometry Mahr (Marsurf GD, 25 Göttingen, Germany), five measurements using pre-established distances from the edge: 2.25; 2.0; 1.75; 1.5 and 1.25 μm were realized (Fig.1).

The erosive challenge was performed immersing each block in 17.6 mL of an acid soft drink (Coca-Cola, Coca-Cola®, Rio de Janeiro, RJ, Brazil) 25ºC, during 5 minutes, three times per day, for 5 days (in a total of 75 minutes), followed by rinsing with deionized water. Abrasive challenge was performed after the second and third erosive cycles using a mechanical brushing simulator during 30s per specimen with 150g load, with 200 oscillations per minute (in a total of 100 oscillations per cycle), with application of the respective dentifrices' slurry (50g of dentifrice : 100ml of deionized water) and use of a soft toothbrush (Colgate Twister, Colgate-Palmolive Company, São Bernardo do Campo, São Paulo, Brazil)(Fig.1). After each erosive and abrasive cycles, the samples were rinsed with deionized water and immersed in artificial saliva ($CaCl_2$ -0.70 mmol/L; $MgCl_2.6H_2O$- 0.20 mmol/L; KH_2PO_4 - 4 mmol/L; KCl- 30 mmol/L; NaN_3- 0.30 mmol/L; HEPES- 20 mmol/L) [18].

After the erosive and abrasive challenges, a second profilometry was performed (as described before), and the software MarSurf XCR 20 (Göttingen, Germany) was used to quantify the wear through superposition of initial and final profiles (Fig.1).

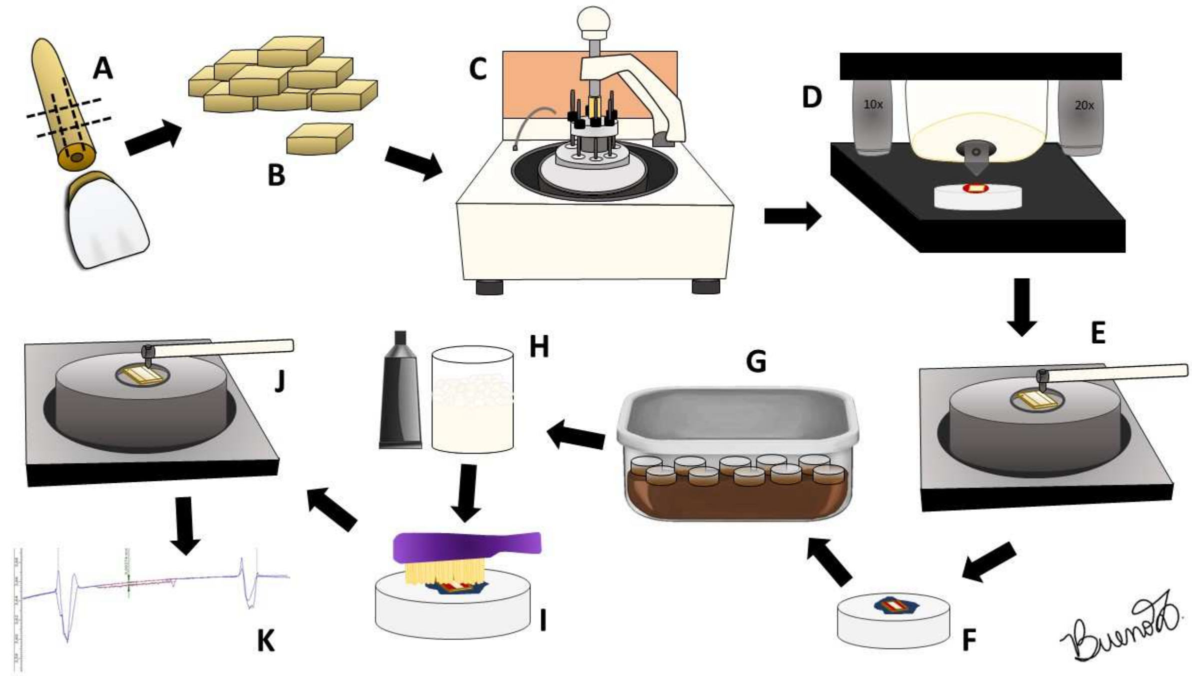

Fig. 1- Illustration of the experimental design. (A) The roots were sectioned with diamond disc. (B) Fifty bovine dentin blocks (4×4×2mm). (C) Specimens were ground flat with silicon carbide discs (320, 600, and 1200 grit) polished with felt discs impregnated with diamond solution. (D) Knop microhardness test for standardizing the sample. (E) Initial profilometry. (F) Protection of 2/3 with nail polish. (G) Erosive challenge (5min/3x/5days). (H) Dentifrices' slurry (50g of dentifrice/ 100ml of deionized water). (I) After 2nd and 3rd erosive cycles, abrasion with brushing (30s /150g load, with 200 oscillations). (J) Final profilometry. (K) Superposition profiles.

2.3. Statistical analysis

Data was assessed for normality using Shapiro-Wilk test, and analyzed with Kruskal-Wallis, followed by Tukey's test were applied and the level of significance adopted was. Data were analyzed by ANOVA followed by Fisher test (LSD) ($p<0.05$).

3. Results

The ANOVA one-way reveled differences for median values with an interquartile interval (Q1 and Q3) in dentin loss among the treatment groups (p = <0.001). The group placebo presented the higher values of median in dentin wear, however, not have statistically significant difference when compared with group of CHX. PA+F not had difference with dentifrice of fluoride (F). Just as PA and PA+F not presented statistically significant difference between them, showing the lowest values found of median (Table.1). All null hypothesis was rejected.

Table. 1- Median, Q1-and Q3 of dentin erosive-abrasive loss (mm) of the different groups (n = 10).

TREATMENT	MEDIAN (Q1-Q3)
PLACEBO	1.68 (1.52-2.08) a
CHX	1.25 (1.05-1.44) a, b
F	0.87 (0.74-1.03) b, c
PA	0.48 (0.40-0.60) d
PA+F	0.58 (0.55-0.62) c, d

*Distinct lower cases letter between the groups show statistically significant difference (ANOVA and Tukey's test, p < 0.001).

4. Discussion

Non-carious lesions such as dental erosion and abrasion have become more frequent in the last decade, and are often related to dentin hypersensitivity. This said, it is crucial to develop preventive treatments to avoid erosive tooth wear other than removing deleterious habits by itself, and the incorporation of natural cross-linkers and MMPs inhibitors to gels, mouthwashes and dentifrices could help to avoid dentin mineral loss, as well as to maintain the demineralized organic matrix DOM [6, 11].

Dentifrices could consist in a wide-spread and cost-efficient alternative to deliver such treatments and was the method proposed by the present study. As suggested by other authors [16], placebo group/G1 served as reference for the maximum wear which could be generated with the present erosion/abrasion protocols: 1.68 (Q1-1.52/Q3-2.08)], as it did not present any active principle in its composition.

CHX is considered the principal inhibitor of MMPs and cathepsins cysteine activities through chelation of metallic ions with dentin[4, 13]. Sodium fluoride also inhibits MMPs through reactivity of F^- with Ca^{2+} ion, causing a competition with MMPs, which depends of Ca^{2+} and Zn^{2+} [13, 19, 20].

Although CHX often shows lower dentin wear when compared with placebo, being similar to other inhibitors, [16, 21] the present study showed similar results between groups 1 and 2, probably due to the lower concentration used. Nevertheless, its results were similar to fluoride-containing dentifrices (G3) as reported in the literature [16].

The PA had been used as a natural cross-linker in preventive and reparative treatments [9, 22]. In the present study, PA was extracted from grape seeds and is capable of promoting dentine remineralization and modifications in the collagen matrix by formation of Calcium- PA [9, 12, 23], preventing it cleavage by collagenase and it solubilization, generating a biomodification intra and inter-molecular of the dentin collagen, as also synthesis of collagen[9, 12, 24, 25]. The present study's results showed lower values of mineral loss after erosive and abrasive cycles [0.48 (Q1-0.40/Q3-0.60)].

The association between both active principles (PA and fluoride) was also investigate in order to assess a potential increased effect (G5). Nevertheless, the dentin wear was similar to the dentifrices containing only Fluorides (G3) and only PA (G4).

Based on the above, both active principles were able to reduce dentin wear, although PA presented the best results both when used by itself or in combination with fluorides. Thus, the null hypothesis was rejected.

The erosive protocol was based on immersion in Coca Cola soft drink (pH 2.5) during 5min, in order to promote demineralization and activation of MMPs from dentin [6]. The abrasive challenges were performed using a mechanical brushing simulator in order to standardize the protocol. The specimens were stored in artificial saliva all the time, a remineralization protocol was performed for at least 2 hours prior to the next challenge [26]. Finally, bovine teeth were chosen since they its MMPs activities are similar to the human teeth; and they are readily available for research [27].

In vitro studies are important in dental erosion to support future studies in situ, are less expensive and not dependent of voluntaries [28]. But it is noteworthy that an in vitro study is not capable of fully simulate intraoral condition, especially the presence of salivary MMPs which might contribute for a faster degradation of dentin. This said, further studies should focus in the interaction of the active principles with additional enzymatic activity from saliva.

In conclusion, Proanthocyanidin's dentifrices, associated or not with fluorides reduced wear of bovine dentin after erosion and abrasion challenges. Fluoride-containing dentifrices are also capable of reducing dentin wear, although results are inferior to PA-containing dentifrices.

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2.2- Article 2

Evaluation of Proanthocyanidin-based dentifrices on dentin-wear after erosion and dental abrasion - *In situ* study

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Evaluation of Proanthocyanidin-based dentifrices on dentin-wear after erosion and dental abrasion - *In situ* study

Abstract

Objective: The aim of this in situ study was to evaluate the effect of proanthocyanidin (PA)-based dentifrices on dentin wear after erosive and abrasive challenges.

Methods: This crossover double-blinded study was performed in 5 phases of 5 days each, with 10 healthy volunteers who wore 5 palatal devices (1 for each phase) with 4 dentin specimens each. The groups under study were: G1 – placebo dentifrice (negative control); G2 – 0.012% chlorhexidine dentifrice (1st positive control); G3 – NaF 1110 ppm fluoride dentifrice (2nd positive control); G4 – 10% purified PA dentifrice (1st test); G5 – fluoride + PAdentifrice (2nd test). Erosion was performed by palatal device immersion in acid beverage (Coca-cola®), 3x per day/ 5min/ 5 days. Abrasion was applied after the first and third erosive cycles using an electric toothbrush during 30 seconds in each specimen associated with the respective dentifrices' slurry. The dentin wear (in µm) was assessed using profilometry.

Results: Repeated Measures Analysis of Variance (ANOVA) followed by LSD Fisher's test identified that G4 and G5 presented the lowest wear 0.93 (± 0.38) and 0.82 (± 0.34) respectively, followed by G2 – 1.19 (± 0,42) and G3-1.29 (± 0.34), and G1- 1.76 (± 0.55) showing the highest wear.

Significance: The PA-based dentifrice associated or not with fluorides could be a promising alternative for prevention of mineral losses due to erosion and abrasion.

Keywords: dentin, erosion, cross-liking agent, metalloproteinases, toothpastes.

1. Introduction

Non carious lesions such as dental erosion have become more prevalent in dental offices as a result of increased life expectance and better maintenance of teeth in the oral cavity, associated with dietary habits/external acid (i.e. wide consumption of acidic foods and beverages), as well as stomach acids/internal acid, leading to tooth structure dissolution [1-3].

Usually such lesions are recognized when the patient started to experience dentin hypersensitivity due to exposure of dentin in the oral cavity [4]. The lesion's rate of progression depends on frequency and duration of exposure, adjuvant factors such as dental abrasion resulting of toothbrush and/or parafunctional habits [1, 2, 5].

Non carious lesions in dentin tend to progress at a faster rate since dentin presents a higher critical pH (the dentin demineralizes at any pH below 5.5), lower surface hardness (when compared to enamel), presence of Matrix Metalloproteinases/MMPs and cysteine cathepsins which degrades the exposed collagen, contributing to dentin wear[6-9]. Within this context, demineralized organic matrix (DOM) acts as a barrier for acid penetration, and has some capabilities in preventing the advance of non-carious lesions, including some resistance to abrasion [6, 10, 11].

Many products have been studied with goal of avoid the collapse of the DOM as well as increase its resistance to wear. Proanthocyanidins (PA) are found in natural products and many studies have shown its capacity of remineralization, decrease of demineralization, interaction and synthesis of collagen in dentin and increase in its mechanical proprieties [12, 13]. The PA interaction with collagen in dentin probably occurs due to chemical linkage, by hydrogen bonding between phenolic hydroxyl part of PA with the protein amide carbonyl of collagen generating a cross-linkers mechanism, but also by hydrophobic and covalent linkage [14, 15]. This component has been tested in different ways, concentrations and time of application and in all studies presented good results [16, 17]. The use of PA could contribute in the prevention of mineral loss, especially if it becomes widely available in products of daily use (i.e. dentifrices) [18].

Due to greater difficulty of quantity the mineral loss in clinical research, many *in vitro* and *in situ* studies are developed [19] with aim reduce the dental wear, find answers for solve dental erosion problem, and evaluate new products may be clinically used [3]. However, *in situ* studies allow simulation inside of mouth, executed after in vitro studies, being considered an accurate methodology [19].

Based on the above considerations, the aim of this present study was to evaluate the effects of dentifrices containing PA on the dentin mineral loss after erosive and abrasive challenges, using in situ conditions. The null hypothesis tested was that PA dentifrices would not minimize dentin wear when compared with other tested dentifrices.

2. Materials and Methods

2.1. Experimental design

The present crossover, double-blinded, in situ study was performed in 5 phases of 5 days each, with 10 volunteers that wore 5 palatal devices (1 for each phase) with 4 dentin blocks each. The study factor was the dentifrices divided in 5 levels: G1 – placebo dentifrice (negative control group); G2 – 0.012% chlorhexidine dentifrice (1st positive control group); G3 – NaF 1110 ppm fluoride dentifrice (2nd positive control group); G4 – 10% purified proanthocyanidin dentifrice (1st test group); G5 – fluoride + proanthocyanidin dentifrice (2nd test group), having as response variable the depth of dentin loss (µm) measured using profilometry.

2.2. Specimens Preparation and Analysis

Two hundred dentin blocks (4×4×2mm) were extracted from bovine roots using 2 diamond discs (Extec Corp, Enfield, CT, USA) separated by a spacer of 4mm, adapted on an ISOMET low-speed saw machine (Buehler Ltd, Lake Bluff, IL, USA). Specimens were ground flat with 320, 600 and 1200 grit Al_2O_3 papers (Buehler) using a polishing machine (APL 4, Arotec, Cotia, SP, Brazil). Between each paper grit, samples were cleaned in ultrasonic T7 Thornton (Unique Ind. e Com. de produtos Eletrônicos Ltda., São Paulo, SP, Brasil) with deionized water for 2 minutes. Samples were stored in 0.1% thymol solution (pH 7.0) at 4°C until used.

After specimens' preparation, Knoop microhardness was performed with 25g load for 10 seconds (HMV-2000; Shimadzu Corporation, Tokyo, Japan) in order to ensure similar initial conditions among all groups (range of values 32). All specimens were sterilized by gamma radiation, randomized and divided into 5 groups. Two reference marks were created on the dentin surface using a scalpel blade and an initial profilometry was performed (Mahr Perthometer, Göttingen, Germany) as follows: five measurements were made for each specimen at pre-established distances from the edge: 2.25; 2.0; 1.75; 1.5 and 1.25 µm. Two-thirds of the surface was covered with red nail varnish maintaining 2 mm of exposed dentin area in the center.

After approved by the local ethics committee (CAAE: #65055317.7.0000.5417) and conformed 1964 Helsinki Declaration for experiments involving humans, ten volunteers were selected based on the inclusion and exclusion criteria (Table.1). For each of the 10 volunteers, an alginate impression (Jeltrate, Dentisply/ Sirona, Pirassununga, SP, Brazil) of the maxillary arch was performed using a stock metal tray, the casts were poured with type IV stone (Durone IV, Dentisply/ Sirona, Pirassununga, SP, Brazil) and the appliances were designed using selfcuring acrylic resin (JET, Artigos Odontológicos Clássico Ltda, São Paulo, SP, Brazil) with 4 sites (4 x 4 x 3 mm) where the randomized specimens were positioned and fixed with wax.

Table. 1- Criteria of inclusion and exclusion for selection of volunteers

INCLUSION CRITERIA	EXCLUSION CRITERIA
• Post graduate student • Age 21-35 years • Adequate oral health No caries or erosion lesions • Stimulated salivary flow rate >1 ml/min • Accept informed written consent.	• Systemic illness • Pregnancy or breastfeeding • Under orthodontic intervention

The ten volunteers wore 5 removable palatal appliances with 4 dentin specimens each, 1 appliance for each phase. Each study's phase lasted 5 days, had 7 days of interval before the following phase, and corresponded to each evaluated group. Erosive challenges were performed by palatal appliances immersion in 17.6 mL of coca cola soft drink (Coca-Cola®) at room temperature, 3 times per day x 5 minutes x 5 days (75 minutes total). Abrasive challenges were performed after the first and third erosive cycles using an electric toothbrush (Oral-B Vitality D12, P&G Ltad, Louveira, São Paulo, Brazil) during 30 seconds in each specimen associated with the respective dentifrices' slurry. The volunteers wore the appliances during 12 hours per day, removing it only for meals, oral hygiene, erosive/abrasive tests, and overnight (maintained in containers with humidity).

After the end of each study's phase, a new profilometer analysis was performed following the previously described protocol. Superimposition of initial and final profiles were performed using MarSurf XCR 20 (Göttingen, Germany) and the dentin loss was quantified.

2.3. Statistical Analysis

Data were analyzed by Repeated Measures Analysis of Variance followed by Fisher's LSD test ($p<0.05$).

3. Results

The wear results for the different tested groups are listed in Figure 1.

All groups presented dentin wear. G1 (1.76 ± 0.55) showed the highest values, followed by G2 (1.19 ± 0.42) and G3 (1.29 ± 0.34). G4 (0.93 ± 0.38) and G5 (0.82 ± 0.34) showed the lowest dentin wear among the tested groups.

Table 2 - Mean and standard deviation of dentin loss after erosive tooth wear and treated with different dentifrices (ANOVA $p<0.05$).

TREATMENT	**MEAN (STANDAR DEVIATION)**
G1	1.76 (± 0.55) a
G2	1.19 (± 0.42) b
G3	1.29 (± 0.34) b
G4	0.93 (± 0.38) c
G5	0.82 (± 0.34) c

4. Discussion

Many factors may interfere with dental wear. I*n situ* studies are capable of simulate what occur in patient’s mouths, overcoming the limitations with in vitro studies (do not fully reproduce intraoral situation) and in vivo studies (do not quantify the mineral loss) [19]. This said, a crossover protocol is preferred in order to minimize the effect of different volunteers on the final result, resulting in higher statistics reliability [19].

The placebo dentifrice (G1) showed the highest wear rate (1.76 ± 0.55μm), as reported by other authors [20, 21], not contributing for a increase in wear resistance when compared with other groups, probably as a result of the absence of a MMPs inhibitor.

Chlorhexidine is a gold-standard inhibitor of MMPs and widely used for in vitro and in situ studies [22, 23]. Chlorhexidine is by chelation of metallic ion blocking the linked of MMPs with these ions and avoid the degradation of collagen, but the CHX also inhibits cathepsins cysteine [22, 23]. In the present study, CHX was used at 0.012% in a dentifrice (G2), considering that CHX in lower concentration showed effective in inhibit MMPs and minimize the mineral loss in dentin [20].

Fluoride-containing dentifrices (G3) are used as remineralization agent and, although fluorides present an extremely low potential, they inhibit MMPs- 2 and 9 enzymes [24], avoiding the degradation of collagen fibrils and maintaining the DOM. Its action happens through competition with MMPs for Ca^{2+} and Zn^{2+} [22, 24]. In this study represented the second positive control.

Based on the present study's results, both groups G2 and G3 were able to reduce the dentin wear, respectively 1.19 ± 0.42μm and 1.29 ± 0.34μm, when compared with the placebo dentifrice (1.76 ± 0.55μm). Similar results were reported by another in vitro study [20].

It is noteworthy that dentifrices containing chlorhexidine can cause side effects, such as teeth staining and/or loss of taste sensitivity [25].

Proanthocyanidin (PA) consists in a natural, renewable, extract [15, 26], which presents little toxicity [22] and is capable of readily inhibit MMPs and cathepsins cysteine [27], as well as prevent mineral loss through cross-linking action which biomodifies the collagen fibrils of the dentin organic matrix, improving its mechanical proprieties [13, 16, 28, 29].

Based on the present study results, PA containing dentifrices showed the lowest dentin wear when associated (G5) or not (G4) with fluorides, respectively 0.82 ± 0.34μm and 0.93 ± 0.38μm. The association with fluorides might be beneficial because of the increased MMPs inhibition and above-mentioned remineralization capabilities [7, 20, 30, 31]. This said, the null hypothesis was rejected.

The protocol chosen was use of devices during the day, due to difficulty collaboration of volunteers for use during the night [19], as also due to the limited remineralization activity during the nighttime [32], and the remineralization intervals consisted of a minimum of 2 hours until the following planned erosion and/or abrasion cycle. The intervals to remineralization adopting during the use were of minimum 2 hours for next erosion and abrasion cycle.

Coca Cola was adopted as the erosive challenge due to its lower pH, potential wear, and wide consumption by the society [33]. Bovine teeth were selected as it is similar to human dentin when considering evaluation of MMPs activity [34].

In the present study, dentifrice was chosen as a treatment delivery system since it is easy to implement and widely used by the population. In summary, with this study Proanthocyanidin's dentifrices and its combination with fluoride dentifrices showed reduce the mineral loss of dentin tissue after erosion and dental abrasion. Based on that, these news formulations of dentifrice can be an interesting choice for patients who present clinical dentin lesions resulting from erosion and abrasion.

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3 Discussion

3 DISCUSSION

Dental erosion has been extensively studied due to its increased prevalence in the population and associated clinical complications (i.e. dentin hypersensitivity). However, clinical studies present limitations related to quantification of the mineral loss (WEST *et al.*, 2011). Therefore, *in vitro* and *in situ* studies have been performed to overcome those limitations in a intraoral-like environment (WEST *et al.*, 2011).

The in vitro portion had as advantages to be cheaper, quicker, and more controlled than other studies designs, serving as a pilot study previous *in situ* study. It also served as reference for sample size calculation and diagnosis of possible outcomes (WEST *et al.*, 2011; WEST *et al.*, 2017). The *in situ* portion was designed to prove the results obtained during the in vitro portion, but using an intraoral environment after the erosive and/or abrasive challenges (KLONT; TEN CATE, 1991; WEST *et al.*, 2011). The adopted protocol consisting of not using the appliances overnight was selected in order to improve results' reliability and volunteers' comfort (WEST *et al.*, 2011; ALENCAR *et al.*, 2016; MENDONCA *et al.*, 2017). Both analysis (*in vitro* and *in situ*) showed that PA-based dentifrices, associated or not with fluorides reduced dentin wear. The null hypothesis was rejected using both study's designs.

Dentifrices containing inhibitory agents could consist in an easily adopted, widespread, and cost-effective treatment modality to increase the resistance to erosive and abrasive challenges (GANSS *et al.*, 2014; MAGALHAES *et al.*, 2014).

Different acids and protocols are used to promote dental wear and simulate erosive and abrasive challenges (LUSSI *et al.*, 1995; REDDY *et al.*, 2016). The Cola Cola soft drink presents a lower pH of 2.5 and is widely consumed acid drink (RIOS *et al.*, 2006), and was used in both studies. Between erosive and abrasive challenges, the samples were stored in artificial saliva (*in vitro*), and in natural saliva (*in situ*), during a minimum of 2h before the following erosive and/or abrasive cycles. Although the results had been similar between both studies, the natural saliva can be influenced the small difference at values of wear as the salivary MMPs are active in lower pH and contribute with dentin MMPs for degradation of DOM (BUZALAF *et al.*, 2015). Abrasives cycles were performed twice during 30s per cycle. Although the in vitro study allowed standardization of load at 150g, the *in situ* study relied on the pressure applied by the volunteers.

Root bovine dentin were used in both studies as it is considered similar to human dentin when considering MMPs-2 and 9 activity, as well as due for being easily available (KATO *et al.*, 2011).

The mainly response variable was profilometry, which allows graphic representation of the sample surface (GANSS *et al.*, 2007), however, the measure in dentin must be under wet conditions for maintenance the dentin collagen of matrix (ATTIN *et al.*, 2009).

The G1/placebo dentifrice showed the highest dentin wear for both studies confirming that the absence of active principles capable of inhibit the MMPs in fact does not prevent wear (HANNAS *et al.*, 2016). In contrast, CHX is considered gold standard for dental erosion in dentine inhibiting the degradation of collagen by MMPs (TJADERHANE *et al.*, 2015). CHX chelates with calcium with metal ions, competing with the MMPs (GENDRON *et al.*, 1999; BRESCHI *et al.*, 2018), and avoiding the degradation of fibrillar collagen by MMPs enzymes and cysteine cathepsins (SCAFFA *et al.*, 2012).

Sodium fluoride present in dentifrices is well known for prevention of dental caries, but it also presents action to prevent dental erosion through remineralization and inhibition of MMPs (especially MMPs- 2 and 9) through competition with Ca2+ ion (KATO *et al.*, 2014; BUZALAF *et al.*, 2014; MAGALHAES *et al.*, 2014; BRESCHI *et al.*, 2018). Although the higher the concentration of NaF, the lower the degradation of the demineralized dentin (BRACKETT *et al.*, 2015), the resent study used 1100ppm as it is commonly commercialized. For both studies, CHX and Fluoride-containing dentifrices reduced dentin wear when compared with the control group.

Nevertheless, PA-containing dentifrices showed the lowest dentin wear among all groups for both studies, probably due to its capabilities of stabilizing the DOM and avoid the collagen degradation (BOTEON *et al.*, 2017) through crosslinking and biomodifications of dentin structure (BEDRAN-RUSSO *et al.*, 2011; CASTELLAN *et al.*, 2011; AYDIN *et al.*, 2019). Such biomodifications improve the mechanical proprieties, promotes dentin mineralization, and stimulate collagen synthesis (CASTELLAN *et al.*, 2010; BEDRAN-RUSSO *et al.*, 2014; BEDRAN-RUSSO *et al.*, 2014; EPASINGHE *et al.*, 2017; BALALAIE *et al.*, 2018; AYDIN *et al.*, 2019). In addition, it can inactivate cathepsins cysteines and approximately 89% of the MMPs (EPASINGHE *et al.*, 2013; SCHEFFEL *et al.*, 2014).

PA can be extracted from several natural sources and, for the present study, it was derived from grape seeds as proposed previously (BEDRAN-RUSSO et al., 2011). The association of MMP inhibitors with fluoridated dentifrice as an attempt to reduce dental erosion (BUZALAF et al., 2012) has been confirmed with these studies. The PA dentifrice was effective for reduction of dentin wear being an excellent solution for patients suffering erosion and/or abrasion used daily.

4 Conclusion

4 CONCLUSION

It can be concluded that PA dentifrices, associated or not with fluorides, promoted the reduction of dentin mineral loss after erosion and abrasion challenges both in situ and in vitro.

Incorporation of PA in dentifrices may be interesting as a widespread, cost-effective preventive treatment against dental wear.

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Appendixes

APPENDIX A- DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS-PAPER-1

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article EFFECT OF PROANTHOCYANIDIN-BASED DENTIFRICES ON DENTIN-WEAR – IN VITRO STUDY will be included in Thesis of the student Tamires de Luccas Bueno was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 04 de junho de 2019.

Tamires de Luccas Bueno
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APPENDIX B- DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS-PAPER-2

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article EVALUATION OF PROANTHOCYANIDIN-BASED DENTIFRICES ON DENTIN-WEAR AFTER EROSION AND DENTAL ABRASION - IN SITU STUDY will be included in Thesis of the student Tamires de Luccas Bueno was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 04 de junho de 2019.

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Daniela Rios
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Author

APPENDIX C- TERM OF CONSENTING

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Nós Prof. Dr Heitor Marques Honório e Tamires de Luccas Bueno, responsáveis pela pesquisa intitulada **“Efeito in vitro e in situ de dentifrícios na matriz orgânica desmineralizada submetida a erosão e abrasão dentária”,** convidamos você aluno de Pós- Graduação (mestrado e doutorado) na faixa etária de 21- 35 anos, a participar desse nosso estudo.

Esta pesquisa tem como objetivo desenvolver uma “pasta de dente” a base de compostos naturais para diminuir desgaste da estrutura do dente para pacientes que fazem uso de alimentos ácidos, que tenham problemas gástricos ou que são expostos a produtos químicos em seu ambiente de trabalho.

A pesquisa terá 5 (cinco) etapas de 5 (cinco) dias cada, tendo um intervalo de descanso de 7 (sete) dias entre elas.

Aceitando participar dessa pesquisa você será moldado para a realização de uma placa que irá no “céu da boca”. Esta plaquinha terá 4 (quatro) pedaços de dente de boi, devidamente esterilizados e que você fará durante cinco dias sem interrupção. O aparelho será instalado um dia antes do início da pesquisa, à noite, após a última higiene, para permitir formação de película adquirida. Você terá que usá-lo durante o dia e noite, no entanto, você poderá tirá-lo por meia hora no período da manhã, uma hora no almoço, meia hora no meio da tarde e uma hora no período da janta para se alimentar.

Durante o período experimental você deverá colocar o aparelho por inteiro, quatro vezes ao dia, durante os períodos disponíveis para alimentação, por 5 minutos, em um copo contendo 200ml da bebida do estudo (Coca-Cola). Sendo o líquido trocado a cada imersão e mantido em temperatura ambiente.

Você deverá fazer escovação dos blocos com uma escova elétrica por 30 segundo em cada bloco, após a imersão no líquido, realizando nos períodos destinados ao almoço e a janta, com a pasta testada que estará presente em seu Kit.

Terminando cada etapa os aparelhos serão devolvidos ao responsável da pesquisa, e você ficará sete dias sem o uso do aparelho para que a próxima etapa seja iniciada, sendo os mesmos procedimentos realizados em cada etapa, conforme descrito à cima, no entanto somente a pasta será diferente.

Para realizar as etapas você receberá um kit contendo: uma escova, pasta de dente e fio dental para higiene bucal quando estiver com o aparelho fora da boca (que serão de seu domínio ao final da pesquisa). Para escovação dos blocos presentes na placa, você receberá uma escova elétrica à bateria, pasta de dente a ser testada (que serão devolvidos no final de cada etapa). O kit contará com um copo para imersão em solução erosiva (Coca-cola), gaze para envolver o aparelho, que será umedecida com água de abastecimento (Bauru- 0.7 ppm F) e um estojo para armazenar o aparelho, quando este estiver fora da boca.

Você utilizará o aparelho durante 25 dias no total, não havendo nenhum risco de dano aos seus dentes, já que o desafio erosivo será realizado com o dispositivo fora da sua boca. Durante o período de uso do aparelho, você não deverá consumir bebidas ou alimentos ácidos (exceto água). O uso do mesmo não causará dor à você, mas poderá ocorrer algum desconforto ou dificuldade na pronúncia de algumas palavras nas primeiras horas, que se restabelecerá normalmente após a adaptação à condição de existência de aparelhos dentro da boca. A utilização do aparelho não afetará sua qualidade de vida ou rotina, pois será semelhante ao uso de aparelhos que consertam dentes tortos.

Você **não** terá benefício direto, já que todo o tratamento será realizado na placa utilizada por você. Porém, **tendo resultado satisfatório** com as pastas testadas, e em caso de necessidade de uso, este **será disponibilizado e prescrito** a você. Os resultados obtidos com essa pesquisa poderão colaborar com desenvolvimentos de protocolos que ajudarão em futuras pesquisas sobre dentes com desgaste (erosão). Embora **não seja previsto nenhum risco**, incômodos no momento da moldagem e do uso do dispositivo podem ocorrer, no entanto, em caso de eventuais danos decorrentes da pesquisa, **os pesquisadores garantem indenização aos convidados**. Verificando-se a necessidade de algum tratamento não relacionado com a pesquisa, você receberá tratamento nas dependências dessa instituição.

O uso do dispositivo é de suma importância, pois permite realizar o estudo imitando os eventos que ocorrem na boca de forma fiel, sem trazer nenhum efeito colateral aos dentes dos convidados por que os aparelhos são utilizados.

Rubrica do Pesquisador Responsável:

Rubrica do Participante da Pesquisa :

A sua **participação será voluntária**, isto é, a qualquer momento você poderá recusar –se a participar da pesquisa interrompendo o uso do aparelho e retirar seu consentimento. Sua recusa **não** terá nenhum prejuízo em sua relação com o pesquisador, nem com a instituição. Asseguro que **sua participação nessa pesquisa será sigilosa durante toda a pesquisa**, assim como todos os seus dados pessoais não serão divulgados em eventos, congressos ou em revista. Você **não terá nenhum custo** por participar dessa pesquisa, pois todo o material será devidamente fornecido por mim, e esta será realizada enquanto o convidado estiver presente na Faculdade, em horários que não interfiram em suas atividades.

Estando de acordo em participar da pesquisa o aceite será formalizado através da assinatura do presente “TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO”, em dua vias, sendo que uma delas será entregue a você, convidado da pesquisa e outra com o pesquisador responsável. Qualquer dúvida sobre os procedimentos e sua participação nessa pesquisa poderá entrar em contato com os pesquisadores por meio dos telefones e endereços eletrônicos, respectivamente: Tamires de Luccas Bueno (14) 99677-2440 e tamireslbueno@gmail.com ; Heitor Marques Honório (14) 98112- 7777 e heitorhonorio@usp.br e, para denúncias e/ou reclamações poderá entrar em contato com Comitê de Ética em Pesquisa-FOB/USP, à Alameda Dr. Octávio Pinheiro Brisolla, 9-75, Vila Universitária, ou pelo telefone (14)3235-8356, e-mail: cep@fob.usp.br, e a forma de contato com CONEP (endereço, telefone, e-mail), quando pertinente.

Pelo presente instrumento que atende às exigências legais, o Sr. (a) ____, portador da cédula de identidade ______________________, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3, item IV.5.a e item IV.6b na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o participante da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Rubrica do Pesquisador Responsável:

Rubrica do Participante da Pesquisa :

Bauru, SP, ___ de ___________de 2017.

______________________________ Assinatura do Participante da Pesquisa

______________________________ Tamires de Luccas Bueno
Assinatura do Pesquisadora Responsável

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução CNS nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.

Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

Horário e local de funcionamento:
Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **14hs às 17 horas**, em dias úteis.
Alameda Dr. Octávio Pinheiro Brisolla, 9-75
Vila Universitária – Bauru – SP – CEP 17012-901
Telefone/FAX(14)3235-8356
e-mail: cep@fob.usp.br

Rubrica do Pesquisador Responsável:

Rubrica do Participante da Pesquisa :

Annex

ANNEX A- GUIDELINES FOR AUTHORS USED IN PREPARATION OF PAPER 1 AND 2- DENTAL MATERIALS

Introduction

Authors are requested to submit their original manuscript and figures via the online submission and editorial system for *Dental Materials*. Using this online system, authors may submit manuscripts and track their progress through the system to publication. Reviewers can download manuscripts and submit their opinions to the editor. Editors can manage the whole submission/review/revise/publish process. Please register at: https://www.evise.com/profile/api/navigate/DEMA.
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