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The effect of TiF_4 varnish after pre-treatment with MMP inhibitors on dentin erosion with or without demineralized organic matrix

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FOLHA DE APROVAÇÃO

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"Mãe é quem fica. Depois que todos vão. Depois que a luz apaga.

Mãe fica. Espremida no canto da cama durante a noite. Fica com o resto da comida do filho, fica preocupada se ainda não foi dormir.

Quando a gente fica que nasce a mãe. Na presença inteira. No olhar atento. Nos braços que embalam. No colo que acolhe.

Mãe é quem fica quando o chão some sob os pés. Quando todo mundo vai embora. Quando as certezas se desfazem. Mãe fica.

Mãe é a teimosia do amor, que insiste em permanecer e ocupar todos os cantos. É caminho de cura. E no filho que vai, sempre fica um pouco da mãe.

No coração do filho, mãe fica."

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ABSTRACT

ABSTRACT

The effect of TiF_4 varnish after pre-treatment with MMP inhibitors on dentin erosion with or without demineralized organic matrix

This study tested the protective effect of TiF_4 varnish, after pre-treatment with proanthocyanidin (PA) on the progression of erosive dentin wear, under the presence or absence of the demineralized organic matrix (DOM). For that, 360 bovine root dentin samples were polished and subjected to the analysis of the baseline profile. The samples were then eroded for 30 minutes (0.1% citric acid, pH 2.5) and the profile was measured again (erosion profile). Half of the samples were subjected to the DOM removal using collagenase for 4 days, while the other half remained immersed in water (with DOM). The removal of DOM was checked by profilometry (DOM profile). Samples were randomly divided into 24 groups (n= 15) according to the factors: 1 – With or without DOM; 2 – Pre-treatment with 0.012% chlorhexidine gel, 10% proanthocyanidin gel or untreated (control) for 1min; 3 – Final Treatment with TiF_4 varnish, NaF varnish, placebo varnish or untreated (control) for 6h. The samples were submitted to a pH cycling for 5 days: 0.1% citric acid (4x90s/day) and immersion in artificial saliva between the challenges. The final profile was obtained for the calculation of dentin wear (µm, three-way ANOVA/Tukey test). When DOM was preserved, the erosive wear was lower (7.08µm) compared to the condition without DOM (9.80µm), regardless of the treatments (p=0.00). The pre-treatment had no influence on the amount of dentin wear (p=0.63), while the final treatment was effective in reducing the progression of dentin wear (about 18% compared to control, p=0.00). TiF_4 was the only agent capable of reducing erosive dentin wear progression.

Key words: Dentin erosion. Fluoride varnish. Proanthocyanidin.

RESUMO

RESUMO

Efeito do verniz de TiF_4 após o tratamento inicial com inibidores de MMP na erosão da dentina com e sem matriz orgânica desmineralizada

Este estudo teve por objetivo testar o efeito protetor do verniz de TiF_4, após pré-tratamento com proantocianidina (PA) na progressão do desgaste erosivo da dentina, sob a presença ou ausência da matriz orgânica desmineralizada (DOM). Para isso, 360 amostras de dentina radicular bovina foram polidas e submetidas à análise do perfil baseline. As amostras foram então erodidas durante 30 minutos (0,1% de ácido cítrico, pH 2,5) e o perfil foi novamente medido (perfil pós erosão). Metade das amostras foram submetidas à remoção do DOM usando colagenase por 4 dias, enquanto a outra metade permaneceu imersa em água (com DOM). A remoção do DOM foi verificada por perfilometria (perfil DOM). As amostras foram divididas aleatoriamente em 24 grupos (n=15) de acordo com os fatores: 1 - Com ou sem DOM; 2 - Pré-tratamento com gel de clorexidina 0,012%, gel de proantocianidina a 10% ou não tratado (controle) por 1min; 3 - Tratamento final com verniz TiF_4, verniz NaF, verniz placebo ou não tratado (controle) por 6h. As amostras foram submetidas a ciclagem de pH por 5 dias: 0,1% de ácido cítrico (4x90s/dia) e imersão em saliva artificial entre os desafios. O perfil final foi obtido para o cálculo do desgaste da dentina (µm, teste ANOVA de três fatores / Tukey). Quando a DOM foi preservada, o desgaste erosivo foi menor (7,08µm) em comparação com a condição sem DOM (9,80µm), independentemente dos tratamentos (p=0,00). O pré-tratamento não teve influência na perda de estrutura da dentina (p=0,63), enquanto o tratamento final foi eficaz na redução da progressão do desgaste da dentina (cerca de 18% comparado ao controle, p=0,00). Porém, o TiF_4 foi o único agente capaz de reduzir a progressão do desgaste erosivo da dentina.

Palavras-chaves: Erosão dentinária. Verniz Fluoretado. Proantocianidina

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1 INTRODUCTION

1 INTRODUCTION

Dentin has a high organic contente, consisting mainly of collagen fibrils (BUTLER, MUNKSGAARD, RICHARDSON, 1979; PASHLEY, 1991; HOULLÉ et al., 1997). Dental erosion is defined as the loss of tooth structure due to chemical process that does not involve bactéria (LUSSI, 2006; LUSSI, CARVALHO, 2014), which can affect the dentin when the enamel is lost or when there is radicular exposure. Studies indicate a high prevalence of tooth erosion in its early stages among adolescents (AGUIAR et al., 2014), with an estimated prevalence of 30.4% (SALAS et al., 2015a).

The decrease of pH in the presence of extrinsic and/or intrinsic acids induce to demineralization with organic matrix exposure as well as the activation of matrix metalloproteinases (MMPs), enzymes responsible for the degradation of extracellular matrix components during remodeling and degradation processes, which are present in the dentin itself and saliva. Subsequent neutralization by salivary buffers appears to increase the degradative activity of MMPs (TJÄDERHANE et al., 1998; MAZZONI et al., 2007; SULKALA et al., 2007; MAZZONI et al., 2011; KATO et al., 2011; SALAS et al. 2015b; FEMIANO et al., 2016).

The demineralized organic matrix (DOM) present at the lesion surface interferes with the diffusion of ions outside and inside the demineralized area, reducing the progression of erosion during subsequent acid attacks (GANSS, KLIMEK, STARCK, 2004). Therefore, collagen fibrils degradation by MMPs might speed up the progression of tooth erosive loss. It is expected that, clinically, erosive lesions have active degradation of the DOM by the action of proteases (SCHLUETER et al., 2012). The loss of the DOM might explain why tooth erosion seems to be harder clinically than that produced in the laboratory (GANSS, SCHULZE, SCHLUETER, 2013).

To minimize the erosive tooth loss, fluoride has been employed (WIEGAND, ATTIN, 2003; GANSS et al., 2010; MAGALHÃES et al., 2011; LUSSI, CARVALHO, 2015). The NaF mechanism of action is based on CaF_2 precipitation on the tooth surface that would act as a mechanical barrier against acid and as a fluoride reservoir reducing demineralization and accelerating remineralization (GANSS et al., 2004; GANSS, SCHLUETER, KLIMEK, 2007; MAGALHÃES et al., 2011; FLURY et al., 2013; LUSSI, CARVALHO, 2015). However,

besides the low acid resistance of the CaF_2 globules, another limitation is the lack of protective effect on dentin when the DOM is enzymatically removed (GANSS et al., 2004; SCHLUETER et al., 2007).

On the other hand, fluoride containing polyvalent metals, such as TiF_4, have shown a good efficacy against dental erosion (HOVE et al. 2006; MAGALHÃES et al. 2008; HOVE et al., 2008; MAGALHÃES et al., 2010; MAGALHÃES et al., 2011; LEVY et al., 2012; SOUZA et al., 2014; COMAR et al., 2015; CASTILHO et al., 2015; MAGALHÃES et al., 2016; MARTINES DE SOUZA et al., 2017). The better efficacy of TiF_4, comparede to other fluoride salts, is due to the additional effect of titanium, which can minimize demineralization by interacting with the phosphate group to form a "*glaze*" layer over the surface (TEZEL, ERGUCU, ONAL, 2002; RIBEIRO, GIBSON, BARBOSA, 2006), rich in titanium phosphate dihydrate, titanium oxide and calcium fluoride (MAGALHÃES et al., 2013). In addition, it is suggest that TiF_4 varnish interacts with the dental surface, due to the low pH of the agent, leading to an increase fluoride incorporation (GU, LI, SÖREMARK, 1996) as seen in the literature in one work involving enamel (COMAR et al., 2013).

MARTINES DE SOUZA et al. (2017) evaluated the effect of TiF_4 varnish when applied in the presence or absence of DOM and show that this varnish obtained better results when compared to NaF varnish, especially when DOM was preserved, being superior to placebo and control varnishes (which did not differ statistically among themselves) (MARTINES DE SOUZA et al., 2017).

However, in order to preserve the DOM, several other studies have investigated the effect of enzyme inhibitors against tooth erosion (GENDRON et al., 1999; KATO et al., 2009; MAGALHÃES et al., 2009; KATO et al., 2010a; KATO et al., 2010b; KIM et al., 2011; KATO et al., 2012; KATO et al., 2014; BRACKETT et al., 2015). Inhibitors such as chlorhexidine (GENDRON et al., 1999; MAGALHÃES et al., 2009; KATO et al., 2010b; KIM et al., 2011) have shown excellent results in this process, but the search for natural protease inhibitors has attracted increasing attention (BEDRAN-RUSSO et al., 2008; BEDRAN-RUSSO et al., 2011; CASTELLAN et al., 2011; BEDRAN-RUSSO et al., 2014; SESEOGULLARI-DIRIHAN, 2015; BOTEON, 2017a; EPASINGHE, BURROW, YIU, 2017; NANDAKUMAR, NASIM, 2018). The main reasons for this are the lower toxicity of natural agents, minimal side effects, their characteristic of renewable/sustainable resource (BEDRAN-RUSSO et al., 2014) and less interaction with other products, such as MDP present in some adhesive systems (LIU et al., 2013).

Among these natural agents, stands out the proanthocyanidin (PA), which is richly found in natural products such as cocoa, grapes, cranberry and peanuts (BEDRAN-RUSSO et al., 2014). Many studies have shown the inhibitory effect of MMPs on proanthocyanidin-rich agents, confirmed their dose-response effect and also that the PA provided less wear of the dentin submitted to erosive challenge (HAM et al., 2003; BEDRAN-RUSSO et al., 2008; RUEL et al., 2009; NAGPAL, MANUJA, PANDIT, 2013; BEDRAN-RUSSO et al., 2014; KHADDAM et al., 2014; LIU et al., 2014; BOTEON et al., 2017a; BOTEON et al., 2017b; SESEOGULLARI-DIRIHAN, 2015; EPASINGHE, BURROW, YIU, 2017; NANDAKUMAR, NASIM, 2018). Proanthocyanidin is considered a cross-linking agent, its effect has been attributed to its interaction with proline-rich proteins such as collagen (HIRAISHI et al., 2013) and has the ability to improve the mechanical properties of the dentin matrix by enhancing the bonds of the collagen fibrils and the bonds between them by inactivating the bonds with the MMPs (but although several cross-linking agents are available, their specific anti-MMPs are still unclear) (HIRAISHI et al., 2013; SESEOGULLARI-DIRIHAN et al., 2016).

Therefore, the aim of this study was to compare the effect of TiF_4 and NaF, with equivalente fluoride concentrations, applied on pre-eroded dentin samples on reducing the progression of dentin erosive loss caused by an acid frequently found in the most known erosive beverages, when applied proanthocyanidin and chlorhexidine as inhibitors of MMPs, in order to preserv the demineralized organic matrix and to maximize the effect of the fluoride varnishes. Considering that clinically the DOM may be lost by enzymatic action, the results of the present study can guide clinical studies on preventive strategies to control dentin erosion.

This work tested the following null hypotheses: (1) there is no difference between the fluoride varnish on the progression of dentin erosion, regardless of the condition of the treatment and the presence or absence of the DOM; (2) there is no difference in the progression of dentin erosion to the different types of MMP inhibitors used, regardless of dentin condition (with or without DOM) and regardless of the type of fluoride used; (3) there is no difference in the progression of erosion between dentin, with or without MOD, regardless of the type of fluoride tested and treatment with MMP inhibitors.

2 ARTICLE

2 ARTICLE

The article presented in this Dissertation was written according to Journal of Dentistry instructions and guidelines for article submission.

The effect of TiF_4 varnish after pre-treatment with MMP inhibitors on dentin erosion with or without demineralized organic matrix

INTRODUCTION

Dentin is a dental hard tissue rich in organic content, consisting mainly of collagen fibrils [1-3], which may be exposed to oral cavity, when enamel is lost or in case of gingival recession. Once exposed, dentin becomes susceptible to chemical and mechanical challenges that may result in wear. Studies have shown that the prevalence of tooth erosive wear affecting dentin is relevant in children (about 50%) and adolescents (about 30%) [4,5], which negative impact on the quality of life when associated with hypersensitivity [6,7].

When dentin is subjected to erosive challenges and some minerals are lost, the organic content is exposed and proteolytic enzymes, such as matrix metalloproteinases (MMPs), are activate and may contribute fasten the progression of wear by degrading the organic content [8]. Accordingly, some MMPs inhibitors have been applied, such as chlorhexidine-CHX and natural agents, to reduce the effect of organic degradation on the progression of erosive wear [8,9]. Some studies have shown a great effect of CHX mouthrinse applied daily or CHX gel at high concentration applied at once on the protection against dentin erosion [8,10]. Other agents claiming to be natural have been tested in replacement of CHX, such as green tea (active principle: EGCG) due to their lower cytotoxic potential and side effects [10-12].

Among the natural agents, this work has studied proanthocyanidin (PA), which is richly found in natural products such as cocoa, grapes, cranberry and peanuts [13,14]. Many studies have shown the MMPs inhibitory effect of PA-rich agents under erosive challenges [14-25]. PA is considered a cross-linking agent, acting on proline-rich proteins such as collagen [26]. It has shown ability to improve the mechanical properties of the dentin matrix by enhancing the bonds between collagen fibrils due to the inactivation of bonds of collagen fibrils with the MMPs [26,27].

Despite the high interest in MMPs inhibitors, the most applied agent to protect the tooth against erosive wear is still fluoride [28-31]. NaF mechanism of action is based on CaF_2 precipitation on the tooth surface creating a mechanical barrier against acids [30-34]. However, CaF_2 globules present low acid resistance and fail in protecting against dentine

erosive wear, when the demineralized organic matrix (DOM) is removed by enzymes [32,35].

On the other hand, fluoride containing polyvalent metals, such as TiF_4, have shown better protective effect against tooth erosion compared to NaF [30,37-45]. Its mechanism of action is based on the formation of "*glaze*" like-layer over the surface [46,47], rich in titanium phosphate dihydrate, titanium oxide and calcium fluoride [48].

Recently, Martines de Souza et al. (2017) [45] evaluated the effect of TiF_4 varnish when applied in the presence or absence of DOM (removed enzymatically) with respect to the progression of erosive wear. TiF_4 varnish was able to significantly reduce the progression of dentin wear compared to NaF varnish, regardless of the presence or not of DOM [45].

Considering the possibility of DOM degradation under *in vivo* conditions and also the protective effect of MMP inhibitors and fluoride agents, it would be very interesting to combine both in order to improve the protection against dentin erosive wear. So the aim of this *in vitro* study was to compare the protective effect of fluoride agents, after pre-treatment with MMP inhibitors on the progression of erosive dentin wear, in the presence or absence of the DOM.

This work tested the following null hypotheses: (1) there is no significant difference in the progression of dentin erosive wear, with or without DOM, regardless of the type of treatment (MMP inhibitors and fluoride agents); (2) there is no significant difference in the progression of dentin erosive wear between the different types of MMP inhibitors, regardless of the dentin condition (with or without DOM) and the type of fluoride; (3) there is no significant difference between the fluoride varnishes on the progression of dentin erosive wear, regardless of the dentin condition (with or without DOM) and the type of MMP inhibitors.

MATERIALS AND METHODS

Sample preparation

Three hundred and sixty dentin samples were prepared from bovine incisors recently extracted, which had been previously stored in 0.1% thymol solution (pH 7.0). The root and crown were separated using a cutting machine (Maruto, Tokyo, Japan) and a diamond disk (Maruto, Tokyo, Japan). The roots were coupled to a prefabricated silicone mold (Biopdi, São Carlos, Brazil) and embedded in autopolymerizing acrylic resin, allowing the cervical buccal surface was exposed. The samples were polished using silicon carbide sandpapers (320, 600 and 1,200 grades of Al_2O_3 papers; Extec Corp., USA). Thereafter, the baseline profile was measured by using a non-contact laser profilometry (PROSCAN 2100 3D, Scantron, Taunton, UK), to evaluate the curvature of the samples and obtain a control surface of dentin, later two thirds of the samples' surfaces were protected with nail varnish (Risqué, São Paulo, Brazil), to obtain 2 control areas (non-eroded areas).

All dentin samples were eroded for 30 minutes, using a 0.1% citric acid solution (pH 2.5) at room temperature and under stirring (60 rpm). The acid solution was replaced every 6 minutes (30 mL/sample). Dentin erosive wear (erosion profile) was measured at the same area as the baseline profile was done.

Thereafter, half of the samples (n= 180) were submitted to the removal of the DOM by exposing them to a solution containing collagenase. The enzyme collagenase from *Clostridium histolyticum* type VII (product No. Co773, Sigma-Aldrich, St. Louis, MO, USA with collagen digestion activity of 1.98 U/µg solid at 25°C, pH 7.5, in the presence of calcium ions) was dissolved in a mineral solution containing 4.08 mM H_3PO_4, 20.10 mM KCl, 11.90 mM Na_2CO_3, and 1.98 mM $CaCl_2$ (all chemicals from Merck, Darmstadt, Germany) with a pH of 6.7 [49], at 37ºC for 4 days. Matrix remnants were removed under a microscope view using a cotton pellet soaked with NaOCl (Carl Roth GmbH & Co.). For the samples without DOM, the profile was again measured (DOM profile). On the other hand, the other half of the samples (n= 180) were exposed to deionized water to preserve the DOM during this period and they were not subjected to the analysis of DOM profile.

Experimental Treatment Groups

Eroded dentin samples, with and without DOM, were randomly divided into 24 groups (n=15 per group), which were evaluated by 3 study factors:

• DOM (Demineralized Organic Matrix), on 2 levels:

- With DOM
- Without DOM (collagenase treatment)

• Pre-treatment, on 3 levels:

- No treatment (negative control)
- 0.012% chlorhexidine gel (positive control)
- 10% proanthocyanidin gel (test group)

Both gels presented the same basic composition, with the exception of the active principle: natrosol gel 2%, CMC 0.5%, phenoxyethanol 1% and water (formulated in a manipulation pharmacy) [15].

• Final treatment, on 4 levels:

- TiF_4 varnish (2.45% F, pH 1) (test group)
- NaF varnish (2.45% F, pH 5) (positive control)
- Placebo varnish (without fluoride) (pH 5) (negative control 1)
- No treatment (negative control 2).

All experimental varnishes had the same basic composition (except the type of fluoride): colophonium, synthetic resin, thickening polymer, essence, artificial sweetener and ethanol (FGM-Denstcare, Joinvile, Brazil) [45].

Treatment and pH Cycling

The pre-treatment using PA or CHX gel was done on the center of dentin surface (1/3 surface) by using microbrush for 1 minute [50]. The samples from control group were not treated and remained moistened in ultrapure water. After gel application, the gel was removed by using cotton swab and all the sample were immersed in artificial saliva for 24 hours. Thereafter, the varnishes (TiF_4, NaF, placebo) were applied on the same dentin surface as it was done for pre-

treatment by using microbrush, and the samples were immediately exposed to artificial saliva for 6 hours [37]. After 6 hours, the varnishes were carefully removed using a surgical blade and a swab soaked with acetone solution (acetone:water 1:1).

The samples were then submitted to a pH cycling during 5 days [37]. The pH cycling was performed 4 times a day, as following: (1) demineralization by using 0.1% citric acid solution (pH 2.5) simulating extrinsic erosion, for 90 seconds (30 mL/sample) at 25°C; (2) washing in deionized water for 5 seconds; (3) remineralization by immersion in an artificial saliva (pH 6.8) for 2 hours [51] (30 mL/sample) at 25°C; (4) washing in deionized water again for 5 seconds. The saliva was composed of the following reagents: 0.2 mM glucose, 9.9 mM NaCl, 1.5 mM $CaCl_2 \cdot 2H_2O$, 3 mM NH_4Cl, 17 mM KCl, 2 mM NaSCN, 2.4 mM K_2HPO_4, 3.3 mM urea, 2.4 mM NaH_2PO_4 and traces of ascorbic acid (pH 6.8) [51]. The samples were kept in artificial saliva overnight completing 24 hours of cycling. After 5 days, the dentin erosive wear (final profile) was measured as described below. Figure 1 shows a flowchart of the study.

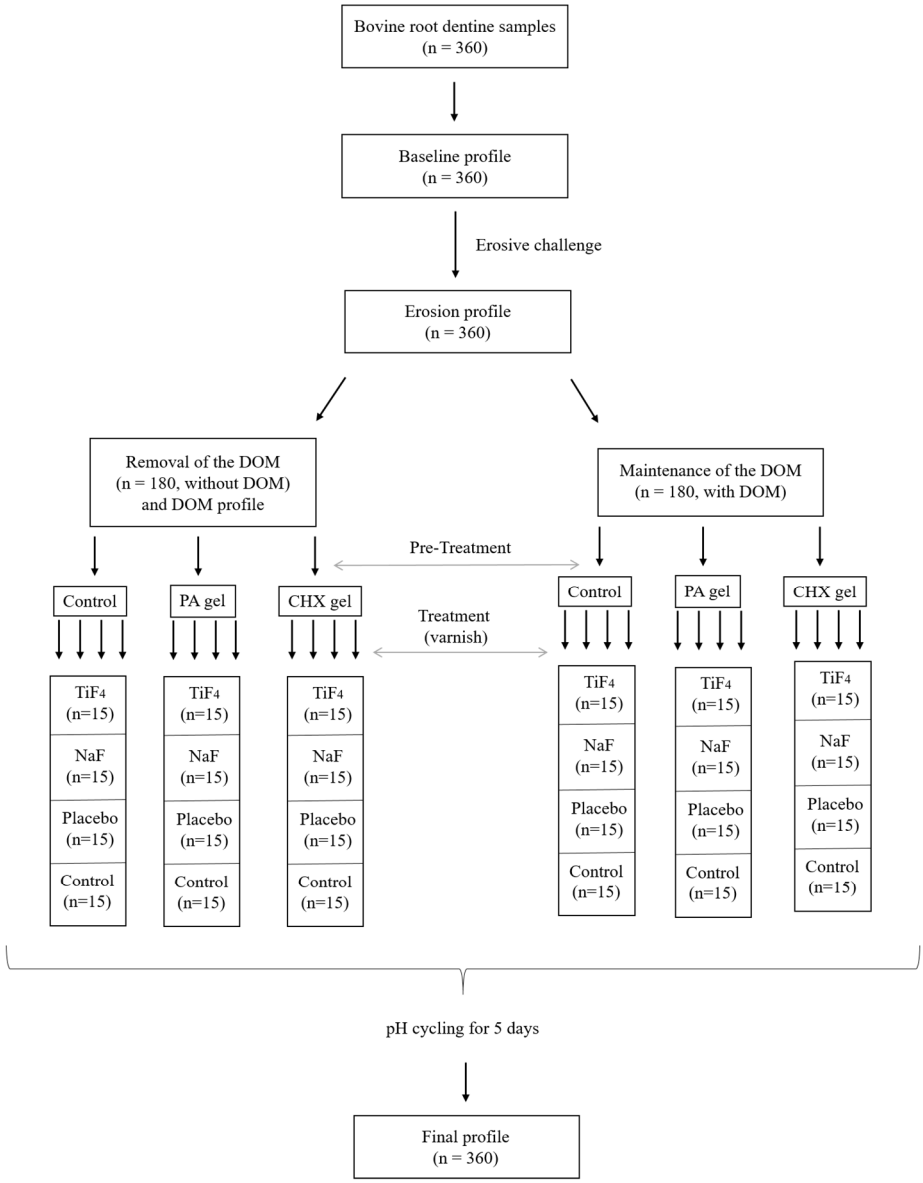

Figure 1. Flowchart of the study

Non-Contact Laser Profile

Dentin erosive wear was determined using a non-contact laser profilometer (PROSCAN 2100 3D, Scantron, Taunton, Reino Unido) at the baseline, after erosion, after DOM removal and after treatments/pH cycling (final). At the beginning of the experimental phase (baseline), the samples were taken to the optical profilometer to determine the curvature of the surface, and those with curvature higher than 0.3 µm were excluded [52]. The sensor of the instrument was programmed to scan a central area of the specimen 4 mm long (on the X axis) by 2 mm wide (Y axis) in the center of the sample, constructing an image that included the exposed and the reference surfaces of both sides. The equipment was adjusted to cover 200 steps with a size

of 0.01 mm on the X axis and 10 steps of 0.1 mm on the Y axis. The depth of the lesion (3-pt step height) was calculated by using a specific software (ProscanApplication software version 2.0,17), based on the subtraction of the mean height of the exposed area, relative to the mean height of the reference surfaces. The samples were kept in relative humidity until the moment of the reading in order to avoid the dryness of the dentin. Immediately prior to analysis, the samples were dried carefully with an absorbent paper.

Statistical Analysis

The mean dentin erosive wear was tabulated in Excel datasheet. The data analysis showed homogeneity of the variances (Levene test) and normality (Shapiro-Wilks test), so that the data can be submitted to parametric statistical analysis. The data were compared using three-way ANOVA, considering the following factors: 1) dentin condition (DOM factor in 2 levels), pre-treatment with MMPs inhibitor (pre-treatment in 3 levels) and different types of fluoride varnishes (final treatment in 4 levels). A post hoc test (Tukey) was applied for multiple comparisons. The level of significance adopted was 5%. Statistica 11.0 (Stat Soft Inc.) was used.

RESULTS

Table 1. Mean values (±SD) of dentin erosive wear (µm) from samples submitted to the pre-treatments and final treatments in the presence or absence of DOM (non-contact laser profilometer)

Groups	Demineralized organic matrix (DOM)	Initial treatment	Final treatment	Mean values ± standard deviation
1	With DOM	No treatment	TiF_4	$6.2 \pm 4.4^{aA\#}$
2	With DOM	No treatment	NaF	$9.1 \pm 4.6^{aA\bullet}$
3	With DOM	No treatment	Placebo	$6.8 \pm 3.7^{aA\bullet}$
4	With DOM	No treatment	Control	$5.4 \pm 2.7^{aA\bullet}$
5	With DOM	Chlorhexidine	TiF_4	$6.0 \pm 3.7^{aA\#}$
6	With DOM	Chlorhexidine	NaF	$7.8 \pm 4.2^{aA\bullet}$
7	With DOM	Chlorhexidine	Placebo	$7.2 \pm 3.1^{aA\bullet}$
8	With DOM	Chlorhexidine	Control	$6.1 \pm 3.0^{aA\bullet}$
9	With DOM	Proanthocyanidin	TiF_4	$5.5 \pm 4.7^{aA\#}$
10	With DOM	Proanthocyanidin	NaF	$7.7 \pm 4.0^{aA\bullet}$
11	With DOM	Proanthocyanidin	Placebo	$7.3 \pm 4.4^{aA\bullet}$
12	With DOM	Proanthocyanidin	Control	$9.7 \pm 4.2^{aA\bullet}$
13	Without DOM	No treatment	TiF_4	$7.4 \pm 3.4^{bA\#}$
14	Without DOM	No treatment	NaF	$11.0 \pm 3.1^{bA\bullet}$
15	Without DOM	No treatment	Placebo	$10.9 \pm 3.4^{bA\bullet}$
16	Without DOM	No treatment	Control	$11.3 \pm 3.5^{bA\bullet}$
17	Without DOM	Chlorhexidine	TiF_4	$7.9 \pm 3.9^{bA\#}$
18	Without DOM	Chlorhexidine	NaF	$10.4 \pm 3.6^{bA\bullet}$

19	Without DOM	Chlorhexidine	Placebo	$10.3 \pm 4.2^{bA\bullet}$
20	Without DOM	Chlorhexidine	Control	$9.8 \pm 3.0^{bA\bullet}$
21	Without DOM	Proanthocyanidin	TiF_4	$7.7 \pm 4.4^{bA\#}$
22	Without DOM	Proanthocyanidin	NaF	$11.1 \pm 3.6^{bA\bullet}$
23	Without DOM	Proanthocyanidin	Placebo	$9.4 \pm 4.1^{bA\bullet}$
24	Without DOM	Proanthocyanidin	Control	$10.6 \pm 3.6^{bA\bullet}$

***Different superscript small letters show significant differences in the comparison between presence or absence of DOM, in the same initial and final treatment. Different superscript uppercase letters show significant differences in the initial treatment within the same condition (with or without DOM) and within the same final treatment. Different overwritten symbols show significant differences in the comparison between the final treatment within the same condition (with or without DOM) and the same initial treatment (three-way ANOVA and Tukey test, p<0,05).**

According to three-way ANOVA, the dentin condition (with or without DOM) and the final treatments (fluorides) had significant effect on the progression of dentin erosive wear ($p= 0.000$). On the other hand, the pre-treatments (CHX or PA) did not have any influence on the progression of dentin erosive wear ($p= 0.63$) as well as no interaction was found between the factors ($p> 0.05$).

Dentin with DOM presented a significant lower dentin wear compared to those without DOM, regardless of the treatments. This fact can not be considered a surprise, because it is something expected since when you remove DOM the tendency is for a greater structural loss of dentin and, therefore, greater wear.

The treatment with TiF_4 varnish showed significant protective effect against dentin erosive wear, differing from the other groups, which in turn did not differ from each other. This protective effect of TiF_4 was seen for all dentin conditions and pre-treatments.

DISCUSSION

The first hypothesis of this paper was rejected once dentin with demineralized organic layer (DOM) presented a significant lower dentin wear compared to those without DOM, regardless of the treatments. But DOM can be destroyed or damaged easily as already known [8,10,24,54], because acidic attacks provided by dental erosion results in pH drop and demineralization of dental hard tissue [8,55,56] with consequent exposure of the organic matrix and MMPs activation [56, 57]. So DOM may be degraded by the matrix metalloproteinases (MMPs), present in both saliva and dentin [15,16,50,58], which are activated in acid pH and degrade the exposed collagen when the pH is neutralized [10,15,16,59]. After an erosive challenge, the length of acidic pH is small and the time required for saliva neutralization of the tooth surface ranges from 2 to 7 minutes, with a individuals variations [15]; with neutral pH, the activated MMPs start to degrade collagen [10,15,16,59]. Thus, enzymatic removal of the organic matrix by MMPs increases the demineralization process. Ganss et al. (2004) [32] showed that after fluoridation with collagenase, a linear increase in mineral loss (73.3 ± 17.6 μm on day 5) was observed, which statistically exceeded the control group (45.9 ± 14.3 μm on day 5). Fluoridation, without collagenase, was able to significantly reduce mineral loss from the second day (12.2 ± 10.2 μm on day 5) compared to the other groups and the results indicated that the organic matrix is essential for fluoride efficacy in dental erosion [32]. Thinking about this, our research group hypothesizes that the maintenance of the DOM by using MMP inhibitors associated or not with fluoride agentes would be an effective way to reducing the wear progression.

The second hypothesis was accepted because the pre-treatments (chlorhexidine-CHX or proanthocyanidin-PA) did not have any influence on the progression of dentin erosive wear. Most of the studies in the literature show a good effect of chlorhexidine as inhibitors of MMPs, decreasing its activity [8,10,15,16,60]. However, the exact mechanism of MMP inhibition is not completely understood, but the most accepted theory is that CHX present their inhibitory effect through divalent chelation of metal ions, especially zinc and calcium [8,61-65]. In this way, MMP inhibitors will prevent MMPs from binding to collagen substrate and its further cleavage [65]. In relation to PA, the exact mechanism of cross-linking is not completely understood also, but four diffetent theories explain PA interactions with proteins which include covalente [13], hydrogen bonding [66], ionic [67] and hydrophobic [68] interactions [65]. Our research found differents results from those presented in the literature: it was not possible verify an effect of MMP inhibitors on the tooth structure wear inhibition. Perhaps, for the fact, this

study did not evaluate these agents alone, but in interaction with fluoridated agents. However, more accurate studies should be conducted to understand the interaction between CHX and PA with MMP inhibitors, until found results about their interactions and action mechanisms, or find optimal protocols and ideal concentrations so that they present a good action on the dentin structure.

Much of the differents results found in these work, for those present in the literature [8,10,15,16,60], could be due to the fact that in our study the analysis of structural loss was performed by non-contact profilometry, while in the other works was used contact profilometry. Contact profilometers use a diamond tip moved across the surface to record the surface profile. However, has the potential risk of affectingthe reading or even damaging the sample as a consequenceof the contact [69,70]. Non-contact profilometers generally use some type of laser to scan the surface to create the profile. Also, allows volumetric loss analysis because generate a surface plane rather than just simple line profiles [69,71]. Schlueter et al. (2016) [72] showed that for dentin samples it depends what conditions we would like to evaluate, but the method most indicated would be non-contact profilometry [72].

And the third hypothesis of this paper was rejected whereas the final treatments (fluorides) had significant effect on the progression of dentin erosive wear, being the best of them the treatment with TiF_4 varnish, which showed significant protective effect against dentin erosive wear due to the action of titanium (Ti) that reacts with the tooth forming a glaze-like layer more acid resistant [48]. To reach the same efficacy of TiF_4 varnish, frequent applications of NaF varnish might be required [45], which in turn might be unfeasible from the clinical point of view. Also varnish application allows a longer time of fluoride contact with dentin surface compared to the other fluoride sources [45]. Martines de Souza et al. (2017) [45] evidenced that despite the best efficacy of the TiF_4 varnish, it partially lost the protective effect when DOM was removal, which seems to have a higher influence on the reaction between Ti and dentin. Through EDX analysis a decrease of 22% in Ti content at dentin surface happened due to the absence of the DOM and, even more pronounced this effect occur ate the subsurface (5-10 μm depth) with a loss of 78.5% of Ti, highlighting the fact that the collagen fibrils might play an important role in the penetration of Ti and its protective effect [45]. SEM analyzes of the surface indicated a deposition of material on and inside the dentinal tubules in the DOM preserved and treated with TiF_4 and NaF varnish, on
the contrary, for the placebo varnish and the control group, the dentin samples presented wide, open and visible tubules [45]. Even if the results showed in this study did not reveal statistical

interaction between the presense or absence of DOM and final treatment with TiF_4 varnish, we believe that Ti need to penetrate into the DOM to achieve its best performance in reducing dentin erosion progression.

Extrapolating the methodology to clinical practice, it would be interesting if there was a way to unite, in a single step, the final and initial treatment (MMPs gels inhibitor plus fluoride varnishes), decreasing the clinical time of the dental surgeon and decreasing patient time in the dental office. More studies should be done to understand better the interactions and mechanisms between MMP inhibitors and fluorided agents on the dentin structure wear.

CONCLUSIONS

Based on the results, it can be concluded that the presence of DOM is an extremely importante factor for the reduction of dentin erosion and the best treatment is the TiF_4 varnish *in vitro*, regardless of the use of MMP inhibitors.

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3 Discussion

3 DISCUSSION

The first hypothesis of this paper was rejected once dentin with demineralized organic layer (DOM) presented a significant lower dentin wear compared to those without DOM, regardless of the treatments. But DOM can be destroyed or damaged easily as already known (KATO et al., 2010; BARBOSA, KATO, BUZALAF, 2011; RUGG-GUNN, 2013; NANDAKUMAR, NASIN, 2018), because acidic attacks provided by dental erosion results in pH drop and demineralization of dental hard tissue (KATO et al., 2010; MAZZONI et al., 2011, KATO et al., 2011) with consequent exposure of the organic matrix and MMPs activation (KATO et al., 2011; TJÄDERHANE et al., 2015). So DOM may be degraded by the matrix metalloproteinases (MMPs), present in both saliva and dentin (BUZALAF, CHARONE, TJÄDERHANE, 2015; BOTEON et al., 2017a; HAN et al., 2017), which are activated in acid pH and degrade the exposed collagen when the pH is neutralized (BARBOSA, KATO, BUZALAF, 2011; KATO et al., 2012; BOTEON et al., 2017a; HAN et al., 2017). After an erosive challenge, the length of acidic pH is small and the time required for saliva neutralization of the tooth surface ranges from 2 to 7 minutes, with a individuals variations (BOTEON et al., 2017a); with neutral pH, the activated MMPs start to degrade collagen (BARBOSA, KATO, BUZALAF, 2011; KATO et al., 2012; BOTEON et al., 2017a; HAN et al., 2017). Thus, enzymatic removal of the organic matrix by MMPs increases the demineralization process. Ganss et al. (2004) (GANSS et al., 2004) showed that after fluoridation with collagenase, a linear increase in mineral loss (73.3 ± 17.6 μm on day 5) was observed, which statistically exceeded the control group (45.9 ± 14.3 μm on day 5). Fluoridation, without collagenase, was able to significantly reduce mineral loss from the second day (12.2 ± 10.2 μm on day 5) compared to the other groups and the results indicated that the organic matrix is essential for fluoride efficacy in dental erosion (GANSS et al., 2004). Thinking about this, our research group hypothesizes that the maintenance of the DOM by using MMP inhibitors associated or not with fluoride agentes would be an effective way to reducing the wear progression.

The second hypothesis was accepted because the pre-treatments (chlorhexidine-CHX or proanthocyanidin-PA) did not have any influence on the progression of dentin erosive wear. Most of the studies in the literature show a good effect of chlorhexidine as inhibitors of MMPs, decreasing its activity (KATO et al., 2010; BARBOSA, KATO, BUZALAF, 2011; BOTEON et al., 2017a). However, the exact mechanism of MMP inhibition is not completely understood,

but the most accepted theory is that CHX present their inhibitory effect through divalent chelation of metal ions, especially zinc and calcium (VISSE, NAGASE, 2003; KATO et al., 2010; BALALAIE, REZVANI, MOHAMMADI BASIR, 2018). In this way, MMP inhibitors will prevent MMPs from binding to collagen substrate and its further cleavage (TJÄDERHANE et al., 2013). In relation to PA, the exact mechanism of cross-linking is not completely understood also, but four diffetent theories explain PA interactions with proteins which include covalente (SESEOGULLARI-DIRIHAN et al., 2015), hydrogen bonding (PIERPOINT, 1969), ionic (HAN et al., 2013) and hydrophobic (PAEPEGAEY, 2013) interactions (TJÄDERHANE et al., 2013). Our research found differents results from those presented in the literature: it was not possible verify an effect of MMP inhibitors on the tooth structure wear inhibition. Perhaps, for the fact, that this study did not evaluate these agents alone, but in interaction with fluoridated agents. However, more accurate studies should be conducted to understand the interaction between CLX and PA with MMP inhibitors, until found results about their interactions and action mechanisms, or find optimal protocols and ideal concentrations so that they present a good action on the dentin structure.

Much of the differents results found in these work, for those present in the literature (KATO et al., 2010; BARBOSA, KATO, BUZALAF, 2011; BOTEON et al., 2017a; HAN et al., 2017), is due to the fact that in our study the analysis of structural loss was performed by non-contact profilometry, while in the others works was used contact profilometry. Contact profilometers use a diamond tip moved across the surface to record the surface profile. However, has the potential risk of affectingthe reading or even damaging the sample as a consequenceof the contact (SCHLUETER, JUNG, GANSS, 2016). Non-contact profilometers generally use some type of laser to scan the surface to create the profile. Also, allows volumetric loss analysis because generate a surface plane rather than just simple line profiles (SCHLUETER, JUNG, GANSS, 2016). Schlueter et al. (2016) showed that for dentin samples it depends what conditions we would like to evaluate, but the method most indicated would be non-contact profilometry (SCHLUETER, JUNG, GANSS, 2016).

And the third hypothesis of this paper was rejected whereas the final treatments (fluorides) had significant effect on the progression of dentin erosive wear, being the best of them the treatment with TiF_4 varnish, which showed significant protective effect against dentin erosive wear due to the action of titanium (Ti) that reacts with the tooth forming a glaze-like layer more acid resistant (SCHLUETER, JUNG, GANSS, 2016). To reach the same efficacy

of TiF_4 varnish, frequent applications of NaF varnish might be required (MARTINES DE SOUZA et al., 2017), which in turn might be unfeasible from the clinical point of view. Also varnish application allows a longer time of fluoride contact with dentin surface compared to the other fluoride sources (MARTINES DE SOUZA et al., 2017). Martines de Souza et al. (2017) (MARTINES DE SOUZA et al., 2017) evidenced that despite the best efficacy of the TiF_4 varnish, it partially lost the protective effect when DOM was removal, which seems to have a higher influence on the reaction between Ti and dentin. Through EDX analysis a decrease of 22% in Ti content at dentin surface happened due to the absence of the DOM and, even more pronounced this effect occur ate the subsurface (5-10 μm depth) with a loss of 78.5% of Ti, highlighting the fact that the collagen fibrils might play an important role in the penetration of Ti and its protective effect (MARTINES DE SOUZA et al., 2017). SEM analyzes of the surface indicated a deposition of material on and inside the dentinal tubules in the DOM preserved and treated with TiF_4 and NaF varnish, on the contrary, for the placebo varnish and the control group, the dentin samples presented wide, open and visible tubules (MARTINES DE SOUZA et al., 2017). Even if the results showed in this study did not reveal statistical interaction between the presense or absence of DOM and final treatment with TiF_4 varnish, we believe that Ti need to penetrate into the DOM to achieve its best performance in reducing dentin erosion progression.

Extrapolating the methodology to clinical practice, it would be interesting if there was a way to unite, in a single step, the final and initial treatment (MMPs gels inhibitor plus fluoride varnishes), decreasing the clinical time of the dental surgeon and decreasing patient time in the dental office. More studies should be done to understand better the interactions and mechanisms between MMP inhibitors and fluorided agents on the dentin structure wear.

Based on the results, it can be concluded that the presence of DOM is an extremely important factor for the reduction of dentin erosion and the best treatment is the TiF_4 varnish *in vitro*, regardless of the use of MMP inhibitors.

4 CONCLUSIONS

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Based on the results, it can be concluded that the presence of DOM is an extremely importante factor for the reduction of dentin erosion and the best treatment is the TiF_4 varnish *in vitro*, regardless of the use of MMP inhibitors.

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