UNIVERSIDADE DE SÃO PAULO
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In vitro and *in situ* assessment of Proanthocyanidin on enamel erosion prevention

Avaliação *in vitro* e *in situ* da Proantocianidina na prevenção da erosão do esmalte

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ABSTRACT

In vitro and *in situ* assessment of Proanthocyanidin on enamel erosion prevention

Proanthocyanidin is a natural agent that has been extensively tested in Dentistry in different fields with promising results, including on dental erosion. It has an interesting performance on dental tissues, especially on dentin, in which interacts with collagen matrix, inducing cross-linker formation and improving the dentin mechanical properties. However, as Proanthocyanidin can also act on de-remineralization process as well as interacting with salivary proteins, it might play a role on enamel erosion prevention. Thus, the aim of this thesis was to evaluate the Proanthocianidin on enamel erosion by *in vitro* and *in situ* studies, not necessarily in that order. The first study evaluated the effect of Proanthocyanidin applied over acquired enamel pellicle on initial erosion. The acquired pellicle was formed in situ by the placement of intraoral palatal devices in two healthy volunteers for 2 hours. The enamel blocks of each group were treated in vitro: G1: 6.5% proanthocyanidin gel and acquired pellicle formed in situ; G2: only 6.5% proanthocyanidin gel; G3: only acquired pellicle formed in situ; and G4: no intervention. Gels were applied for 1 minute. Then, enamel blocks were immersed in 0.5% citric acid, pH 2.5, for 30 seconds to promote a short erosive challenge. The response variable was the percentage of surface hardness loss was carried out. Data analysis showed that the G1 group showed the lowest value of hardness loss compared to other groups (G2, G3 and G4), which exhibited a greater hardness loss with no significant difference among them. The article 2 evaluated the in vitro effect of Proanthocyanidin on noneroded and eroded enamel compared to fluoride submitted to 5-day erosive cycling. Gels was applied were applied once every day before the first erosive cycling. The enamel erosion was carried out with cola drink for 5 minutes, 3 times per day. The enamel loss determination was performed by profilometry analyses. Results showed that the tested gels were not able to prevent the enamel wear. Finally, the article 3 evaluated the effect of Proanthocyanidin applied over acquired enamel pellicle, but on 5-day erosive cycling. The studied groups were Proanthocyanidin gel on acquired enamel pellicle, only Proanthocyanidin gel and only acquired enamel pellicle. Three volunteers were responsible to the placement of intraoral palatal devices for the acquired pellicle groups. The erosive cycling consisted by immersion of the enamel blocks in the same kind of acid from article 1, but for 2

minutes, 3 times per day. The enamel loss was determined by profilometry analysis. Data analysis suggested that Proanthocyanidin can prevent enamel loss only on acquired pellicle presence.

Keywords: Preventive dentistry. Dental enamel. Tooth erosion

RESUMO

Avaliação in vitro e in situ da Proantocianidina na prevenção da erosão do esmalte

A Proantocianidina é um agente natural que vem sendo amplamente testado em Odontologia em diferentes campos com resultados promissores, inclusive na erosão dentária. Ela possui um desempenho interessante nos tecidos dentários, principalmente na dentina, na qual interage com a matriz de colágeno, induzindo a formação de cross-linkers o que melhora suas propriedades mecânicas. No entanto, como a Proantocianidina também pode atuar no processo de des-remineralização, além de interagir com proteínas salivares, ela poderia desempenhar um papel na prevenção da erosão do esmalte. Dessa forma, o objetivo desta tese foi avaliar a Proantocianidina na erosão do esmalte através de estudos *in vitro* e *in situ*, não necessariamente nesta ordem. O primeiro estudo avaliou o efeito da Proantocianidina aplicada sobre a película adquirida do esmalte na erosão inicial. A película adquirida foi formada *in situ* pelo uso de dispositivos palatinos em dois voluntários saudáveis por 2 horas. Os blocos de esmalte de cada grupo foram tratados *in vitro*: G1: gel de proantocianidina a 6,5% e película adquirida formada *in situ*; G2: apenas 6,5% de gel de proantocianidina; G3: apenas película adquirida formada *in situ*; e G4: sem intervenção. Os géis foram aplicados por 1 minuto. Em seguida, os blocos de esmalte foram imersos em ácido cítrico a 0,5%, pH 2,5, por 30 segundos para promover um curto desafio erosivo. A variável de resposta foi a porcentagem de perda de dureza superficial. A análise dos dados mostrou que o grupo G1 apresentou o menor valor de perda de dureza comparado aos outros grupos (G2, G3 e G4), os quais apresentaram maior perda de dureza, sem diferença significativa entre eles. O artigo 2 avaliou o efeito *in vitro* da Proantocianidina no esmalte hígido e erodido comparado ao fluoreto, após ciclagem erosiva de 5 dias. Os géis foram aplicados uma vez por dia antes da primeira ciclagem erosiva. A erosão do esmalte foi realizada com Coca-Cola® por 5 minutos, 3 vezes ao dia. A perda de esmalte foi avaliada por perfilometria. Os resultados mostraram que os géis testados não evitaram o desgaste do esmalte. Finalmente, no artigo 3 foi avaliado o efeito da Proantocianidina aplicada sobre a película adquirida do esmalte, mas sob desafio erosivo de 5 dias. Os grupos estudados foram gel de Proantocianidina + película adquirida, apenas gel de Proantocianidina e apenas película adquirida. Três voluntários foram responsáveis

pelo uso dos dispositivos palatinos para os grupos com películas adquirida. A ciclagem erosiva foi feita através da imersão dos blocos de esmalte no mesmo tipo de ácido do artigo 1, mas por 2 minutos, 3 vezes por dia. A perda de esmalte foi determinada por perfilometria. A análise dos dados sugeriu que a Proantocianidina pode prevenir a perda de esmalte apenas na presença de película adquirida.

Palavras-chave: Odontologia preventiva. Esmalte dentário. Erosão dentária

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1 INTRODUCTION

1 INTRODUCTION

Dental erosion is the term that refers exclusively to the process of chemical softening of the dental surface under acidic exposure in which minerals are devoid[1,2]. When mechanical forces are associated, the process advances causing effectively the tissue loss. In this stage, the process is called tooth erosive wear and implies in irreversible mechanism[1,2]. Early diagnosis and intervention are definitely important, especially due to high prevalence[3] and the fact that the erosive tooth wear can also be considered the third oral condition most commonly observed, after caries and periodontal disease, with a similar prevalence than dentin hypersensitivity[4].

The preventive management of dental erosion/erosive tooth wear is quite complex, mainly because there are many factors related to patient's nutrition and behavior which stimulates the lesions progression[5]. In some cases, this progression can be slow and gradual, while in others there may be rapid changes, compromising the integrity of dental tissue and the longevity of the restorations if presented[4]. Considering that the rehabilitation of the severe cases is expensive and time-consuming[3,6], the adoption of preventive measures to avoid this event and to arrest it in case of tissue loss is clearly necessary and must be installed at the appropriate time[5].

Saliva and acquired pellicle are considered as the most important biological protective factors, since they can influence the acid effects on tooth surface[7,8]. Saliva acts directly on the erosive agent by diluting, clearing, neutralizing and buffering acids. It also reduces demineralization rate and enhance remineralization by providing calcium, phosphate and fluoride to eroded surface. In addition, saliva plays a role in forming acquired enamel pellicle[7]. The latter is formed by adsorption of proteins, peptides, lipids and other macromolecules, acting as a barrier between the tooth surface and the oral environment[8,9]. Acquired enamel pellicle consists of two distinct layers: a basal layer formed by initial adsorbed proteins and globular layer composed of proteins aggregates[8,10]. As the basal layer is not totally removed during acid attack, but gradually dissolved from its external to basal components[11], the acquired enamel pellicle acts as perm-selective membrane, reducing and retarding direct contact

between acids and enamel surface[10]. Then, several studies have tried to improve this biological protection by modifying its composition[12-16].

Simultaneously to control of the dental erosion etiological factors, it is also possible to use different strategies and anti-erosion agents that have a certain ability to retain the acid effects on the tooth surface, such as fluoride[17-22], casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP)[23-25] and resin-based materials[26-29]. However, natural agents have attracted the attention of researchers due to the absence of side effects, very low toxicity[30] and by their renewable and/or sustainable qualities[31]. Among these agents is Proanthocyanidin, which derived from fruits, vegetables, nuts, seeds and flowers[30], but grape seed is one of the richest sources of Proanthocyanidins[32]. This natural agent has already been extensively tested in dentistry, mainly in studies conducted on dentin tissue, exhibiting satisfactory effects in root caries[33-35], in experimental dental adhesives or as pre-treatment to improve dentin mechanical properties[36-39] and on dentin erosion[40,41].

In relation to enamel, the action of Proanthocyanidin is still controversial, since there are some certain promising results only in specific situations[42,43]. As it is known that Proanthocyanidin can interact with salivary proteins[12,30,44], it would be interesting to investigate if this interaction might influence enamel loss under greater erosive conditions. Therefore, the aims of this thesis were to analyze the role of Proanthocyanidin on enamel erosion.

2 ARTICLES

2 ARTICLES

2.1 ARTICLE 1 - Proanthocyanidin protects the enamel against initial erosive challenge when applied over acquired pellicle.

2.2 ARTICLE 2 - *In vitro* effect of 6.5% Proanthocyanidin gel on preventive and arresting erosive potential on enamel

2.3 ARTICLE 3 - Influence of Proanthocyanidin applied over acquired enamel pellicle on erosive tooth wear

ARTICLE 1 - The article listed below was accepted for publication and cannot be reproduced in this thesis for copyright reasons.

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The manuscript's acceptance e-mail is presented in Annex 1.

ARTICLE 2 - The article presented in this thesis was written according to the Journal Applied of Oral Science instructions and guidelines for article submission.

In vitro effect of 6.5% Proanthocyanidin gel on preventive and arresting erosive potential on enamel

ABSTRACT

Proanthocyanidin is a natural agent that has been extensively tested in Dentistry in different fields with promising results, including on dental erosion. Objective: the aim of this *in vitro* study was to evaluate the effect of Proanthocyanidin on noneroded and eroded enamel submitted to erosive cycling. Material and methods: The enamel blocks were randomly divided among 3 groups taking preventive and arrest potential into account. For both situations, the tested treatments involved no treatment (control), 6.5% PA gel and 1.23% NaF, respectively for noneroded and eroded enamel. For eroded enamel groups, the erosion was previously induced by immersion of enamel blocks in an acid beverage for 5 minutes. The erosive challenge was performed 3 times during 5 days. The response variable was depth of enamel loss (µm) by using a contact profilometer. Results: The gels cannot prevent enamel loss after erosive cycling, since there was no difference between gels groups and control groups, for both initial enamel conditions. Conclusion: 6.5% Proanthocyanidin and 1.23% NaF gels were no effective to prevent and arrest erosive damages on enamel substrate.

Keywords: Dental enamel. Grape seed proanthocyanidins. Tooth wear

INTRODUCTION

Dental erosion is defined by the tooth structure loss induced by intrinsic or extrinsic acids, with no microorganism's involvement[1]. As in recent years there has been an increase in its prevalence[2], the search for preventive measures has become more important, especially as they reduce the chance of the lesion progressing, avoiding a more invasive and expensive treatment[3]. Among these measures is the use of remineralizing agents such as monovalent fluorides in low-to-moderate concentrations, fluorides in high concentration, acid fluoride formulations and polyvalent metal fluorides[4]. All these formulations exhibit protective protection against dental erosion[4-8].

Other researches have tested natural agents in order to restrain the dental erosion effects[9-12], mainly due to their biocompatibility, low toxicity and no adverse effects. Proanthocyanidin is a natural agent which can be found in many fruits, nuts and in a greater amount in the grape seed extract[13]. This agent presents interesting properties on dental tissues, mainly on dentin, such as collagen biomodification which improves dentin mechanical properties[14] and it also acts in reducing demineralization and promoting remineralization in root caries lesions[15-17]. Additionally, studies with erosive protocols showed that Proanthocyanidin was able to inhibit the wear and the degradation of demineralized organic matrix[10,11].

On the other hand, the action of Proanthocyanidin on enamel is still controversial, since there are some certain promising results only in specific situations such as cariogenic challenge[12] and on initial enamel erosion model (softening stage) in acquire pellicle presence[18]. Then, it would be interesting to know whether Proanthocyanidin could have any action on enamel in an erosive tooth wear conditions. Therefore, the aim of this study was to evaluate the *in vitro* effect of Proanthocyanidin gel compared to fluoride gel, in preventing erosion developing (noneroded enamel) as well as in inhibition erosion progression (eroded enamel). The null hypotheses tested were that (1) the initial enamel condition (noneroded and eroded enamel) will not influence the enamel loss and (2) the tested gels will not reduce enamel loss.

MATERIAL AND METHODS

Experimental design

This study was conducted *in vitro* and the factors under investigation were tested gels (6.5% Proanthocyanidin gel, and 1.23% NaF gel and no treatment) and initial enamel condition (noneroded and eroded enamel). The enamel blocks were randomly divided among 3 groups considering preventive and arrest potential. The tested treatments involved no treatment (control), 6.5% PA gel and 1.23% NaF respectively for noneroded and eroded enamel, i.e., a total of 6 groups, with n = 15. For eroded enamel groups, the erosion was previously induced by immersion of enamel blocks in an acid beverage for 5 minutes. The erosive challenge was performed during 5 days. The response variable was depth of enamel loss (µm) by using a contact profilometer (Figure 1).

Enamel blocks preparation

The enamel blocks were obtained from bovine incisors and the crowns were separated from their roots and embedded into self-curing acrylic resin (JET, Campo Limpo Paulista, SP, Brazil)[19]. Then, the surface of the blocks was ground flat with water-cooled silicon carbide paper discs (300, 600, and 1200 grade papers; Extec Corp, Enfield, USA) using a Metallographic Polishing Machine (APL 4, Arotec, Cotia, SP, Brazil) and polished in the same equipment with a felt paper wetted with 1µm diamond spray (Buehler, Ltd., Lake Bluff, IL, USA). An ultrasonic device (USC2500, Merse, Campinas, SP, Brazil) was used for 2 minutes to clean enamel blocks, after each type of silicon carbide discs and at the end of polishing. Surface Knoop hardness tests were performed at five sites in different regions of the blocks (25 g for 10 s - Micromet® 5114 hardness tester; Buehler Ltd., Lake Bluff, Illinois, United States) and 90 blocks were selected and randomly divided in the 6 groups described above. Prior to treatment, identification marks were made on the enamel blocks surfaces using a scalpel blade (Embramac, Itapira, SP, Brazil) in order to divided the enamel block surface in three areas, in which the central area corresponding to treatment and the outer areas corresponding to control areas. One of the control areas was marked with a 1/4 drill to assure that final and initial profiles were measured at exactly the same sites. Then, the enamel blocks were placed in the profilometer and their X and Y-axis positions were noted, allowing a subsequent accurate repositioning of the contact

profilometer' stylus (at the final profilometry). Subsequently, five baseline surface profiles were obtained from each enamel block using a contact profilometer (MarSurf GD 25, Göttingen, Germany) linked to a computer with a specific software (MarSurf XCR20, Göttingen, Germany) at determined distances (2.25, 2.0, 1.75, 1.5 e 1.25 µm)[10]. After that, the marks which was made by scalpel blade and the two outer areas were covered with a nail varnish (Maybelline Colorama, Cosbra Cosmetics Ltda, São Paulo, SP, Brazil) in order to allow references surfaces for enamel loss analysis.

Treatment and erosive cycling

The studied gels were applied once over enamel daily, before the first erosive challenge, during the 5 days of this experiment. All gels formulations presented the same composition (hydroxyethylcellulose, propyleneglycol, methylparaben, imidazolidinyl urea and de-ionized water, pH 7.0)[10], except for the Proanthocyanidin (Purified Grape Seed Oligomeric Proanthocyanidins, 1298219, Sigma-Aldrich Co. ®,St. Louis, MO, USA) and NaF (Via Farma®, São Paulo, SP, Brazil). Enamel blocks were immersed in an acid beverage (Coca-Cola®, SPAL-Beverages Brazilian Industry S/A; Jundiaí, SP, Brazil) for 5 minutes, followed by rinsing with deionized water for 20 seconds and immersion in artificial saliva (0.33 g KH2 PO4, 0.34 g Na2 HPO4, 1.27 g KCl, 0.16 g NaSCN, 0.58 g NaCl, 0.17 g CaCl2, 0.16 g NH4 Cl, 0.2 g urea, 0.03 g glucose, 0.002 g ascorbic acid, pH 7.0)[20] for 2 hours. This erosive cycling was repeated 3 times per day, during 5 days.

Final profilometry and enamel wear analyses

Before the final profilometric measurement the nail varnish was carefully removed from de enamel blocks with the aid of a scalpel blade. As the enamel blocks were precisely repositioned in the profilometer through the drill mark reference and x and y-axis positions' notes, five surface profiles were performed again at the same sites as the baseline profiles. Then, the enamel loss was quantitatively determined using the MarSurf XCR 20 software by calculating the mean depth of the eroded surface relative to the baseline surface profiles[10].

Statistical analysis

Statistical data analysis was performed in software statistica 10.0 (Stat Soft Inc.). Since the principles of normality and homogeneity of variances were satisfied, Two Way Analysis of Variance (ANOVA) followed by Tukey's test were applied, considering a significant level of 5%.

RESULTS

The results of studied groups are presented in table 1. Two-way ANOVA showed a significant different only for initial enamel condition (noneroded or eroded, p=0,0003), in which the noneroded groups presented a lower enamel loss than eroded group. For the tested gels factor there was no significant interaction (p=0,065) between the groups (PA gel, NaF gel and control group). There was no significant interaction between both studied factors.

DISCUSSION

A previous study reported no difference in susceptibility to new acid challenges between noneroded and eroded bovine enamel[19], differently from the present study where the initial enamel condition influenced the enamel wear, since the noneroded enamel blocks showed a significant lower wear compared to eroded ones - then, the first null hypothesis was rejected. Some factors can explain these conflicting results such as initial erosion intensity of eroded group and the aggressiveness of erosive challenge. In the present study the enamel blocks of eroded group were previously immersed in an acid beverage (pH around 2.3 – 2.5) for 5 minutes, while in the study conducted by Oliveira et al. (2017) the enamel blocks were immersed in hydrochloric acid (0.01 M, pH 2.3) for 30s[19]. After 4 minutes immersion into citric acid 0.034 M, pH 3.6, the enamel loss can already be seen[21,22]. In addition, there are a fast hardness loss at the first minutes of erosion, followed by a stabilization period in which hardness remained constant, characterizing the enamel wear[22]. Considering these findings, the protocol used in the present study to form the initial erosion lesion had a severe intensity, impacting the enamel loss after the 5-day erosive cycling. The erosive cycling protocols were also different, since the present study the enamel blocks were submitted to three erosive cycles per day (5 minutes in acid beverage) for 5 days, while

the other study the enamel blocks were exposed to hydrochloric acid for 2 minutes, four times a day, also during 5 days[19].Then, as it is known that an erosive time of about 4 minutes with HCl (pH 3.0) might simulate one day of erosion on a clinical situation[23], it can estimate that present study might simulate around 18 *in vivo* erosion days compared to 10 *in vivo* erosion days from the other study, and therefore, characterizing a stronger erosive challenge.

When enamel is exposed to acids it loses mineral from the surface, a process called softening and if the erosive challenges continue, this softened tissue is easily lost by mechanical factors[24]. Therefore, shorter time was allowed to fluoride plays the remineralization on the softened enamel surface. As the thickness of this layer is smaller than that loosed, fluoride mainly aims to prevent continuous progression of wear rather promote its remineralization[25]. Fluoride induces the precipitation of CaF_2 on tooth surface, which can act as mechanical barrier against acids[4,25]. However, this layer presents a low resistance to acid challenges, dissolving quickly, it means that, the fluoride protection is limited[4,25]. It can explain the results of 1.23% NaF group in the present study, which was not able to reduce the enamel wear, exhibiting no significant difference compared to control group even with a daily gel application.

Despite the fact that fluoride acidic formulations were more effective than pH-neutral gels in reducing erosion[26], both gels 6.5% Proanthocyanidin and 1.23% NaF were made in neutral pH in order to avoid any influence of the tested gels' pH in the erosive protocol of this study. The kind and concentration of fluoride can interfere with the agents' protection abilities. The 1.23% NaF presented a good effect in preventing erosion when applied over dentin tissue[27], different from that occurred on enamel, since the 1.23% NaF group cannot minimize the enamel wear, as it showed by the results of this study. The concentration may have influenced the NaF group results, since studies have shown that a concentration equal or up to 2% of this monovalent fluoride was able to prevent and / or reduce the enamel erosion progression[5-7]. Moreover, alternatives fluorides to control tooth erosion, such as polyvalent metal fluorides (TiF_4 or SnF_2) attracted the attention of researchers, mainly by formation of a metal-rich enamel layer, which renders higher resistance against acids than the one that contains only CaF_2 [4,28]. Despite these promising alternatives, fluoride effects appear to be reduced when the clinical situation was simulated, and also when the erosive challenge is more severe[4].

In a previous report, a single application of Proanthocyanidin gel was sufficient to reduce the dentin wear during a 5-day erosive cycling[10]. However, in the present study in which the same gel was daily applied on enamel, it was not able to reduce the enamel loss, with a similar performance that the control groups (no treatment). Considering that the erosive protocol was the same for both studies, the distinct results can be related to Proanthocyanidin's action on each substrate – enamel and dentin – due to the great differences between them[29]. On dentin, Proanthocyanidin can act in reducing demineralization and enhancing remineralization[15] through a different mechanism from fluoride, by formation of insoluble complex that remains stable in acid pH[30]. Additionally, as Proanthocyanidin can induce collagen cross-linker formation[14], it maintains collagen matrix intact, protecting the underline tissue against structure loss[11]. Therefore, there are two possible mechanisms acting on dentin tissue and it is difficult to analyze them separately, since they can occur simultaneously. Due to enamel composition is predominantly inorganic[29], it was possible to evaluate the Proanthocyanidin's influence only for one these mechanisms, the de-remineralization process.

A study tested the Proanthocyanidin effect on enamel and it was conducted in an artificial cariogenic challenge, the Proanthocyanidin inhibited the demineralization and promoted remineralization, but with a lower performance than fluoride[12]. On the other hand, when Proanthocyanidin gel was *in vitro* applied on enamel submitted to a short-term erosive challenge with citric acid it exhibited no effect in preventing demineralization[18]. Thus, it is possible to speculate that Proanthocyanidin has a restricted action on enamel or its action was limited by acid challenge aggressiveness. Despite both caries and erosion result in a demineralization process, there are a quantitively difference between these processes[31]. While in caries the plaque fluid always contains Ca, P and the pH rarely drops below 4.0 - 4.5, in an erosive protocol, the acids (intrinsic or extrinsic) have very low pH and not presenting significant Ca, P and F concentrations, so there are a highly undersaturated environment[31,32]. Thus, the erosive protocol can result in a faster dissolution rate, favoring a greater loss of enamel from the tooth surface[29].

Both tested gels (Proanthocyanidin and NaF) cannot able to prevent (noneroded) and/ or reduce enamel erosion progression (eroded enamel) in this protocol for different reasons. Nevertheless, it appears that the severity of erosive challenge can also limit the preventive agents' efficiency. In addition, it can also

increase the chances of enamel loss for previously eroded groups. However, as the action of Proanthocyanin on initial enamel erosion in the acquired pellicle presence has already known[18], further studies are necessary to investigate its effects at the same protocols that mimic the intraoral environment, but in prolonged erosive challenges in order to evaluate the enamel wear.

CONCLUSION

Considering the limitations of this study, the results suggest that the initial enamel condition can influence the enamel loss under a stronger erosive protocol, since the eroded enamel blocks presented a greater enamel loss than noneroded ones. Furthermore, Proanthocyanidin and NaF gels had no effect on preventing enamel loss for both enamel initial conditions.

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FIGURES

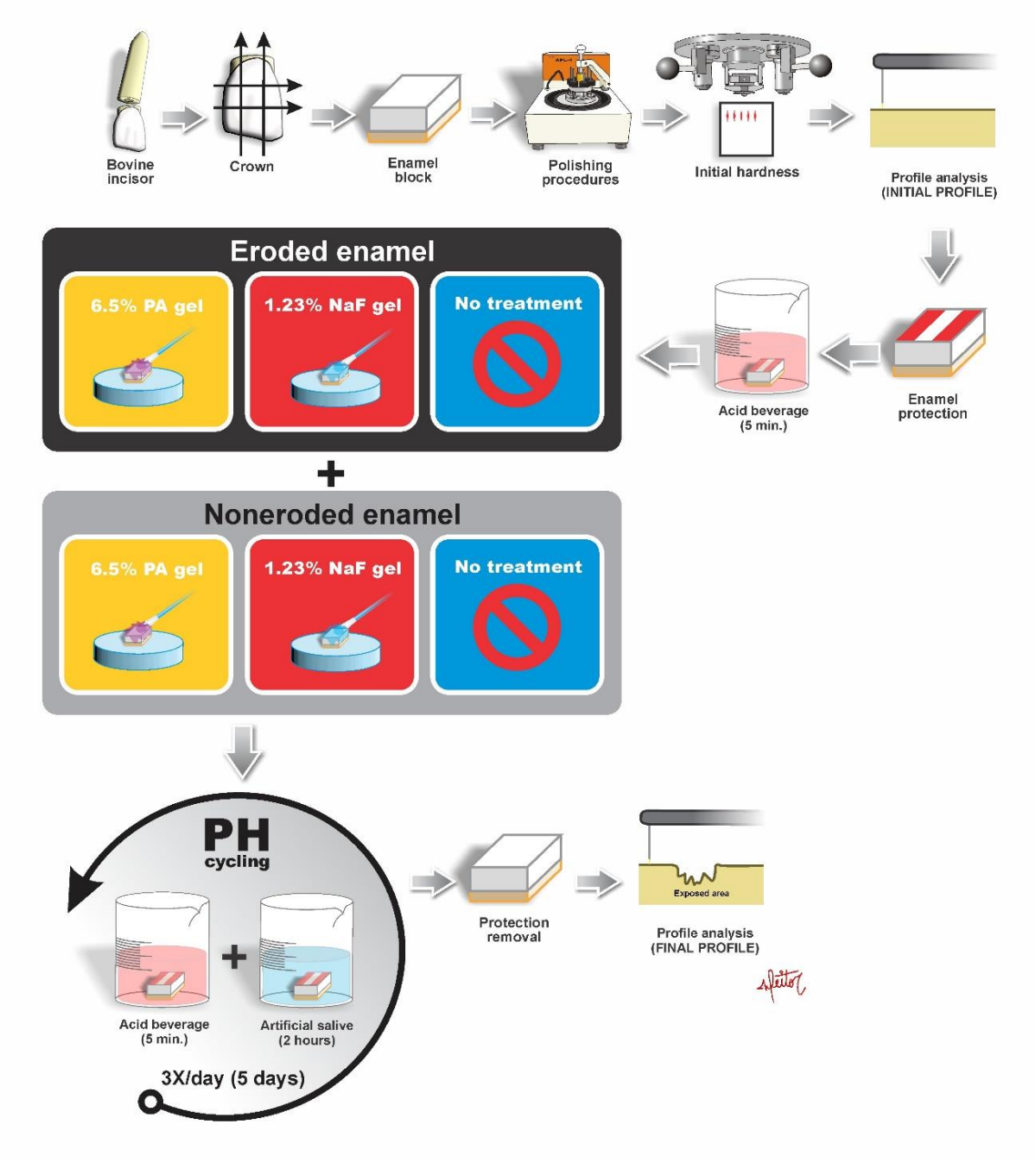

Figure 1- Experimental design

TABLES

Table 1 – Means values (±sd) of enamel loss (µm) of studied groups.

		Initial Enamel Condition	
	GROUPS	*Noneroded* [A]	*Eroded* [B]
Tested gels/control	6. 5% Proanthocyanidin gel	1.30±0.34[a]	1.60±0.27[a]
	1.23% NaF gel	1.38±0.36[a]	2.07±0.42[a]
	Control – no treatment	1.47±0.42[a]	1.60±0.40[a]

*Equal lowercase letters indicate no statistically significant difference when comparing tested gels (Two Way ANOVA and Tukey's Test, p=0,065) and different capital letters indicate significant differences when comparing initial enamel condition (Two Way ANOVA and Tukey's Test, p=0,0003).

ARTICLE 3 - The article presented in this Thesis was written according to the Archives of Oral Biology instructions and guidelines for article submission

Influence of Proanthocyanidin applied over acquired enamel pellicle on erosive tooth wear

Highlights:

1. The isolated effect of Proanthocyanidin on enamel erosion appears to be restricted
2. Proanthocyanidin can improve acquired enamel pellicle properties
3. Interaction between Proanthocyanidin and acquired pellicle can inhibit enamel wear

ABSTRACT

Objectives: The aim of this study was to evaluate the effect of Proanthocyanidin gel applied over acquired pellicle in reducing enamel erosive loss.

Design: 63 enamel blocks obtained from bovine incisors were randomly allocated into 3 groups (n=21): G1- PA + AEP: 6.5% Proanthocyanidin gel and acquired pellicle formed *in situ*, G2- PA: only 6.5% Proanthocyanidin gel and G3- AEP: only acquired pellicle formed *in situ*. Gel was applied for 1 minute twice daily. Then, enamel blocks were subjected to erosive cycling by immersion in citric acid, three times per day. Acquired pellicle formation, treatment and erosive cycling were repeated for 5 days. The response variable used was the contact profilometry to determine the enamel loss (µm).

Results: All groups showed a statistically significant difference ($p < 0.001$). G1 group exhibited the lowest wear value after erosive cycling (0.28 ± 0.19 µm), followed by the G3 group (1.45 ± 0.87 µm) and the PA group (2.49 ± 0.79 µm).

Conclusion: Proanthocyanidin acts synergically with acquired pellicle on enamel to reduce the enamel wear in erosive conditions.

Keywords: Enamel. Preventive dentistry. Grape seed proanthocyanidins. Salivary acquired pellicle. Tooth erosion. Tooth wear

INTRODUCTION

Recently, studies have showed the potential use of natural agents that can be extracted from renewable resources, drawing wide attention to dentistry researchers (Bedran-Russo et al., 2014). There are two reasons for this increased interest: natural agents have very low toxicity when compared to synthetic agents (Han, Jaurequi, Tang, & Nimni, 2003) and they can be considered renewable and/or sustainable resource (Bedran-Russo et al., 2014). Among these natural agents are Proanthocyanidins, which are characterized as polyphenols that are available in fruits, vegetables, nut, seeds, flowers, and barks (Han et al., 2003), but cocoa and grape seed are among the richest sources of Proanthocyanidins (de Pascual-Teresa, Santos-Buelga, & Rivas Gonzalo, 2000). These structures are able to form an insoluble complex with carbohydrates and proteins (Cao, Fu, & He, 2007) and are also considered as a natural cross-linker due to their ability of precipitate proline rich proteins by hydrogen and covalent bonds (Ku, Sathishkumar & Mun, 2007; Castellan, Pereira, Grande, & Bedran-Russo, 2010).

As previously mentioned, Proanthocyanidins have been extensively tested in dentistry in studies related to caries (Xie, Bedran-Russo, & Wu, 2008; Silva, Gonçalves, Borges, Bedran-Russo, & Shinohara, 2015), in experimental dental adhesives or as pre-treatment to improve dentin mechanical properties (Castellan et al., 2010; Zhou et al., 2016; Leme-Kraus et al., 2017; Dias et al., 2020) and also on tooth erosion (Boteon, Prakki, Buzalaf, Rios, & Honório, 2017; Boteon et al., 2017; Boteon et al., 2020). Special attention should be given to the latter because the numbers about dental erosion are not low, since in primary teeth the mean prevalence is between 30% and 50% and in permanent teeth between 20% and 45% (Schlueter & Luka, 2018). Therefore, preventive approaches are required in order to control dental erosion, especially those that influence saliva and acquired enamel pellicle (AEP), which are considered as the most important biological protective factors (Hara & Zero, 2014; Hannig & Hannig, 2014).

During the erosive challenge, the AEP is not completely removed from the enamel surface, inhibiting acids effects (Hannig et al., 2004; Hannig et al., 2007). Despite the effectiveness of this biological protection (Hannig & Hannig, 2014), AEP properties may be improved by modifying its composition through specific agents such as chlorhexidine (Joiner, Elofsson, & Arnebrant, 2006), mucin and casein (Cheaib &

Lussi, 2011), vegetables oils (Hanning et al., 2012; Ionta et al., 2017), including the Proanthocyanidin (Joiner, Muller, Elofsson, Malmsten, & Arnebrant, 2003; Boteon et al., 2020). Recently, Proanthocyanidin applied over AEP was able to reduce enamel demineralization on initial erosion (Boteon et al., 2020), when just softening was present. Then, it would be interesting to know as this natural agent would behave in the enamel loss condition. Therefore, the aim of this study was to evaluate the influence of Proanthocyanidin applied over AEP subjected to erosive wear, in order to assess this interaction on enamel structure loss. The null hypothesis tested was that the Proanthocyanidin application over AEP will not reduce the enamel loss.

MATERIAL AND METHODS

Experimental design

The research protocol of this study was approved by the local Research Ethics Committee (Protocol 22092819.7.0000.5417). All volunteers signed an informed consent form before the confirmation of their eligibility for the study.

The acquired pellicle was formed *in situ* by the use of intraoral palatal device by three healthy volunteers for 2 hours. The enamel blocks of each group (n = 21) were randomly divided in three groups: G1- PA + AEP: 6.5% Proanthocyanidin gel and acquired pellicle formed *in situ*, G2- PA: only 6.5% Proanthocyanidin gel and G3- AEP: only acquired pellicle formed *in situ*. Gel was applied for 1 minute twice daily. Then, enamel blocks were subjected to erosive cycling by immersion in citric acid, three times per day. Acquired pellicle formation, treatment and erosive cycling were repeated for 5 days. The response variable used was the contact profilometry to determine the enamel loss (µm) (Figure 1).

Enamel blocks preparation

80 enamel blocks (4x4x3 mm^2) were obtained from bovine incisors, using a IsoMet® low speed saw cutting machine (Buehler Ltd.; Lake Bluff, Illinois, United States) with two diamond disks (Extec Corp.; Enfield, Connecticut, United States), which were separated by a 4-mm thickness spacer. Then, the enamel blocks surface was ground flat with water-cooled silicon carbide discs (320, 600, and 1200 grade papers; Buehler Ltd.; Lake Bluff, Illinois, United States), and polished with felt paper wetted with 1 µm diamond spray (Buehler Ltd.; Lake Bluff, Illinois, United States). An

ultrasonic device (USC2500, Merse, Campinas, SP, Brazil) was used for 2 minutes to clean enamel blocks. Surface Knoop hardness tests were performed at three sites 100µm away from each other (25 g for 10 s - Micromet® 5114 hardness tester; Buehler Ltd., Lake Bluff, Illinois, United States) in order to select 63 enamel blocks. Then, the blocks were randomly divided in 3 groups using Microsoft Excel to distribute blocks with lower and higher initial hardness values equally into all groups. The selected enamel blocks were wrapped in surgical grade envelope (VedaMax Wenceslas President, SP, Brazil) and sent for sterilization by ethylene oxide (Central Sterilization Acecil Trade and Industry Ltd., Campinas, Brazil).

Initial profilometry

Enamel blocks were marked with a scalpel blade (Embramac, Itapira, SP, Brazil) to determine the control and test areas (Santos et al., 2018). One of the control areas was marked with a 1/4 drill to ensure that profiles were measured at exactly the same sites (Santos et al., 2018). Subsequently, three baselines surface profiles were obtained from each of enamel blocks using a profilometer (Marh, MarSurf GD 25, Göettingen, Germany) with a specific software (XCR 20, MarSurf GD 25, Göettingen, Germany) at certain distances from the mark over the y-axis: 0.5, 1.0 and 1.5 µm. The marks were made by scalpel blade and the control areas were covered with a nail varnish (Maybelline Colorama, Cosbra Cosmetics Ltda, São Paulo, SP, Brazil) in order to allow references surfaces for enamel loss analysis.

Acquired enamel pellicle (AEP) formation

The enamel blocks were exposed to the oral cavity of three healthy volunteers, who satisfied the following inclusion criteria: physiological salivary parameters (stimulated >1 ml/min; unstimulated >0.1 ml/min; neutral pH 7.0-7.5); absence of erosive tooth wear, untreated carious lesions, or periodontitis; residing in the same fluoridated area (0.70 mg F/l). The exclusion criteria were: use of medicines that affect the salivary characteristics; undergoing radiation or chemotherapy; presence of systemic diseases; smoking; frequent regurgitation and/or vomiting; gastro-esophageal reflux; pregnancy or breastfeeding; practicing pool activities; working in low pH environment or fluoride topical application in the past two months. The intraoral palatal devices were made with acrylic resin containing 7 sites (5 x 5 x 3 mm). Enamel blocks were fixed in the intraoral device and exposed simultaneously to the oral cavity

of the volunteers. The position of the enamel blocks in the sites was randomized. The volunteers used the intraoral devices for the AEP formation from PA+AEP and AEP groups. In order to avoid overlapping phase, they used the intraoral device on different weeks (Figure 2). Thus, on each of the 5-day erosive cycling, the use of intraoral device of PA+AEP and AEP groups were initiated in the morning, after the volunteer to feed and brush their teeth with a dentifrice containing 1450 ppm F of sodium Monofluorophosphate (Colgate Triple Action, Colgate Palmolive Ltda, São Paulo, SP, Brazil) and soft toothbrush (Curaprox Ultra soft CS 5460, Curaprox, Kriens, Switzerland). After brushing, the oral cavity was rinsed and the volunteer waited 1 hour to insert the appliance into the oral cavity (Alencar et al., 2016), where it remained for 2 hours for the AEP formation (Mendonça et al., 2017). The volunteers were instructed not to eat or drink in this period.

Enamel blocks treatment and erosive cycling

After the time of 2 hours to AEP formation (only for PA+AEP and AEP), the enamel blocks were subjected to three erosive cycles per day for 5 days. The intraoral device corresponding to PA+AEP group was removed of the oral cavity for the application of 6.5% Proanthocyanidin gel (Boteon et al., 2020). The gel was applied over enamel for 1 minute twice a day (before de first and the third erosive cycling) on each of the 5-day erosive cycling. The gel formulation presented the following composition: hydroxyethylcellulose, propyleneglycol, methylparaben, imidazolidinyl urea and de-ionized water, pH 7.0) (Boteon et al., 2017). The Proanthocyanidin was from a purified grape seed extract (Purified Grape Seed Oligomeric Proanthocyanidins, 1298219, Sigma-Aldrich Co. ®, St. Louis, MO, USA). After the application time, the gel was carefully removed with the aid of a microbrush. Afterwards, the erosive challenge was performed *in vitro* by the immersion of the intraoral device in 0.5% citric acid (pH 2.5) for 2 minutes. Then, each enamel block was rinsed with a deionized water to cease the demineralization process. Right after, the intraoral device was reinserted in the oral cavity, where it remained until the next acid immersion. The time interval between the erosive challenges was 2 hours. After the end of the third and last erosive cycling of the day, the intraoral device was removed from the oral cavity and stored in wet gauze and kept refrigerated until the following day. The enamel blocks belonging to AEP group followed the same procedures, except for the gel application, this means that, after 2 hours in oral cavity to form the acquired enamel pellicle, they were directly

immersed in citric acid for 2 minutes and then, they were rinsed and reinserted in the oral cavity for 2 hours. Therefore, for the PA+AEP and AEP groups, the intraoral device was intermittently used by the volunteers for a total of 8 hours per day, during the 5 days. The volunteers had 2 hours available for lunch and oral hygiene, including waiting 1 hour after brushing to insert the intraoral device in the oral cavity. In this period, the device was kept on wet gauze to avoid dehydration of the enamel blocks.

For the enamel blocks corresponding to PA group (only 6.5% Proanthocyanidin gel), all the procedures were carried out *in vitro.* Firstly, the same gel was also applied on enamel for 1 minute twice a day (before de first and the third erosive cycling) on each of the 5-day erosive cycling. After the gel removing, the enamel blocks were immersed in 0.5% citric acid (pH 2.5) for 2 minutes, after that, they were rinsed in deionized water. Then, the enamel blocks were immersed in artificial saliva (0.33 g $KH_2 PO_4$, 0.34 g $Na_2 HPO_4$, 1.27 g KCl, 0.16 g NaSCN, 0.58 g NaCl, 0.17 g $CaCl_2$, 0.16 g NH_4 Cl, 0.2 g urea, 0.03 g glucose, 0.002 g ascorbic acid, pH 7.0 – (Klimek, Hellwig, & Ahrens, 1982 adapted) for 2 hours. The citric acid and artificial saliva solutions were renewed daily.

Final profilometry and enamel loss analysis

After the end of the experimental phases, the nail varnish from enamel blocks was carefully removed with the aid of scalpel blade and three surfaces profiles from each enamel block were obtained at the same sites as the baseline profiles using the same profilometer and the distances described in the topic *Initial profilometry*. Those marks were made with a drill helped to check whether the enamel blocks were placed in the correct position for the measurements.

The graphs generated by the initial and final profilometries were superimposed by XCR 20 software (MarSurf GD 25, Göettingen, Germany), considering three graphs per enamel block since three surface profiles were made per block. During the superimposition procedure, two parallel lines corresponding to the initial and final profiles were obtained. The enamel loss was calculated by the vertical distance between these two parallel lines (Santos et al., 2018) and it was expressed as the mean values of three superimposed graphs.

Statistical analysis

The data were initially submitted to normality tests (Shapiro-Wilk) and homogeneity of variances (Levene) and as they obeyed the principles of normality, the

one-way ANOVA test was applied for comparison among the groups, followed by Tukey's post-test. The level of significance was 5%. Statistical analysis was performed with STATISTICA 10.0 software (Stat Soft Inc.).

RESULTS

The results of this study are shown in table 1. The statistical analysis revealed that all groups showed a statistically significant difference ($p < 0.001$). The PA+AEP group exhibited the lowest wear value after erosive cycling, followed by the AEP group and the PA group, which presented the highest wear value.

Table 1: Means and standard deviation values of enamel loss (µm) after erosive cycling.

Groups	Enamel loss (µm)
G1- PA+AEP: 6.5% Proanthocyanidin gel + Acquired pellicle	0.28 (± 0.19) [a]
G2- PA: only 6.5% Proanthocyanidin gel	2.49 (± 0.79) [b]
G3- AEP: only Acquired pellicle	1.45 (± 0.87) [c]

* Different letters indicate a statistically significant difference when comparing the different groups (ANOVA and Tukey Test, $p < 0.001$).

DISCUSSION

This study was carried out to investigate the influence of Proanthocyanidin on AEP on erosive tooth wear, but there was no ultrastructural assessment of the AEP's presence and its characteristics by the application or not of a Proanthocyanidin-based gel. Then, the Proanthocyanidin/AEP protective effect against erosive wear was indirectly studied through the enamel loss caused after the 5-day erosive cycling by a contact profilometry analyses.

Enamel blocks were immersed in citric acid three times a day followed by remineralization by human (PA+AEP and AEP groups) or artificial saliva (PA group), in the 5-day period. This cycling pattern is not very common in studies that evaluate anti-erosive agents. Usually, erosive cycling is performed through four (Santos et al., 2018; Ionta et al., 2018; Ionta et al., 2019; Jordão et al., 2019) or six (Schlueter, Klimek, & Ganss, 2009; Ganss, Neutard, von Hinckeldey, Klimek, & Schlueter; Schlueter, Klimek, & Ganss, 2011; Schlueter, Klimek, & Ganss 2013) demineralization-remineralization cycles. However, this study was designed to ensure that the AEP would be present when the Proanthocyanidin gel (PA+AEP group) was applied (around 10 am). Thus, the experimental phase initiated at 8 am with the intraoral device insertion in the oral cavity, which remained there for 2 hours for the AEP formation (Mendonça et al., 2017) and it finished at 6 pm, including 2-hour lunch break and brushing. Therefore, the volunteers used the intraoral device for a total of eight hours per day, which characterizes the intermittent use of the appliance (Santos et al., 2018). This kind of protocol results in a similar enamel loss compared to continuous use of the appliance, being a simplified reliable protocol appropriated for *in situ* erosion studies in enamel (Santos et al., 2018). Although the present study shows a less erosive cycle, there was an erosive challenge corresponding to 7.5 *in vivo* erosion days. This estimate can be performed using a simulation reported by Bartlett, Evans, Anggiansh & Smith et al. (1996). According to these authors, an erosive time about 4 minutes with HCl might correspond to one day of erosion on clinical situation, because the pH drops below 5.5 in 4.3 minutes during 24 hours, in gastro-esophageal reflux patients (Bartlett et al., 1996). When extrapolating this values to the present study, even knowing that citric acid is an extrinsic acid, the enamel was subjected to erosion for 30 minutes, which might simulate the *in vivo* erosion-day previously mentioned. Therefore, the adjustment in the intermittent use (from four to three de-remineralization

cycles) was important to ensure the AEP presence with protection ability at the first daily acid immersion, no exceeding the 8-hour-daily of the appliance use by the volunteers. It can also provide a less discomfort and consequently more acceptance by the ones (Santos et al., 2018), allowing an adequate use of the intraoral device, which implies directly in obtaining reliable results in any *in situ* study.

The time interval between each erosive challenge in this study was fixed in 2 hours, in order to allow the enamel partial remineralization by salivary action (Alencar et al., 2016) and to enable the AEP formation. In summary, this dynamic process starts with single peptides and proteins adsorption, following by salivary proteins that can interact with Ca^{2+} and PO_4 ions of the apatite surface. Finally, there are protein-protein interactions and adsorption of single proteins, protein agglomerates and other biomacromolecules (Hara & Zero, 2014). Moreover, AEP consists of two distinct layers: a basal layer formed by initial adsorbed proteins and globular layer composed of proteins aggregates, is deposited in a time- and site-dependent manner (Hara & Zero, 2014). The presence of this complex structure allows the enamel protection against erosive challenges compared to its absence, as reported by some studies (Hannig et al.,2004; Wiegand, Bliggenstorfer, Magalhães, Sener, & Attin, 2008; Moazzes et al., 2014). The results of the present study confirmed these findings, since AEP group showed a less enamel wear, statistically different compared to PA group ($p < 0.001$), which received the Proanthocyanidin gel treatment but did not present AEP formation. Proanthocyanidin was able to reduce enamel demineralization in artificial cariogenic challenge (Silva et al., 2015), but for erosion prevention, it was not effective in the acquired pellicle absence on initial erosion (Boteon et al., 2020). The present results confirm this finding, since PA group showed the greatest enamel loss. Thus, the AEP is essential for enamel erosion prevention because it acts as perm-selective membrane, reducing and retarding direct contact between acids and enamel surface (Hannig & Balz 1999). It is possible because AEP is not totally removed during acid attack, but gradually dissolved from its external to basal components (Hannig et al., 2007). Therefore, the AEP basal layer is not affected by the acids (Hannig, Berndt, Hoth-Hannig, & Hannig, 2009).

Considering the erosive cycling protocol used in the present study, in which the enamel blocks were carried out further 2 hours *in situ* after erosive challenge, there might be a protection of the eroded nanolacunae underneath the basal layer by proteinaceous structures (Hannig et al., 2009). These structures can represent a repair

process of superficial defects due to infiltration and adsorption of salivary proteins. Then, two possible protective mechanisms could benefit the AEP group in this study: a safety barrier formation to remineralization by these proteinaceous structures and the latter could also act as 'additional' pellicle reducing the enamel solubility, increasing the tooth protection (Hara & Zero, 2014).

For the PA+AEP group, in which Proanthocyanidin gel applied over AEP 2-hour formed *in situ*, the enamel loss has been significantly reduced compared to other studied groups, so the null hypothesis was rejected. Proanthocyanidin can interact with salivary proteins, such as proline-rich proteins (PRP) (Han et al., 2003; Hagerman & Butler 1981) and histatins (Hagerman & Butler, 1981) which are considered AEP precursor proteins by their high affinity for hydroxyapatite, forming the AEP basal layer (Jensen, Lamkin, & Oppenheim, 1992). These salivary proteins also maintain the Ca^{2+} and PO_4 ions saturation state in the oral fluids, by inhibiting their precipitation at neutral pH, releasing these ions after acid attack (Vukosavljevic, Custodio, Buzalaf, Hara, & Siqueira, 2014). Moreover, PRPs and histatins are present in greater numbers in the AEP from individuals who have gastroesophageal reflux disease but not present erosive tooth wear (Martini et al., 2019), which means that these proteins may be responsible in part for protection against wear (Martini et al., 2019). As Proanthocyanidin can also induce the aggregation and precipitation of the salivary proteins (Joiner et al., 2003; Ku et al., 2007; Castellan et al., 2010), it may have positively affected the PA+AEP group, enhancing this partial protection offered by PRPs and histatins against erosive wear as well as the AEP natural protective mechanisms (mentioned in the previous paragraph). The interaction among Proanthocyanidins and these salivary proteins occurred through strong hydrogen bonds (Hagerman & Butler, 1981) and the type of Proanthocyanidin can influence this kind of linkages. In this study, it was used Proanthocyanidin from grape seed extract (Purified Grape Seed Oligomeric Proanthocyanidins, 1298219, Sigma-Aldrich Co. ®, USA) which was characterized as B-type proanthocyanidin (Wu, Wang, & Simon, 2005). It presents a higher molecular weight compared to A-type (from Cranberry extract, for example) (Lazarus, Adamson, Hammerstone, & Schmitz, 1999), then it is more polar due to additional hydroxyl groups and hence potential for hydrogen bonding (Bedran-Russo et al., 2014).

Considering these findings, PA+AEP approach can be a promising alternative for preventing enamel wear by potentializing acquired enamel pellicle protective

properties. However, further studies are necessary before clinical application of the 6.5% Proanthocyanidin gel as protective agent for enamel erosion. As this natural agent can induce discolorations (Bedran-Russo et al., 2014), it is interesting to investigate the relevance of enamel discoloration, evaluating if it is permanent or temporary and the influence of the different application vehicles on it.

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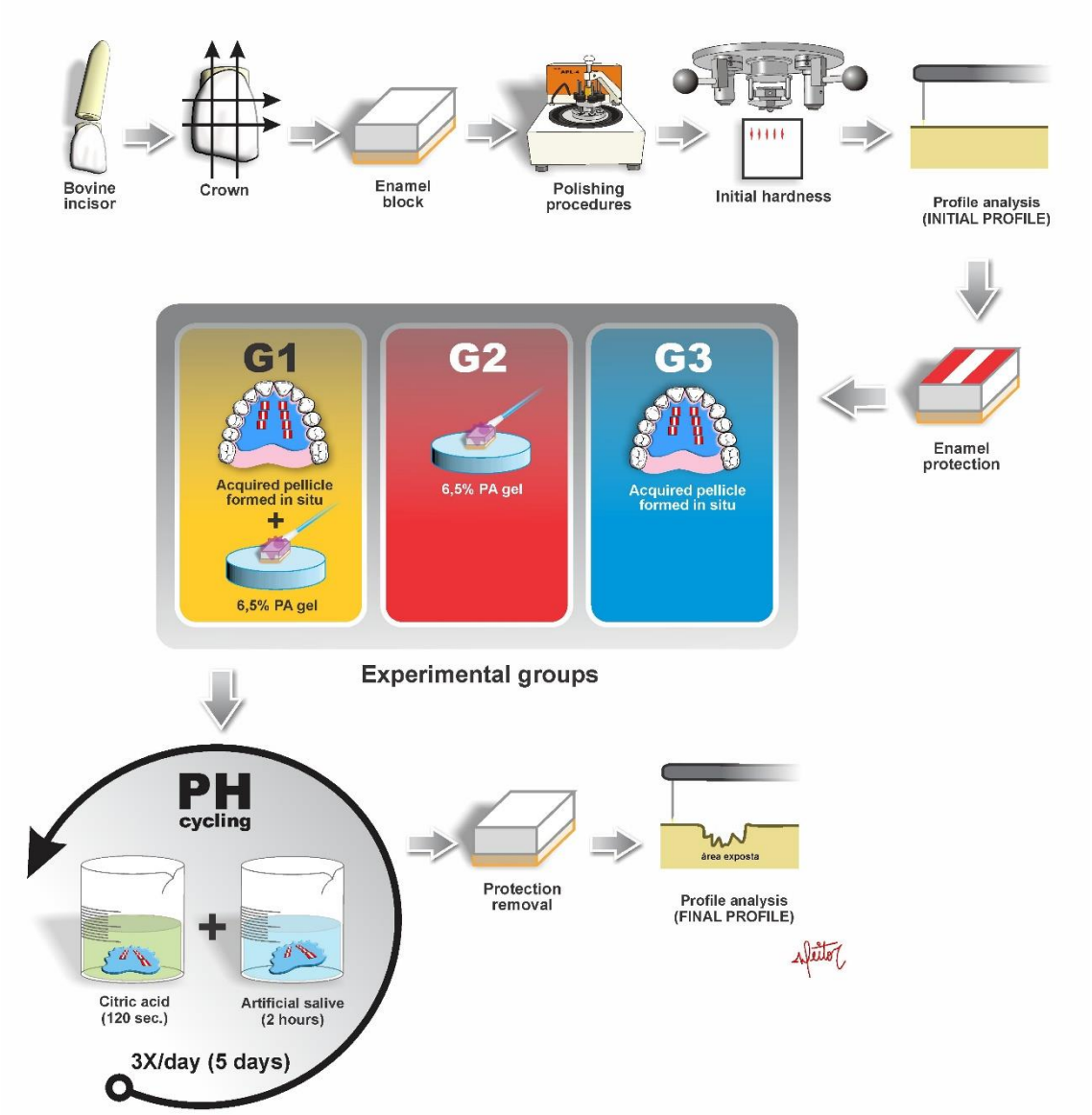
FIGURES


Bovine incisor
Crown
Enamel block
Polishing procedures
Initial hardness
Profile analysis (INITIAL PROFILE)
Enamel protection
G1
Acquired pellicle formed in situ
+
6,5% PA gel
G2
6,5% PA gel
G3
Acquired pellicle formed in situ
Experimental groups
PH cycling
Citric acid (120 sec.)
+
Artificial salive (2 hours)
3X/day (5 days)
Protection removal
área exposta
Profile analysis (FINAL PROFILE)

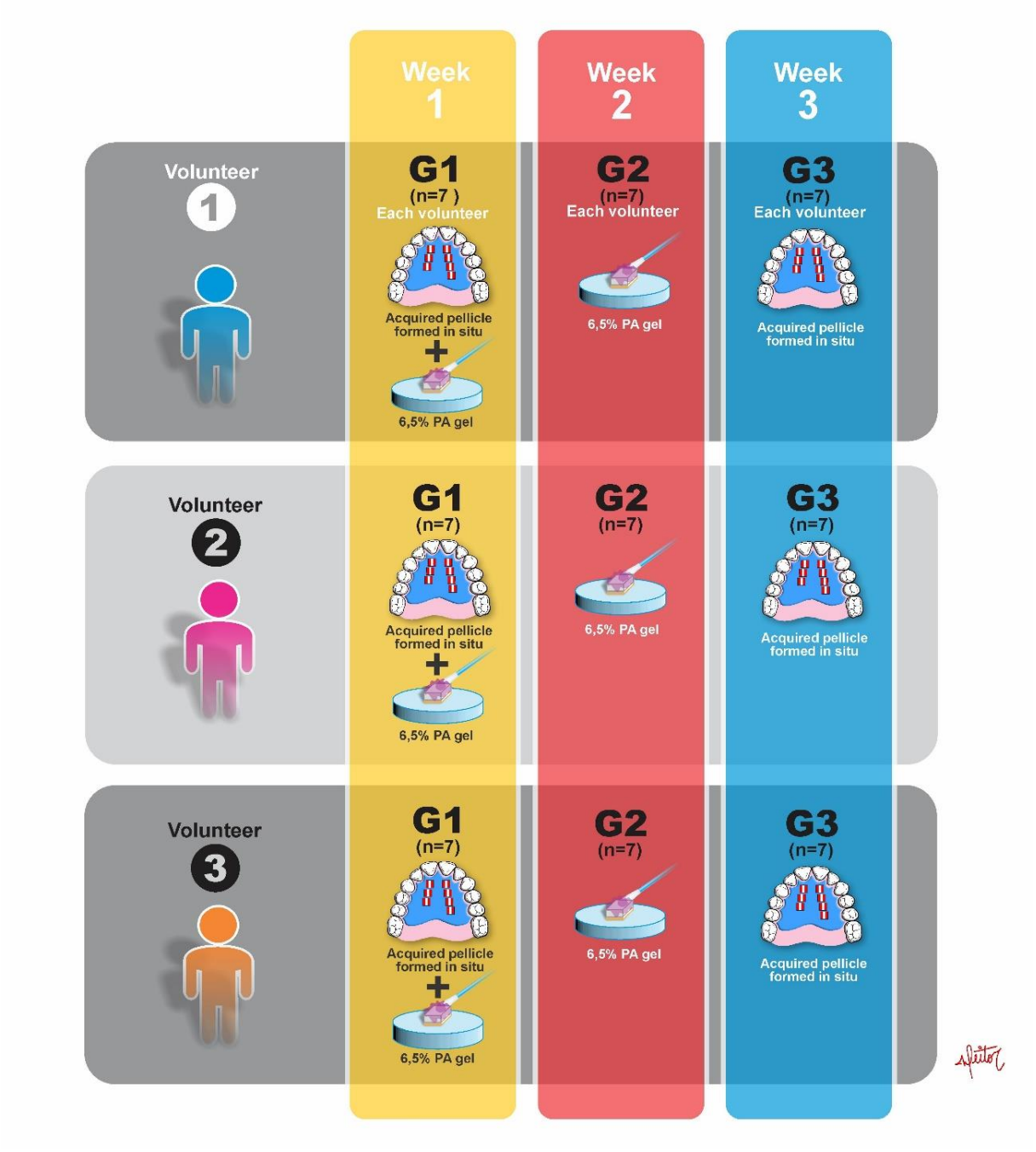


Figure 2- In situ phase protocol

3 DISCUSSION

3 DISCUSSION

The assessment of the protective ability of anti-erosion agents should ideally be carried out by *in vivo* studies[45]. However, this type of study requires long interventions, and it is not possible to measure the erosive tooth wear, nor to evaluate the erosion progression pattern[45]. Therefore, in order to overcome these complications, *in vitro* and *in situ* studies can be used. *In vitro* studies can be conducted in a short period, requiring low operational cost and they are not depending on the volunteers' collaboration[46]. On the other hand, they are not able to simulate the oral cavity conditions with the biological factors, which can interfere on dental erosion, such as saliva and acquired enamel pellicle[46]. Then, the model chosen for the article 2 could influence the results of Proanthocyanidin and NaF groups, since was not possible evaluate these agents in the oral conditions, in which there could be some interaction that would benefit the tested gels, especially for Proanthocyanidin.

Meanwhile, *in situ* studies allow a controlled erosive challenge and they can expose the samples to oral environment[45]. Additionally, *in situ* protocols can be used to simulate two different stages of dental erosion: when tooth surface is exposed to short-term erosive challenge, causing the processes called softening[47,48] and the advance stage of dental erosion, when occurs an irreversible tissue loss[49]. Therefore, this thesis includes the articles 1 and 3, in order to simulate these both stages, initial erosion and tooth wear, respectively. In fact, it should also be noted that the article 3 was designed to assess the Proanthocyanidin behavior on acquired pellicle presence or absence, hence the three groups (PA + AEP, PA and AEP). Articles 1 and 3 showed that the Proanthocyanidin may have positively affected the acquired enamel pellicle, confirming that the *in situ* protocol was the better type of study to evaluate the Proanthocyanidin's abilities on enamel erosion. This positive effect is probably due to it interaction between salivary proteins[12,30,44] which enhances the acquired enamel pellicle protective properties. It can also be noted that articles 1 and 3 confirmed the results obtained by article 2, since the application of Proanthocyanidin gel with no acquired pellicle did not prevent the enamel loss.

The erosive challenges carried out in the three articles of this thesis could simulate the extrinsic erosion, since it was used cola drink (article 2) and citric acid

(articles 1 e 3). In the article 1, as the samples were larger and numerous (90 enamel blocks embedded in acrylic resin which divided in 6 groups), it was necessary to adapt the solutions for the erosive cycling to make the study more practical and with a lower operational cost, since a larger volume of solution would be necessary (about 10 l for each, acid and artificial saliva). In the article 1 and 3, the volume of solution was lower due to the use of the *in situ* model (samples: 4x4x3 mm^2 enamel blocks), which was only necessary to prepare the acid solution, enabling the use of citric acid.

Erosive drinks as cola drink contain one or more types of weak acids in their composition, which are responsible for their low pH (cola drink = 2.2 – 2.6)[50] and high buffer capacity[4]. In addition, the concentrations of soluble calcium and phosphate are usually low, which makes them undersaturated in respect to tooth apatite, causing surface demineralization[5]. Thus, these factors can justify the use of cola drink as an acid solution in erosive cycling, especially when a greater volume of solution is needed such as article 1.

Citric acid is present in most erosive drinks (soft drinks and fruit juices) and it is considered a weak acid[51], however, as it can release or absorb H^+ ions, depending on acid or base addition, it can be resistant for pH changes[50]. Additionally, citric acid is more erosive than hydrochloric and phosphoric acids[52-55]. Considering that the phosphoric acid is the most important erosive ingredient in the cola drink used in the article 2[51], it is possible to explain the difference in the enamel wear pattern of the Proanthocyanidin gel on eroded enamel group from article 2 and G2 group from article 1 (1.60±0.27 µm and 2.49 ± 0.79, respectively). Both groups had no acquired pellicle formation and, in its absence, there is a greater calcium loss due to citric acid action than by phosphoric acid[55]. When comparing the phosphoric acid curve to the citric acid curve, in the common pH range, citric acid is more erosive than phosphoric at any pH [52]. The citric acid greater erosive potential may be related to its specific interaction with hydroxyapatite[53,56]. Different mono-, di- and tri-carboxylic acids are chemically absorbed and bonded to hydroxyapatite by ionic interactions, independent on the pH or acid concentration[56]. Another explanation would be the citric acid ability to form chelating complexes with calcium by citrate ions[57]. However, this property does not contribute to erosion progression at a typical erosion pH (around 3), since only a few citrate ions will be ionized in this lower pH range[50,58]. In a higher pH range (around 6),

93% of the citrate ions are ionized[58], but the erosion caused by the solutions at this pH is slow and clinically irrelevant[5,50,58].

Considering the findings of the three articles presented in this thesis, it was clear that the acquired enamel pellicle plays an important role on enamel erosion, as several studies reported[8,9,11,55,59-61]. As it was previously mentioned, when the acquired pellicle was not present such as article 2, G2 group from the article 1 and PA group from the article 3, the demineralization process was not properly restrained, resulting in enamel loss. Then, this thesis suggests that the Proanthocyanidin positively modified the acquired pellicle, since the groups G1 from article 1 and PA + AEP from article 3 exhibited less hardness loss and enamel wear, respectively. The fact that Proanthocyanidin can interact with salivary proteins[12,30,44] can explain these promising results. This interaction could favor the acquired enamel pellicle thickening, since Proanthocyanidin can induce precipitation and aggregation of salivary proteins (PRPs and histatins)[8,30], which increases the enamel protection against acids. However, as this natural agent can induce discolorations[31], it is interesting to investigate the relevance of enamel discoloration, evaluating if it is permanent or temporary and the influence of the different application vehicles on it. Furthermore, research on prolonged acid challenges and interaction between Proanthocyanidin and others salivary proteins (apart from PRPs and histatins) on erosive conditions may provide better evidence about the Proanthocyanidin's protective ability.

4 CONCLUSION

4 CONCLUSION

This thesis suggests that the aggressiveness of the acid challenge can influence the anti-erosion agents' protective ability. The possibility of previously eroded surfaces presenting a greater enamel wear has also not been discarded. Additionally, it seems that Proanthocyanidin can prevent enamel demineralization only when acquired enamel pellicle is present, then further studies are needed to elucidate the interaction between this natural agent and salivary proteins on erosive conditions, in order to know its actual protective abilities.

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APPENDIX

APPENDIX A – Declaration of exclusive use of the article in dissertation/thesis

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Departamento de Dentística, Endodontia e Materiais Odontológicos

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article "Proanthocyanidin protects the enamel against initial erosive challenge when applied over acquired pellicle" will be included in Thesis of the student Ana Paula Boteon 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, 22 de junho de 2020.

Ana Paula Boteon

Gabriela Guarda Dallavilla

Fabrícia Cardoso

Linda Wang

Daniela Rios

Heitor Marques Honório

ANNEX

ANNEX A - Article accepted for publication

Fwd: Boteon Galleys by email (Proanthocyanidin protects the enamel against initial erosive challenge when applied over acquired pellicle)

Ana Paula Boteon
para mim

Inglês > português Traduzir mensagem

---------- Forwarded message ---------
De: **Heitor Marques Honório** <heitorhonorio@uso.br>
Date: sex, 12 de jun de 2020 12:25
Subject: Re: Boteon Galleys by email (Proanthocyanidin protects the enamel against initial erosive challenge when applied over acquired pellicle)
To: American Journal of Dentistry <amjident@amjident.com>

Dear Katherine Godoy,

As requested, I attach the final files regarding the manuscript: "**Proanthocyanidin protects the enamel against initial erosive challenge when applied over acquired pellicle**".

Please, let me know if there is anything else that I can do.

Warm Regards,

Heitor

Em qui., 11 de jun. de 2020 às 14:07, American Journal of Dentistry <amjident@amjident.com> escreveu:

Dear Dr. Honorio,
Enclosed are:
1. The galleys of the manuscript entitled:

Proanthocyanidin protects the enamel against initial erosive challenge when applied over acquired pellicle

Ana Paula Boteon, DDS, Gabriela Guarda Dallabella, Fabrícia Cardoso, DDS, MS, Linda Wang, DDS, MS, PhD, Daniela Rios, DDS, MS, PhD & Heitor Marques Honório, DDS, MS, PhD

2. The Author Consent Form for the edited version.
3. The Reprint Order Form.
4. The Author Information Form.

Please answer all questions and make any changes in red text and return the manuscript by e-mail within 5 days of receipt.
The Author Consent Form, the Reprint Order Form, and the Author Information Form must all be completed and returned also by email.
Best regards,
Katherine
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