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ABSTRACT

The acquired dental pellicle helps prevent erosion, but the protection level is unknown. This *in situ* study tested whether a two-hour pellicle protects against different erosive challenges by orange juice. Subjects wore palatal appliances loaded with either enamel or dentin specimens. Pellicle was allowed to form, or not (control), on the surfaces of the specimens intra-orally for 2 hrs before the erosive challenges of 0 (control), 10, 20, and 30 minutes' duration. Specimens were randomly removed from the appliances after each challenge. Percentage of surface microhardness change (%SMC) was determined for the enamel specimens, and that of mineral loss and lesion depth for the dentin specimens. Enamel specimens with the pellicle showed a significantly lower %SMC, only after the 10-minute challenge. No protection was found for dentin. It was concluded that the acquired pellicle reduced dental erosion, but that this effect was limited to the less severe erosive challenge on enamel surfaces.

KEY WORDS: erosion, dental pellicle, enamel, dentin.

Protective Effect of the Dental Pellicle against Erosive Challenges *in situ*

INTRODUCTION

Studies have suggested that the high intake of acidic beverages may be related to the occurrence of pathological tooth wear (Millward *et al.*, 1994; Al-Dlaigan *et al.*, 2001; Johansson *et al.*, 2002), usually described in dentistry as dental erosion (Imfeld, 1996). However, some other studies have failed to show this effect clearly (Mathew *et al.*, 2002; van Rijkom *et al.*, 2002). Although the erosive potential of some beverages may be suggested by their acidic nature (Lussi *et al.*, 1993; Larsen and Nyvad, 1999), their actual influence in the erosion process is still unknown, since it can be modified by chemical, biological, and behavioral factors (Lussi *et al.*, 2004). Some of these factors, such as salivary, may potentially inhibit or reduce the occurrence of tooth erosion.

Saliva has a buffering, diluting, and remineralizing capacity (Sreebny, 2000), and also allows for the acquisition of a salivary pellicle on tooth surfaces (Lendenmann *et al.*, 2000). This protein-based pellicle may behave as a diffusion barrier or a perm-selective membrane, preventing direct contact between acids and the tooth surface (Hannig, 1999; Sonju-Clasen *et al.*, 2000), and thus inhibiting its demineralization (Amaechi *et al.*, 1999; Hannig and Balz, 2001). However, the efficacy of this protection under clinical conditions is still unknown, especially in the context of variations in the severity of the erosive attack.

The intent of this *in situ* study was to determine the protective effect of a two-hour salivary pellicle formed on bovine enamel and dentin surfaces against erosive challenges of different levels of severity.

MATERIALS & METHODS

Ethical Aspects

The study protocol was reviewed and approved by the local ethics committee in research (IUPUI Institutional Review Board, process #0310-61). Twelve adult volunteers (four male and eight female), with an average age (SD) of 43.2 (12.3) years, took part in this study after signing an informed, written consent form. They had unstimulated and stimulated (by chewing 3 2.5-cm² sheets of Parafilm M®; SPI Supplies, West Chester, PA, USA) salivary flow rates (SD) of 0.59 (0.24) and 1.69 (0.58) mL/min, respectively, and no evidence of dental erosion, active caries, or periodontal disease.

Experimental Design

This study consisted of 2 independent experiments, one for enamel and one for dentin. Each experiment consisted of a factorial 2 x 4, carried out in 2 independent and blind phases, according to the cross-over design (Fig. 1). The factors under evaluation on each experiment were salivary exposure (at 2 levels: A - with and B - without) and acid challenge (at 4 levels: 0 [control], 10 min, 20 min, and 30 min), resulting in 8 experimental groups. Each group had 24 bovine dental tissue slabs in duplicates or 12 experimental units (n = 12) either of enamel or of root dentin. They were randomly assigned to the 12 participants,

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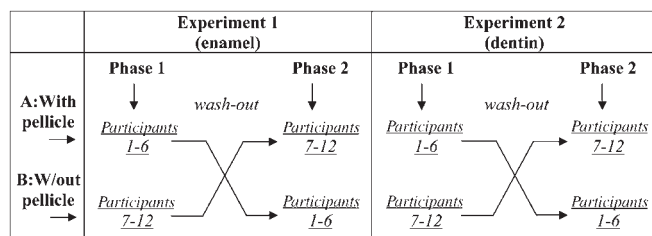


Figure 1. Experimental design.

who were considered as experimental 'blocks'. In each phase, participants were randomly assigned to the levels A or B of the salivary exposure factor. In A, participants started wearing the appliances 2 hrs before the erosive challenges, allowing pellicle to form. In B, the erosive challenges started immediately after the appliance insertion into the mouth. Specimens were removed from the device according to the acid challenges. The percentage of surface microhardness change was calculated for enamel, and the mineral loss and lesion depth were evaluated for dentin. The null hypothesis tested was that previous saliva exposure could not prevent both enamel and dentin demineralization against acid challenges of different levels of severity.

Specimen Preparation

Bovine permanent incisors were extracted from cattle free of bovine spongiform encephalopathy disease and stored in thymol at 4°C. Enamel slabs (5 x 5 mm) were cut from the middle third of the labial coronal surfaces. Dentin slabs (5 x 5 mm) were cut from the cervical third of the root. Initially, the pulpal surfaces of the slabs were flattened in a grinding machine (RotoPol31/RotoForce4 polishing unit; Struers, Cleveland, OH, USA) with #500-, 1200-, and 4000-grit grinding papers (MD-Fuga, Struers). Next, the external/experimental surface was flattened and polished (1- μ m diamond suspension, Struers), resulting in slabs 2 mm thick, which were cleaned ultrasonically in detergent solution (Buehler, Lake Bluff, IL, USA) for 1 min. Specimens with any cracks or scratches were discarded. Those remaining were mounted on acrylic blocks with sticky wax and had their baseline values of surface microhardness (KHNb) determined by the average of 3 indentations—100 μ m apart from each other—made at the center of the specimen, with the use of a Knoop indenter, a load of 10 g, and a dwell time of 5 sec (2100 HT, Wilson Instruments, Norwood, MA, USA). We selected 192 enamel and 192 dentin slabs with average KHNb of 355.5 (14.4) and 47.4 (4.3), respectively, and sterilized them by exposure to ethylene oxide gas for 1 hr, in moist conditions, at 55°C (8XL sterilizer/aerator, 3M Health Care, St. Paul, MN, USA). After aeration for 12 hrs, in the same moist and temperature conditions, specimens were soaked 3 times in de-ionized water, for 15 min each time.

Palatal Device Preparation

Acrylic custom-made palatal devices were made with 2 slots to accommodate 2 plastic holders (5 mm wide x 28 mm long x 2.3 mm high), containing 4 specimens each (Fig. 2). The holders, made of polyetherimide (Ultem®, GE Plastics, Pittsfield, MA, USA), had 4 recesses that kept a 1-mm uniform gap between them and the polished surface of the specimen. This space was designed to allow for the free contact of saliva and acidic beverage with the specimen's surface, and also to protect it from mechanical disturbance (Fig. 2). Prior to the beginning of each experiment, participants had a palatal appliance try-in

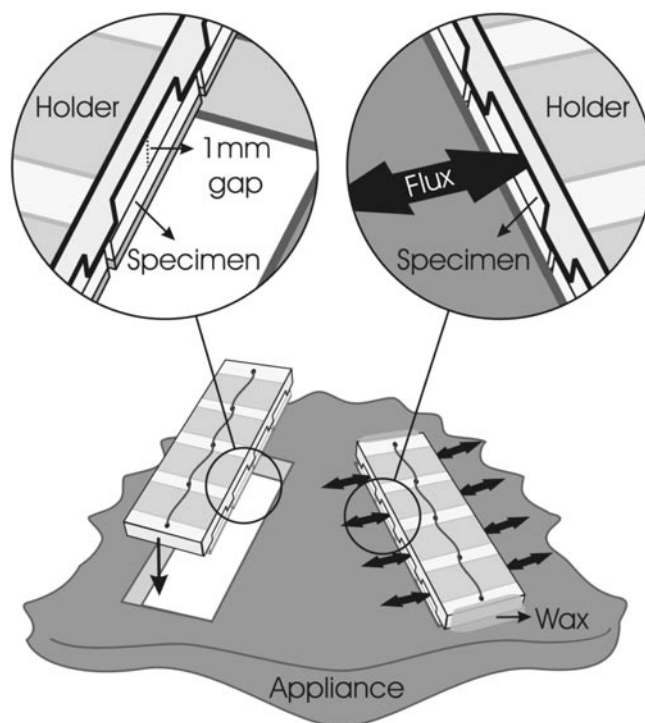


Figure 2. Schematic view of the palatal appliance loaded with the holders.

appointment, when needed adjustments were made.

Intra-oral Phases

Each experiment consisted of 2 phases of 1 day each, with a three-day wash-out period between them. During the experimental periods, participants were instructed to brush their teeth with a silica-based dentifrice (Crest Regular, Procter & Gamble Co., Cincinnati, OH, USA), containing sodium fluoride (0.15% w/v fluoride ion). In phase 1, participants assigned to Group A started wearing the appliances 2 hrs before the erosive challenges, to allow for pellicle formation. No pellicle formation time was allowed for participants of Group B (Fig. 1). Control specimens (0 min) were removed from the appliances before the erosive challenges. Participants were instructed to sip 10 mL of freshly prepared orange juice (pH 3.8, room temperature; Minute Maid Premium Original, 100% pure, no preservatives, lot #AD32329, Coca-Cola Co., Atlanta, GA, USA), hold it for 15 sec against the appliance with the aid of their tongue, expectorate, wait for 15 sec, and repeat the procedures continuously for 40 x (total of 10 min of demineralization and 400 mL of juice). Specimens in the ten-minute group were then removed from the appliances. Specimens in the 20- and 30-minute groups were removed after 10 and 20 additional min (800 and 1200 mL) of exposure to the erosive challenge, respectively. All specimens were individually stored in plastic vials, in moist conditions, at 4°C. Participants assigned to A in phase 1 were assigned to B in phase 2, and vice versa (Fig. 1).

Enamel Analysis

Enamel specimens were re-mounted on acrylic blocks, and the final values of surface microhardness (KHNf) were determined by the average of 5 indentations made 100 μ m apart from the baseline indentations, following the equipment settings previously described. The percentage of surface microhardness change

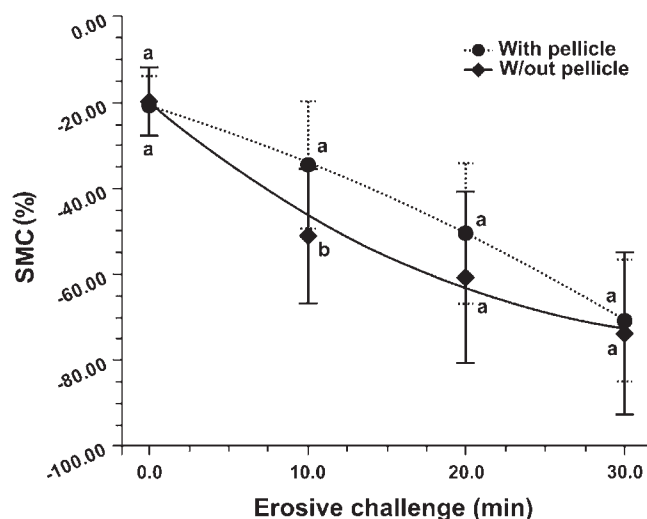


Figure 3. Data of enamel microhardness change. Values adjusted to quadratic fits (with pellicle— $p < 0.05$, $r^2 = 0.66$; w/out pellicle— $p < 0.05$, $r^2 = 0.61$) toward the erosive challenges. Average (SD) values of groups: (with pellicle) 0, -20.92 (7.15); 10, -34.68 (15.14); 20, -50.48 (16.44); 30, -70.94 (14.27); (w/out pellicle) 0, -19.90 (8.13); 10, -49.12 (15.66); 20, -60.51 (20.18); 30, -73.56 (19.06) ($n = 12$). Different letters at each erosive challenge mean statistical difference ($p < 0.05$).

(%SMC) was then calculated, according to the equation: %SMC = (KHNf - KHNb)/KHNb. Average values of the percentage of surface microhardness change of duplicates were obtained and considered as the response variable in the statistical analysis.

Dentin Analysis

Sections ($180 \pm 20 \mu\text{m}$) were cut from dentin specimens and x-rayed together with an aluminum stepwedge (12 steps: 20–240 μm thick), at 20 kV and 30 mA for 65 min. The focus length was set at 42 cm. Microradiographic plates were processed, and images were acquired from the microscope (EOM, Carl Zeiss Inc., Oberkochen, Germany) to the computer with a camera (KP-120U, Hitachi Denshi Ltd, Tokyo, Japan). The images were analyzed with dedicated computer software (TMR 1.26, Inspektor Research Systems BV, Amsterdam, The Netherlands). We obtained mineral loss information by computing the area obtained by plotting the vol% mineral profile toward dentin depth in each dentin section, with the sound dentin set as 48 vol% mineral. Lesion depth was defined as the distance from the surface to the site where mineral content was more than 95% of the sound dentin.

Statistical Analysis

We analyzed the response percentages of surface microhardness change on enamel and mineral loss and lesion depth on dentin to check the presence of carry-over and phase effects, and checked the assumptions of homogeneities of variance and normal distribution. Analyses of variance (ANOVA), followed by Tukey's test and regression analyses, were performed (SAS 6.11, SAS Institute Inc., Cary, NC, USA), at a significance level of 5%.

RESULTS

Neither the carry-over (t test, $p < 0.05$) nor the phase (t test, $p < 0.05$) effects were observed for all responses. Data showed normal distribution ($p < 0.05$) and homogeneity of variance (p

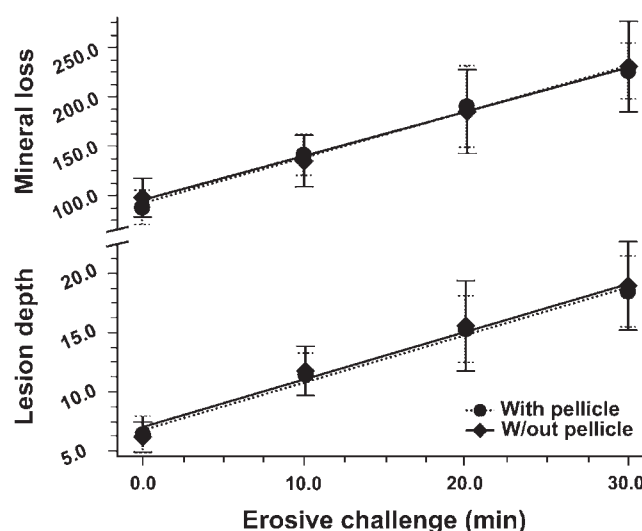


Figure 4. Dentin analysis. **(top)** Data of mineral loss (vol% mineral.μm) in dentin. Values adjusted to linear fits (with pellicle— $p < 0.05$, $r^2 = 0.77$; w/out pellicle— $p < 0.05$, $r^2 = 0.67$) toward the erosive challenges. Average (SD) values of groups: (with pellicle) 0, 88.39 (18.00); 10, 142.00 (20.87); 20, 190.68 (42.33); 30, 226.95 (29.31); (w/out pellicle) 0, 98.62 (20.08); 10, 135.86 (27.03); 20, 185.40 (43.33); 30, 231.09 (46.53) ($n = 12$). **(bottom)** Data on lesion depth (μm) in dentin. Values adjusted to linear fits (with pellicle— $p < 0.05$, $r^2 = 0.78$; w/out pellicle— $p < 0.05$, $r^2 = 0.71$) toward the erosive challenges. Average (SD) values of groups: (with pellicle) 0, 5.95 (1.58); 10, 11.02 (1.86); 20, 14.80 (2.85); 30, 18.02 (3.07); (w/out pellicle) 0, 6.18 (1.33); 10, 11.29 (2.15); 20, 15.20 (3.84); 30, 18.55 (3.77) ($n = 12$).

< 0.05). In Experiment 1, the ANOVA showed a significant interaction between the factors salivary exposure \times acid challenge ($p = 0.0439$). Within the factor acid challenge, the Tukey's tests showed statistical differences between the percentages of surface microhardness change of groups with and without salivary exposure, only at 10 min of acid challenge (Fig. 3). Regression analyses showed quadratic behavior of the percentages of surface microhardness change values toward the increase of acid challenge (Fig. 3). In Experiment 2, the ANOVA showed no significant interactions of the factors for both mineral loss and lesion depth ($p = 0.996$ and 0.621 , respectively), and no significant differences between levels of the saliva exposure factor ($p = 0.4671$ and 0.8852 , respectively). Regression analyses showed linear behavior for both mineral loss and lesion depth values throughout the acid challenges (Fig. 4).

The null hypothesis was rejected for the enamel substrate (Experiment 1) only for the less severe acid challenge (10 min), and it was accepted for dentin for all challenges tested (Experiment 2).

DISCUSSION

The current experimental model was designed for the clinical reproduction of the early stages of dental erosion, which involves the chemical corrosion of the substrate and consequent surface-softening (Mahoney *et al.*, 2003), with no evidence of surface loss. That circumstance allowed us to analyze the protective effect of the acquired salivary pellicle on enamel and dentin against demineralization. Bovine substrates were chosen

because they are easy to obtain and to prepare, and also because they have been accepted as representative of human teeth for the study of demineralization (Mellberg, 1992; Zero, 1995; Hara *et al.*, 2003). The main biological and chemical conditions related to dental erosion were reproduced in this *in situ* model, with natural formation of salivary pellicle, and the presence of physiologically secreted saliva during the orange-juice-induced erosive challenge. The choice for the two-hour pellicle was made based on the equilibrium between protein adsorption and de-sorption being reached within 2 hrs (Lendenmann *et al.*, 2000). Also, a two-hour pellicle has already been proven to be acid-resistant (Meurman and Frank, 1991; Sonju-Clasen *et al.*, 2000). Behavioral conditions—such as the frequency of intake, time of interaction between the beverage and the specimen, and time between intakes—were controlled to allow for the study of different severities of erosive challenge.

Results of Experiment 1 showed that a two-hour acquired pellicle formed on the enamel surface has the potential to protect it from demineralization, as previously reported (Amaechi *et al.*, 1999; Hannig and Balz, 2001; Hannig *et al.*, 2004). However, the protection was limited to the less severe erosive challenge and could not entirely prevent enamel from demineralization. Considering the semi-permeable nature of the pellicle (Lendenmann *et al.*, 2000; Hannig and Balz, 2001), it could be suggested that more than 10 min of demineralization challenge may cause the proteins adsorbed to the enamel surface to be washed away, together with the dissolved mineral (Bennick *et al.*, 1983). It can also be hypothesized that, at low pH, the strong bond between the anionic proteins of the acquired pellicle and the enamel surface is weakened, since the pKa of the acidic amino acids is around 4.0. Those acidic proteins have a high affinity to hydroxyapatite and are thought to drive the adsorption of the pellicle to enamel due to electrostatic interactions (Hannig, 1999). Considering that there was no feasible time to allow for pellicle re-growth during the experiment, no further protection could be expected for enamel with the most severe challenges.

The lack of protection found for dentin substrate in Experiment 2 might confirm the first suggestion cited above. Since dentin is more soluble and porous than enamel, it could be demineralized relatively faster, preventing the pellicle from acting as a protective barrier, even against the initial 10 min of acid exposure. However, direct comparisons of results obtained in Experiment 1 with those in Experiment 2 should be made with caution. Differences in the substrates' nature and characteristics may affect the pellicle composition and properties (Glantz *et al.*, 1996). Additionally, the experiments were conducted independently from each other, and different methods were used to quantify the substrate demineralization. Although the analysis of the percentage of surface microhardness change was initially considered for the analysis of both substrates, because it is recognized as a very sensitive method for detecting initial stages of demineralization (Featherstone, 1996), the deep demineralization found on dentin surfaces made necessary the adoption of a transversal method of analysis, which was elected to be transversal microradiography.

Analysis of the data from Experiment 1 reinforced the importance of the pellicle and showed the influence of behavioral factors on its protective capability. The decrease of the protective effect of the salivary pellicle toward the increase of the acidic beverage challenge suggests a dependent

relationship between erosion prevention and aggressiveness of the acid attack, which were regulated by the frequency and time of acid exposure as well as by the total volume of acid beverage intake. It could be suggested that greater protection to enamel and some protection to dentin might be found if pellicle formation times longer than 2 hrs were adopted (Lendenmann *et al.*, 2000). The maturation process may enhance the acid-resistance of the pellicle, due to structural remodeling by enzymes (Yao *et al.*, 2001; Hannig *et al.*, 2005).

One can speculate about the clinical significance of the results, considering that the intake of 400 mL of orange juice with 10 min of acid exposure and 20 min of total challenge may represent the ingestion of a regular cup of beverage in real conditions. The repetition of this protocol, for 2 and 3 times consecutively, mimicking the consumptions of 2 and 3 cups of acid beverage, and not allowing either pellicle re-organization or remineralization to occur, is thought to be representative of subjects at high risk of erosion. No pellicle interference in the development of dental erosion should be expected in such situations. Since saliva exposure has not been shown to be capable of remineralizing eroded enamel and dentin entirely, even after 24 hrs and in the presence of fluoride (Fushida and Cury, unpublished observations), and since fluoride has not been conclusively proven to prevent erosion (Larsen and Richards, 2002), high-risk behaviors should be avoided. Additional factors influencing the severity of the erosive challenge—such as pH, titratable acidity, and calcium complex capacity of the erosive drink—may potentially affect the protection of the pellicle against erosion and should be further investigated.

Based on the results of the current study, it can be concluded that the two-hour salivary pellicle partially prevented dental erosion by an acid beverage. However, this effect was restricted to the enamel substrate and to the less aggressive challenge.

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