Reduction of Erosion by Protein-Containing Toothpastes

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Key Words

Dental erosion • Casein • Colostrum • Enamel • Pellicle • Toothpaste

Abstract

Aim: To assess the effect of protein-containing toothpastes on the progression of dental erosion in situ (with pellicle) and in vitro (without pellicle). **Methods:** A combined split-mouth (extraoral water or toothpaste brushing) and crossover (type of toothpaste) setup was used. Two protein-containing (high/low concentrations of colostrum) and one nonprotein (placebo) toothpaste were investigated. Sixteen volunteers wore intraoral appliances containing 2 human enamel samples on 3 afternoons for pellicle growth during 90 min. One enamel sample was brushed for 5 s with one of the three toothpastes and subsequently exposed to a slurry of the corresponding toothpaste for 2 min. The other sample was exposed to water. Both samples were subsequently exposed to citric acid (extraorally). Loss of calcium and inorganic phosphate were determined. The same sequence of exposures was applied to 16 enamel samples in an in vitro setup without pellicle. **Results:** With the in situ-formed pellicle, all toothpastes significantly reduced calcium loss compared to water brushing, although no significant differences were

found among toothpastes ($p = 0.073$). For the loss of phosphate, a significant reduction could be found with the use of the high-protein toothpaste compared to the nonprotein toothpaste. Overall there were only slight differences between the toothpastes. Toothpaste effects were less clear in the in vitro experiment. **Conclusion:** The addition of proteins to toothpaste shows some promise for the prevention of erosion. Further research is needed to investigate the performance of the protein-containing toothpastes in longer in situ studies with regard to erosive wear.

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Dental erosion is a growing problem in the Netherlands [El Aidi et al., 2008]. Excessive loss of dental hard tissue due to erosion can result in aesthetic and functional problems [Jaeggi et al., 2006; Vailati and Belser, 2010]. Therefore it is rational, as well as using other preventive measures, to develop oral products that influence the progression of dental erosion. Because of their widespread daily use, toothpastes could be an ideal mode by which protection against dental erosion could be provided. A number of studies have been performed investigating different toothpaste modifications [Newby et al., 2006; Hooper et al., 2007; Rees et al., 2007; Lussi et al.,

2008; Kato et al., 2010]. Examples of these modifications are higher fluoride concentrations and the exclusion of sodium lauryl sulfate (SLS) from the toothpaste. SLS can remove the pellicle and a smear layer present on dentin [Moore and Addy, 2005]. Toothpaste formulations without SLS could be favorable in preventing erosion.

In an *in vitro* study investigating the effect on erosion of toothpastes that claimed to prevent erosion, no significant differences between the toothpastes were found. However, an increase of hardness of enamel after exposure to those toothpastes was found compared to conventional toothpastes [Lussi et al., 2008].

The pellicle is a protein layer present on enamel and has been suggested to be protective against acids by forming a barrier to H^+ ions, thereby preventing the dissolution of hydroxyapatite. The proteins can also act as a buffer by binding H^+ ions and the pellicle can act as a permselective barrier, retarding the movement of positively charged ions such as Ca^{2+} and restricting the approach of H^+ ions [Zahradnik et al., 1976; White et al., 2010]. Therefore, another modification of toothpastes aimed at reducing the loss of enamel could be the addition of proteins to toothpaste, such as are present in colostrum. This has been recently confirmed in the case of casein, one of the components of colostrum, in an *in vitro* study investigating the erosion-inhibiting effect on enamel of the casein protein with and without fluoride compared to a water and fluoride solution [White et al., 2010]. This erosion-inhibiting effect of casein is ascribed to the adsorption of casein on to the hydroxyapatite surface, thus stabilizing the crystal surface and inhibiting ion detachment [Barbour et al., 2008]. In our study we hypothesized that the addition of colostrum proteins to toothpaste would reduce dental erosion. Therefore, the focus of this study was to assess *in situ* the effect of protein-containing toothpastes (different concentrations of proteins) on dental erosion compared to a negative control (brushing with water) and to compare this to a nonprotein (placebo) toothpaste.

Materials and Methods

Three toothpaste formulations were investigated. All the pastes did not contain SLS. The toothpastes were coded as follows:

P-: Zendium Acid Defence without proteins (Sara Lee Household and Bodycare BV, Amersfoort, The Netherlands) (free Ca^{2+} 0.026 mg/g, free inorganic phosphate (Pi) 3 mg/g, 1,450 ppm NaF, pH 6.0 ± 0.1); a specially prepared placebo toothpaste; not commercially available.

P+: Zendium Acid Defence; a commercially available toothpaste with 0.21% w/w protein. This paste contains amyloglucosi-

dase, glucose oxidase, lactoperoxidase, lysozyme, lactoferrin, IgG and casein (free Ca^{2+} 0.029 mg/g, free Pi 3 mg/g, 1,450 ppm NaF, pH 6.0 ± 0.1).

P++: Experimental Zendium Acid Defence: contains the same proteins as toothpaste P+ in higher concentrations (0.57% w/w protein, i.e. 2.7 times more proteins compared to the P+ paste) (free Ca^{2+} 0.041 mg/g, free Pi 3 mg/g, 1,450 ppm NaF, pH 6.0 ± 0.1).

Sample Preparation

Enamel samples were prepared from the buccal surface of human molars and embedded in acrylic resin (De Trey, Self-Cure Acrylic; UK) using a mold that produced blocks of $5 \times 9 \times 3$ mm with an oblique side that was used for the retention of the blocks in the appliance. The human enamel samples (all from impacted 3rd molars) were collected with informed consent at clinics for maxillofacial surgery in the region of Groningen, The Netherlands. The human enamel samples were stored under humid conditions (saline solution). Subsequently, the embedded enamel samples were ground flat on a rotating polishing machine (Phoenix Beta grinder/polisher; Buehler, Germany) under water cooling using SiC grinding paper (P1200; Struers, Copenhagen, Denmark). Sterilization of the samples was performed with ethylene oxide according to the protocol of the Department of Microbiology of the University Medical Center Groningen.

Volunteers

Ethical approval was granted by the Institutional Review Board of the University Medical Center Groningen (UMCG IRB No. 2008/2810). Only participants with a healthy oral environment (i.e. a Dutch Periodontal Screening Index [van der Velden, 2009] score of 1 or lower, no caries activity and no hyposalivation) and with no relevant medical or pharmacotherapy history (American Society of Anesthesiologists score 1 [Owens et al., 1978]) were allowed to participate. All volunteers received verbal and written information concerning the study and gave written consent to participate. Sixteen healthy volunteers (8 females, 8 males) with a mean age of 25 ± 5 years participated. On experience from a pilot study, it was estimated that 11 volunteers would be needed to provide a power of 0.8 [Dupont and Plummer, 1990]. Because of possible dropouts a sample size of 16 volunteers was chosen.

Study Design for the *in situ* Study

The trial was a single-center, double-blind split-mouth (extra-oral water or toothpaste brushing), crossover (type of toothpaste) design. All volunteers wore intraoral appliances containing 2 human enamel samples in the palatal region during 3 afternoons. The appliance was worn from 13.30 to 16.00; each sample had an intraoral time of 90 min. One hour prior to placement and whilst the devices were in place, eating and drinking were not allowed. To prevent cross-contamination with toothpaste remnants, the sample on the right side (water brushed only) was placed in the appliance 30 min earlier than the sample on the left side. After 60 min in the oral cavity this sample was extraorally brushed with water and incubated for 2 min in water, thoroughly rinsed for 30 s with running tap water and returned to the oral cavity for another 30 min. Then both samples were removed; the water-brushed sample was permanently removed for acid exposure, and the toothpaste sample was removed for brushing with one of the toothpastes.

Fig. 1. In situ (pellicle): boxplots showing the reduction of loss of calcium and phosphate after the use of the three toothpastes compared to water. The data for the boxplots are obtained by deducting the loss of calcium/phosphate measured after exposure/brushing with toothpaste from the loss of calcium/phosphate after exposure/brushing with water. Furthermore, the three pastes are mutually compared. * Significant differences between groups.

Brushing was performed by the investigator for 5 s with an electric toothbrush with the toothpaste (Oral B Professional-care 7500 DLX; Braun, Kronberg, Germany) and the sample was subsequently incubated for 2 min in a slurry (1:2 weight ratio toothpaste/water) of the corresponding toothpaste on a shaking table (100 rpm). It was decided to first brush the samples with the pastes for 5 s and then expose the samples for 2 min to the toothpaste/water slurry to mimic the clinical situation. In the clinical situation every tooth is brushed for approximately 5 sec, which results in a combined exposure of the teeth to the toothpaste for about 2 min. This approach resulted in a condition whereby first the pellicle was disturbed by brushing and thereafter proteins present in the toothpaste were introduced into the pellicle. Furthermore, the hand-held electric toothbrush was used as recommended by the manufacturer. No special device was used to control the pressure.

After incubation, the samples were rinsed in the same fashion as the water-brushed samples and replaced in the oral cavity for 30 min. The toothpaste used on a particular test day was randomly chosen for every volunteer so that no order effect could influence the results. The toothpastes were provided by the manufacturer in unmarked containers, coded A–C, to ensure blinding of both the subjects and investigator. The volunteers also used the corresponding toothpaste at home during the week when one of the pastes was tested.

Study Design for the in vitro Control Study

The same procedure as described above was also performed extraorally on enamel samples that were not exposed to the oral cavity, thus without the presence of saliva or pellicle. For this, 16 enamel samples were randomly brushed with toothpastes or water and exposed to the toothpaste slurry or water as described above and subsequently rinsed with water and exposed to citric acid. Instead of placement in the oral cavity, these samples were placed in deionized water (22°C) for the same time period. In between treatments the samples were briefly polished and cleaned to remove the eroded surface layer in order to prevent an influence of the first regime on the next regime with another toothpaste.

Acidic Challenge and Loss of Enamel Measurements

After completion of the in situ/in vitro brushing regime, the samples were exposed under agitation on a shaking table

(100 rpm) to 2 ml citric acid (5 min, 0.05 M, pH 2.3). Calcium and Pi concentrations in the solutions were determined as a measure for loss of enamel. Phosphate concentration is measured by a phosphomolybdate spectrophotometric method as described by Chen et al. [1956]. Lesion depth was calculated from the phosphate loss using the average phosphate content per unit volume for human enamel and the exposed enamel area [Dijkman et al., 1983]. A phosphate concentration of 17.61% in the enamel and an average enamel density of 2.93 g/cm³ were assumed. The calcium concentration was determined by atomic absorption spectroscopy as described in a previous publication [Jäger et al., 2008]. All the samples were digitally photographed and the exposed enamel area was calculated using the software Image J (National Institutes of Health, Bethesda, Mass., USA) on the basis of the number of pixels. The loss of calcium and Pi was expressed in mmol/mm².

Statistical Analysis

The effect of the toothpaste compared to water brushing on one person (in situ) or one sample (in vitro) and the differences between the three toothpastes were analyzed using paired t tests. The above-mentioned analyses were all performed using SPSS software (SPSS 16.0, SPSS Inc., Chicago, Ill., USA). The significance level for all statistical tests was set at $p = 0.05$.

Results

In situ (Pellicle)

Toothpaste P+ was accompanied with the lowest loss of calcium (1.79 mmol/cm², water 2.29 mmol/cm², $p < 0.001$), followed by P- (1.83 mmol/cm², water 2.28 mmol/cm², $p < 0.001$) and P++ (1.85 mmol/cm², water 2.50 mmol/cm², $p < 0.001$). Toothpaste P++ showed the lowest loss of phosphate (0.85 mmol/cm², water 1.94 mmol/cm², $p = 0.025$), followed by P- (1.04 mmol/cm², water 1.46 mmol/cm², $p > 0.05$) and P+ (1.38 mmol/cm², water 2.12 mmol/cm², $p > 0.05$). Figure 1 shows the results of the (paired) comparison of the toothpaste results to the water

Fig. 2. In vitro (no pellicle): boxplots showing the reduction of loss of calcium and phosphate after the use of the three toothpastes compared to water. The data for the boxplots are obtained by deducting the loss of calcium/phosphate measured after exposure/brushing with toothpaste from the loss of calcium/phosphate after exposure/brushing with water. Furthermore, the three pastes are mutually compared. * Significant differences between groups.

results. It can be seen that all three toothpastes generally showed a reduction of erosion compared to water, the effect being significant for calcium loss for all three pastes and for P++ where phosphate loss was concerned. It can also be seen that the phosphate measurements are more variable, leading to lack of power in the analysis.

When reduction of erosion compared to water brushing of the three pastes was mutually compared, a dose-response trend could be observed for both calcium and phosphate measurements (fig. 1). Only P++ showed significantly lower phosphate losses compared to P-. For the loss of calcium the corresponding difference approached significance ($p = 0.073$).

In vitro (No Pellicle)

The results with the in situ-formed pellicle were not quite mirrored in the in vitro experiment without pellicle. In the calcium measurements two of the toothpastes tended to enhance erosion compared to water, with a significant effect for P- and P+ ($p < 0.001$). Toothpaste P++ was accompanied with the lowest loss of calcium (2.28 mmol/cm²), followed by P+ (2.61 mmol/cm²) and P- (2.63 mmol/cm²). Toothpaste P+ showed the lowest loss of phosphate (2.82 mmol/cm²), followed by P++ (3.40 mmol/cm²) and P- (4.42 mmol/cm²). When the three pastes were mutually compared, P++ showed less calcium loss than the other toothpastes ($p < 0.001$), whereas for phosphate loss P+ showed less erosion than P- ($p = 0.04$), but was not significantly different from P++ (fig. 2).

All three toothpastes reduced erosion compared to water (loss of calcium: 2.37 mmol/cm², loss of phosphate: 6.24 mmol/cm²) for the phosphate measurement ($p = 0.04$ to 0.001).

Discussion

In this study the effect of three toothpastes on erosion was tested with an in situ-formed pellicle and in vitro without pellicle. To investigate the loss of enamel we used two outcome measures for erosion: loss of calcium and loss of phosphate. Both are directly related to the dissolution of enamel mineral and obviously closely linked. However, both have inherent limitations and we used both in order to strengthen the results. As can be seen in figure 1, the in situ experiment for both outcome measures showed a very similar trend. For the in vitro experiment this was less clear. The use of toothpastes did reduce the erosion compared to water. However, the hypothesis of this study could only be provisionally accepted because a possible effect of addition of proteins could only be detected for the high concentration paste P++, using the phosphate measurements. A similar effect was found for the loss of calcium but this was (marginally) not significant. The results of the in vitro experiment showed a less clear and consistent effect of the toothpaste. We assume that the interaction with the pellicle is important for the effect.

For years proteins have been used in oral care products to maintain oral health [Lenander-Lumikari et al., 1993; Kirstila et al., 1996; Pedersen et al., 2002; Tenuvuo, 2002], but the addition of proteins to toothpaste is still controversial. Earlier research on these products showed that it was questionable whether these proteins can be immobilized in the acquired pellicle [Hannig et al., 2005]. However, recent studies on the efficacy of enzymatic toothpastes and mouthrinses showed that the immobilization of enzymes in an in situ pellicle can, indeed, be achieved by using toothpaste [Hannig et al., 2010b] but not by using a mouthrinse [Hannig et al., 2010a].

In earlier research it was found that casein significantly reduced the hydroxyapatite dissolution rate when hydroxyapatite was coated with a salivary pellicle. The reduction in dissolution rate is ascribed to the firm adsorption of casein on to the hydroxyapatite surface, which stabilizes the crystal surface and inhibits ion detachment [Barbour et al., 2008]. Moreover, in a recent study it was shown that the efficacy of casein as a barrier to acids in the presence of a pellicle is enhanced [Hemingway et al., 2010]. The absence of a pellicle as a barrier and its role in the augmentation of the efficacy of casein could explain the higher calcium and phosphate losses in our extraoral experiments compared to the intraoral experiments. It can also explain why a protective effect compared to water brushing was not so clear *in vitro*. Furthermore, in this study the enamel samples were exposed to a severe acidic challenge (citric acid, pH 2.3). Exposure to acidic solutions with a higher pH, more commonly encountered for instance in soft drinks, may result in a more intact pellicle and consequently in a better performance of the added proteins.

The P++ paste contains 0.041 mg/g Ca^{2+} compared to 0.026 mg/g Ca^{2+} for the P– and 0.029 Ca^{2+} mg/g for the P+ paste. This extra calcium could have influenced the measurements, resulting in a lower estimate of loss of enamel reduction for the P++ compared to the P+ and P– pastes. This contribution is considered small because the samples were exposed to the toothpaste slurry and brushing extraorally, rinsed with water, and then replaced in the oral cavity for 30 min. Thus, only a very small amount of the paste was left on the samples. It may also be suggested that the higher calcium concentration in itself contributed to the effect of the P++ paste. This could be viewed as an indirect effect of the protein addition, as casein is known to bind calcium, but this is likely to be negligible [Nejad et al., 2010].

The protective effect of fluoride in toothpaste on dental erosion has been studied before. It was shown that fluoride in toothpaste reduces dental erosion or erosive wear compared with a fluoride-free control [Bartlett et al., 1994; Ganss et al., 2007]. We therefore did not study the fluoride effect in this study, but considered it a given fact that fluoride toothpaste would be used. It has been suggested that fluoride and casein can have an additive effect in reducing the dissolution of enamel under caries-like conditions [Weiss and Bibby, 1966]. Recent work by White et al. [2010] confirmed this. In our study all toothpastes contained the same fluoride agent and concentration, and a small additional protein effect was observed.

Similar *in situ* models have been used in studies investigating the erosive potential of soft drinks [West et al., 1998] and the protective effect of fluoride varnish [Vieira et al., 2007].

The main benefit of this system is that the enamel samples are placed in the oral environment with natural pellicle development and can be removed easily. A drawback is the location of the samples on the palate, which can result in a thinner pellicle by abrasion caused by the tongue. The extraoral exposure may reduce clinical relevance.

Although a protective effect of adding protein to toothpaste could only be shown for the highest protein concentration, we conclude that the highest protein concentration toothpaste shows some promise for the prevention of erosion as measured by Pi loss. Further research is needed to investigate the performance of protein-containing toothpastes in longer *in situ* studies with regard to erosive wear and under less aggressive erosive challenge conditions. Moreover, the effect of brushing with protein-containing toothpaste on the protein composition and the acid resistance of the pellicle should be the subject of further investigation.

Acknowledgments

The authors are grateful to Nicole Zwier for her help with data collection. The authors also acknowledge the volunteers for their participation in this study.

Disclosure Statement

The academic investigators employed by the University Medical Center Groningen and the Radboud University Medical Center Nijmegen and researchers employed by Sara Lee Household and Body Care Research BV jointly designed the study. The paper presents research done by all authors and each author participated substantially in the work. This study was funded by an unconditional research grant from Sara Lee Bodycare and Research BV, Amersfoort, The Netherlands. No other conflicts of interest can be identified.

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