Dental erosion

The role of acidic beverages, saliva and toothpastes in the development and reduction of dental erosion

Derk Hendrik Jan
Jager

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Table of content

Chapter 1

Introduction and aim of the study

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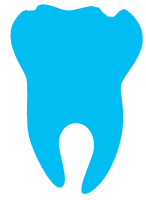


1.1 Dental wear

Dental wear is described as non-carious loss of dental hard tissue (Imfeld, 1996a). Development of dental wear involves multiple processes such as attrition, abrasion and erosion. Attrition (the act of wearing or grinding down enamel or dentin by friction of teeth), abrasion (wearing of tooth substance through interaction between teeth and other materials) and erosion (the progressive loss of tooth substance by chemical processes that does not involve bacterial action) seem to play a major role in the development of wear. All processes can occur at the same time and they all contribute to loss of function and ageing of teeth (The glossary of prosthodontic terms, 2005; Imfeld, 1996a; Nunn, 1996). Finally, the various processes can all be considered physiological or pathological, depending on the amount of dental wear that they caused in relation to the age of the affected patient.
The research described in this PhD-thesis focuses on dental erosion, in particular on the role of beverage parameters, saliva, salivary film/pellicle and toothpaste in the development of dental erosion. Therefore, the current knowledge on the measurement and development of the susceptibility to and the prevention of dental erosion is briefly summarized in this chapter.

1.2 Dental erosion

The early signs of erosive wear appear as a smooth silky-shining glazed surface (figure 1). Initial lesions are located coronal from the enamel-cementum junction with an intact border of enamel along the gingival margin (Ganss and Lussi, 2006). This intact enamel zone, often chamfer shaped, could be the result of plaque remnants which act as a diffusion barrier for acids or as a result of an acid-neutralizing effect of the sulcular fluid (Lussi et al., 2004a). In more advanced stages of erosive wear changes in the original tooth morphology occur (figure 2). On smooth surfaces the convex areas flatten or concavities become apparent.

Prevalence of erosion in the Netherlands

In the Netherlands, 24% of the 12-year-old children demonstrated erosive wear (Truin et al., 2005). Another Dutch study showed even higher figures; in 2008 a prevalence of 32.2% was found in subjects aged between 10-13 years. Even more striking was the observation in the latter study that 24% of the children that were free of erosion at baseline developed erosion over the subsequent 1.5 years (El Aidi et al., 2008).

Aetiology

Dental erosion may be caused by extrinsic and intrinsic factors. Probably the most investigated extrinsic cause of dental erosion is excessive consumption of acidic

Figure 1. *Typical signs of erosion: a smooth silky-glazed appearance, change in colour, cupping and grooving on occlusal surfaces.*

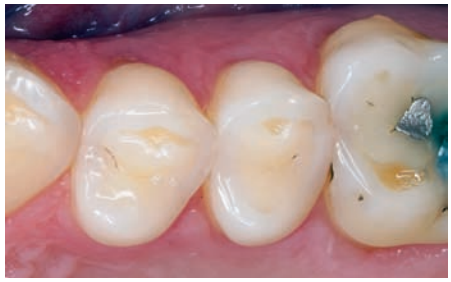


Figure 2. *Advanced stage of dental erosion.*

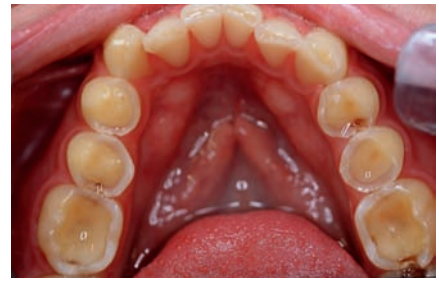
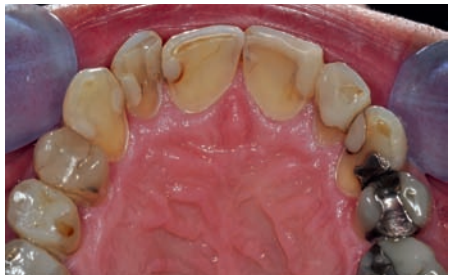


Figure 3. *Palatal dental erosion related to gastric reflux.*



beverages (Ten Cate and Imfeld, 1996; Dugmore and Rock, 2004). The consumption of acidic beverages has risen during the last decades. In the USA, a 300% increase in soft drink consumption has been reported between 1980 and 2000 (Cavadini et al., 2000). One of the intrinsic causes of dental erosion is contact of the teeth with gastric acid during vomiting or reflux. Vomiting and reflux are rather frequently observed in diseases such as anorexia nervosa, bulimia, gastrointestinal disorders, alcoholism and also in pregnancy (Smith and Knight, 1984). A typical clinical sign pointing towards erosion caused by gastric juice is palatal dental erosion (figure 3). Based on only a few reports, it appears that gastric acids are equally likely to induce moderate to severe erosion as dietary acids (Lussi, 2006).

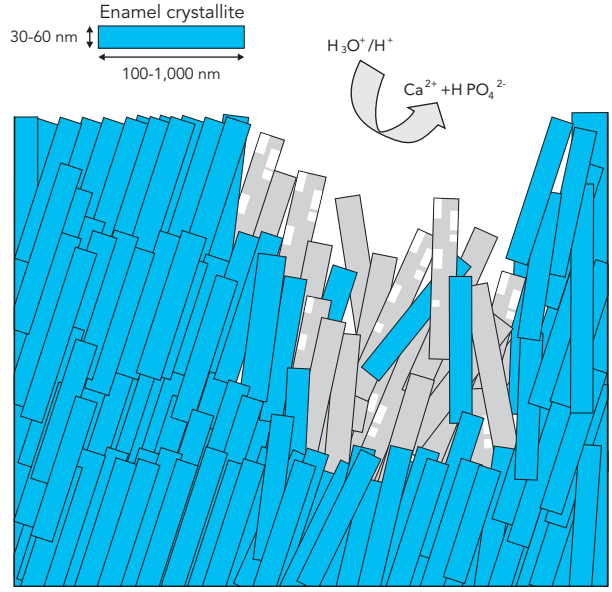
Pathogenesis

Erosion of teeth occurs either by hydrogen ions derived from acids or by anions which can bind or complex calcium (chelating agents). The hydrogen ions are derived from acids as they dissociate in water. The hydrogen ions can combine with

the carbonate ion or the phosphate ion resulting in direct surface etching. Figure 4 illustrates the effect of acidic beverages with a low pH.
In certain acids, e.g. citric acid, anions play a major role next to hydrogen ions. The citrate anion may complex with calcium ions to form for example tricalcium dicitrate tetrahydrate ($Ca_3C_{12}H_{10}O_{14}$*$4H_2O$). This process also results in loss of calcium from the crystal surface (chelating) (Featherstone and Lussi, 2006).
The erosive potential of beverages and food is dependent on several factors. For example, in erosion caused by acidic beverages, the amount of dissolved mineral probably depends on a number of beverage parameters such as pH, titratable acidity and fluoride content. Moreover, the presence of suitable concentrations of calcium and phosphate in the beverage may slow down the dissolution process (Larsen and Nyvad, 1999). Several models have been proposed to predict the erosive potential of beverages based on their chemical properties. Already in 1973, Larsen et al. suggested that the erosive potential of a beverage could be determined by the degree of saturation of a particular beverage with respect to hydroxyapatite or fluorapatite (Larsen, 1973). Later it was found that the erosive potential of a beverage was correlated with its titratable acidity to pH 7.0, its fluoride and phosphate content and its baseline pH (Lussi et al., 1993). Next to the earlier studied beverage parameters, there are additional beverage characteristics, such as viscosity, that presumably influence the erosive potential of a drink.

A variety of techniques are available to assess the erosive potential of acidic beverages of which profilometry and chemical analysis are frequently used techniques. Determination of calcium and phosphorus concentrations in erosive solutions are well established and accurate analytic methods to indirectly measure the loss of minerals from the enamel (Barbour and Rees, 2004). As small concentrations of these ions released from the enamel into a beverage can be measured, it is possible to observe the initial stages of erosion. Moreover, one only needs small volumes of the examined solutions for analysis. This method also allows the use of natural tooth surfaces since polishing is not needed (Barbour and Rees, 2004). However, chemical methods provide only quantitative and no morphological or mechanical data (Grenby, 1996). The method of choice for morphologically measuring the loss of surface layers of enamel, is optical or contact profilometry. In optical profilometry there is no physical contact between the probe and the enamel or dentin surface, so no damage of the surface will occur by scratching of the softened surface (Barbour and Rees, 2004). A drawback of profilometric techniques is that losses of enamel with a depth of less than 2 µm are not measureable. Until now, sparse knowledge is available on how enamel loss as assessed by profilometry and chemical analysis correlate. In addition, the lack of a "golden standard" is a shortcoming in the field of erosion research and the influence of

Figure 4. The development of an erosive lesion (Hannig and Hannig, 2010). Acidic beverages destroy the enamel surface by partial and complete dissolution of the enamel crystallites. The result is a release of Ca^{2+} and HPO_4^{2-} ions that loosens the microstructure of the enamel and hydroxyapatite crystallites (grey). These crystallites become demineralised or are lost.



the composition of the beverages on the erosive wear measurements is unclear. In studies comparing methods for measuring the erosive potential of beverages, a variety of solutions, e.g. citric acid or lactate buffer, has been applied that makes a proper comparison of the results of the various studies difficult (Zero et al., 1990; Ganss et al., 2005). In chapter 2 the influence of beverage composition on the measurement of erosive potential and the influence of exposure to small and large volumes of a beverage are described.

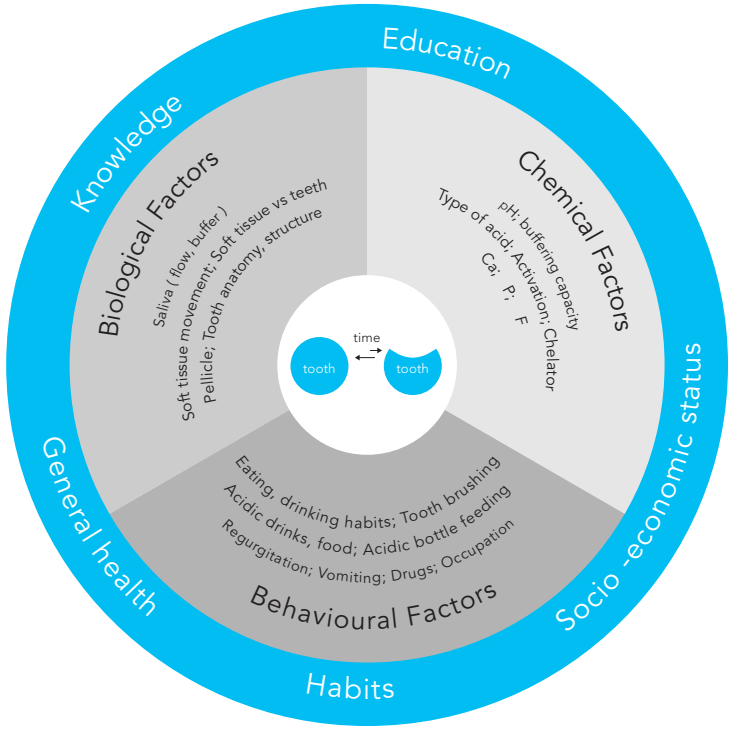
1.4 Susceptibility to erosion

A wide variation among individuals has been reported regarding their susceptibility to develop dental erosion (O'Sullivan and Curzon, 2000; Vieira et al., 2007). To explain why some individuals are more susceptible to erosive wear than others, it is crucial to understand the risk factors and their interactions (figure 5) (Lussi, 2006). The main source of variation in susceptibility of subjects to dental erosion are the biological factors. In in vitro research it was found that saliva from different donors

offers different levels of protection against erosion (Wetton et al., 2007). Furthermore, in an in situ study it was found that the variation between so-called high and low eroders can reach up to ten-fold differences (Hughes et al., 1999b). Moreover, results of in vitro studies investigating the erosive potential of beverages in absence of saliva showed losses of enamel that were many orders of magnitude greater than those recorded on specimens in situ, i.e. under conditions where saliva was present (West et al., 1998; Hughes et al., 1999a). In other words, in the variation in susceptibility of subjects to dental erosion saliva may play an important role (Hall et al., 1999).

Saliva has been assumed to be involved in the protection against erosion in several ways. It dilutes acids and salivary clearance removes the acid gradually from the oral cavity via the swallowing process. In addition, saliva contains protein, bicarbonate and phosphate buffers and saliva is supersaturated with respect to tooth minerals, such as calcium and phosphate. Moreover, saliva contains a wide array of proteins and some of these proteins have protective properties other than a buffering action. Finally, proteins can protect the teeth against acids by the formation of a salivary pellicle when teeth are exposed to saliva (Dawes, 2008). This pellicle acts as a diffusion barrier or a selective permeable membrane, reducing direct contact between acids and tooth surface (Hannig and Balz, 1999), thus reducing demineralization of the tooth surface (Amaechi et al., 1999; Hannig and Balz, 2001). The proteins that form the pellicle affect it's functions, such as ion transport potential, regulation of calcium phosphate crystallization and bacterial adherence (Hannig and Joiner, 2006; Cheaib and Lussi, 2011). Siqueira et al (2007) studied the composition of the pellicle and divided the pellicle proteins in to three groups. The first group consists of calcium binding proteins. These proteins, such as statherin and PRPs, can interact with calcium ions on the enamel surface and are considered pellicle precursor proteins. The second group consists of phosphate binding proteins that are binding to the phosphate ions on the enamel surface. Proteins showing interactions with other proteins form the third group. These proteins, such as MUC 5b are involved in the formation of protein layers (Siqueira et al., 2007). Pellicle proteins can also be grouped according to function such as buffer capacity and remineralization. It is found that there is a partial overlap in proteins that are involved in remineralization processes and those that have a high affinity to enamel surfaces (Siqueira et al., 2007). Based on this information, numerous salivary pellicle proteins could be involved in the protection of teeth against erosion. The importance of saliva is illustrated in hyposalivation where carious destruction and erosive wear are phenomena that occur simultaneously (Jansma et al., 1989; Lajer et al., 2009). In chapter 4 and 5 of this thesis, selected saliva and pellicle parameters are studied to obtain some insight into the presumed role of saliva and pellicle in the inter-individual variation in dental erosion.

Figure 5. *Risk factors for dental erosion and their interactions (Lussi, 2006).*



1.5 Prevention of dental erosion

As mentioned before, dental erosion is a growing problem in the Netherlands (El Aidi et al, 2008). Excessive loss of dental hard material due to erosion can result in aesthetic and functional problems (Jaeggi et al, 2006). Raising awareness of the problem at an early stage and taking adequate preventive measures are both important to prevent extensive and expensive restorative interventions. In two reviews several preventive measures have been suggested (table 1; Lussi and Helwig, 2006; Imfeld, 1996b). Before starting a preventive measure, it is recommended to establish a differential diagnosis of the origin of erosion. This can be based on a thorough anamnesis and clinical inspection. For this, the location and severity must be registered. Also intra-oral photographs and study casts can be helpful. The dental and medical anamnesis must cover, among other items, medication, reflux, heartburn and acid mouth taste. Furthermore, the role of saliva in the development of erosion needs further study. Exposure to saliva has been shown

to be effective for rehardening eroded enamel and to be an important factor in the prevention of erosion (Ameachi and Higham, 2005; Lussi and Helwig, 2006). Therefore, salivary tests, such as assessing the resting and stimulated flow rate as well as buffer capacity, might be worthwhile to include in the physical examination of subjects with signs of dental erosion. Also the role of food and beverages in the aetiology has to be assessed, e.g. by means of a 5-day food diary (3 working days and a weekend). Based on the aetiology, a variety of preventive prophylactic measures can be suggested (table 1).

A complementary measure could be to develop oral care products that slow down the progression of dental erosion. Because of its wide spread use, toothpaste might be an ideal mode by which protection to dental erosion could be provided. Furthermore, it is demonstrated that some of the professional protective measures (application of fluoride varnishes) are effective but only for a limited period (Vieira et al., 2007). Therefore, daily applications of protective products are probably more successful. A number of studies investigated toothpaste modifications such as higher fluoride concentrations and exclusion of sodium lauryl sulphate (SLS) (Newby et al., 2006; Rees et al., 2007; Hooper et al., 2007; Lussi et al., 2008). SLS is able to remove the protective pellicle and the smear layer present on dentin (Moore and Addy, 2005). Therefore, toothpaste formulations without SLS could be favourable in reducing 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, compared to conventional toothpastes, an increase of hardness of enamel after exposure was found compared to conventional toothpastes (Lussi et al., 2008).
Many studies investigated the role of different fluoride formulations and concentrations in reduction of erosion. Common formulations used in caries prevention, such as neutral solutions of sodium fluoride (NaF) have been shown to have a limited effect (Attin et al., 1998; Lussi et al., 2004b; Lussi et al., 2008). Recent research showed a promising effect for stannous fluoride (SnF_2) (Hjortsjö et al., 2010). SnF_2 is already used in toothpastes and mouthrinses, and its effect on plaque and gingivitis is well recognized (Paraskevas and van der Weijden, 2006). Next to SnF_2, stannous chloride ($SnCl_2$) in mouth rinses has been studied before, using SnCl2 as the source of tin with amine fluoride and/or NaF as the source of fluoride. In an in vitro erosive cycling model, such solutions reduced tissue loss significantly, even when using a severe erosion regime (Schlueter et al., 2009). Less is known about the erosion preventive effect of SnF_2 in toothpastes. The concentration of SnF_2 in the toothpastes is usually lower than those used in the solutions, and the abrasive effect of the toothpaste may interfere with the protective effect.
Another modification of toothpaste, aiming for a reduction of the loss of enamel,

could be the addition of proteins such as present in colostrum. Bovine colostrum is a protein source already used in oral care products. It is presumed that the proteins present in bovine colostrum will be incorporated into the pellicle thereby increasing its protective strength to an acidic challenge. In chapter 6 a study is described investigating the effect of stannous fluoride containing toothpaste and in chapter 7 the effect of a protein containing toothpaste on the reduction of erosive wear is dicussed.

Table 1. Recommendations for patients at high risk for dental erosion. (Imfeld, 1996b; Lussi and Helwig, 2006)

-	Reduce acid exposure by reducing the frequency and contact times to acid beverages and food
-	Do not hold or swish acidic beverages
-	Avoid tooth brushing immediately after and before an erosive challenge
-	Use a soft toothbrush and a low abrasion, fluoride containing toothpaste
-	Consider modified acid beverages or non acidic beverages
-	After acid intake, stimulate saliva flow with for example chewing gum
-	Refer patients when intrinsic causes are involved (gasteroenterologist and/or physiologist)

1.6 Aim

The general aim of this PhD research was to obtain insight in the effects of beverage parameters, saliva, salivary film/pellicle and toothpaste on the development of dental erosion. Additionally, the effect of beverage composition on measurement techniques for wear quantification was studied.

The specific aims of this thesis were:

1 To evaluate whether beverage composition and exposure to small and large volumes influences the measurement of erosive potential (chapter 2);

2 To evaluate the erosive potential of beverages, using both short and long exposure times, and to analyze the relationship between erosion and several drink parameters, including viscosity, if possible using a multivariate approach (chapter 3);

3 To investigate the relationship between a selection of salivary parameters and early erosion of hydroxyapatite with an in situ grown saliva film (chapter 4);

4 To investigate the relationship between concentration of carbonic-anhydrase 6, statherin and the total protein concentration in saliva and salivary film/pellicle, and susceptibility of hydroxyapatite to erosion (chapter 5);

5 To evaluate whether protein-containing toothpastes reduce dental erosion in the presence of in situ formed pellicle and in vitro without pellicle (chapter 6);

6 To evaluate the effect of stannous fluoride containing toothpastes in the prevention of erosive enamel wear (chapter 7).

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Chapter 2

Influence of beverage composition on the results of erosive potential measurement by different measurement techniques

D.H.J. Jager, A.M. Vieira, J.L. Ruben and M.C.D.N.J.M. Huysmans.

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Abstract

The influence of beverage composition on the measurement of their erosive potential is unclear. The aim of this study was to evaluate whether beverage composition influences the measurement of erosive potential and to evaluate the influence of exposure in small and large volumes. Eleven beverages were included: water (control); 3 alcopops; 2 beers and 5 softdrinks. For each beverage 15 bovine enamel samples were used: 5 for chemical and 10 for profilometric analysis. After exposure to the beverages (63 min) the resulting solutions were analyzed for Ca and inorganic phosphorus (Pi) content. The samples for optical profilometry were submersed sequentially in 500 ml or in 1 ml of the drinks for 3, 6, 9, 15 and 30 min (total 63 min). For some of the beverages high baseline concentrations of Ca (energy drink) or Pi (cola drink, cola lemon drink, beer, beer lemon) were found. Some of the beverages showed a good correlation between the chemical methods. Profilometry (both for 1 ml and 500 ml) showed generally lower enamel losses than the chemical methods. Lower enamel losses were found for the profilometry 1 ml compared to the profilometry 500 ml only for the cola drinks. It can be concluded that the composition of the beverages had a significant effect on the determination of the erosive potential with chemical analyses. Drink composition also influenced the effect of small vs. large exposure volumes, indicating the need for standardisation of exposure parameters.

Introduction

Dental erosion is defined as an irreversible loss of dental hard tissue due to a chemical process without involvement of micro organisms (Imfeld, 1996). Dental erosion may be caused by either extrinsic or intrinsic factors. One of the extrinsic causes of dental erosion is excessive consumption of acidic beverages (Dugmore and Rock, 2004). Different techniques are available to assess the erosive potential of acidic beverages. Frequently used techniques include profilometry and chemical analysis.

Calcium determination and inorganic phosphorus (Pi) determination are used to measure the loss of minerals from the enamel and are well established and accurate (Barbour and Rees, 2004). Small concentrations of ions released from the enamel can be measured so it is possible to observe the initial stages of erosion and it is possible to use small volumes of the examined solutions. It also allows the use of natural tooth surfaces since polishing is not needed (Barbour and Rees, 2004). However, chemical methods provide only quantitative and not morphological or mechanical data (Grenby, 1996).

For measuring the loss of surface layers the method of choice is optical or contact profilometry. In optical profilometry there is no physical contact between the probe and the surface, so no damage will occur by scratching of the softened surface (Barbour and Rees, 2004). A drawback of profilometric techniques is that enamel losses below 2 µm are difficult to measure.

Little is known about the correlation between different methods and the lack of a "gold standard" is a shortcoming in the field of erosion research. Moreover the influence of the composition of the beverages on the measurements is unclear. In earlier studies comparing methods for measuring the erosive potential of beverages, standard solutions such as citric acid or lactate buffer were used (Ganss et al., 2005; Zero et al., 1990). As a result, the influence of the composition of beverages on the results obtained by the different methods could not be determined. We hypothesised that chemical composition of soft drinks and the volume used influences the determination of erosive potential.

The aim of this study was to evaluate whether beverage composition influences the measurement of erosive potential and to evaluate the influence of exposure in small and large volumes.

Materials and methods

Preparation of samples

A total of 165 buccal surfaces of extracted bovine incisors, stored in water, were ground flat with water-cooled silicon carbide 220 grit grinding discs (SIA siawat P220, Frauenfeld, Switzerland) and cut into blocks of approximally 5 × 3 mm using a vertical sawing machine with a diamond saw blade (11-4243, Buehler, Düsseldorf, Germany). The blocks were embedded in acrylic resin (Autoplast polymer, Candulor AG, Wangen, Switzerland) leaving the enamel surface uncovered and subsequently the samples were polished flat (800-1200 grit grinding paper) and thoroughly rinsed with tap water. The samples were randomly divided into 3 groups of 55 samples each: 1 group for chemical analysis and 2 groups for profilometric analysis. Before inclusion in the experiment the area of exposure of each of the 55 samples used for chemical analyses was measured with a stereomicroscope equipped with a measuring grid (Leitz Durimet, Wetzlar, Germany) fitted out with a digital XY-table (Sony magnescale LY101, Tokyo, Japan).
The 110 samples used for the profilometric analysis were partly covered with PVC tape exposing an area of approximately 3 × 3 mm in the centre of the enamel sample.

Beverages

Eleven beverages, all available in The Netherlands, were included in this study (table 1). Immediately after opening the bottles and degassing (the drinks were placed on a shaking table set at 200 rpm until no bubbles were visible), the pH was measured 5 times using a calibrated glass pH electrode (Radiometer, PHM 84 Research meter, G202C, Copenhagen, Denmark) in 100 ml of the beverages. The temperature in the laboratory was 21 °C with a possible variation of ± 2 °C. Standard buffers, pH 7.01 and 4.00 (20 °C) were used (measurement uncertainty for both ± 0.015 units) (Merck KGaA, Darmstadt, Germany). Calibration was performed with these buffers every day.

Demineralization procedures

Before starting the demineralization procedure the samples for chemical analysis were submersed for 3 min in 3 ml of a standard solution of 50 mM citric acid, 0.4 mM KH_2PO_4, 0.4 mM $CaCl_2$ and 1 mM NaN_3 (pH = 3) to remove the smear layer from the polished surfaces and subsequently rinsed with tap water. All the beverages were decarbonated.
For the chemical analyses each of the 5 enamel samples was submersed in 1 ml of each beverage in a test tube for 3 min under constant agitation on a shaking table at 100 rpm. After 3 min the samples were lifted from the beverages and the enamel surface was rinsed with 3 ml demineralized water, which was collected in the test

tube. From the resulting mixtures 1 ml was used for Ca analysis and 1 ml for Pi analysis. The same procedure was repeated for exposures of 6, 9, 15 and 30 min (total 63 min). The exposures for the different times were made sequentially on the same specimens.
For the profilometric analysis each of the 5 enamel samples was submersed in 1 ml of each beverage in a test tube for 3, 6, 9, 15 and 30 min under constant agitation on a shaking table (100 rpm). The pH of these solutions was measured after each exposure. Another set of 5 samples was submersed in 500 ml of each beverage for 63 min under constant agitation on a shaking table (100 rpm) in beakers with a diameter of 9.5 cm. All experiments were performed at room temperature (21 ± 2 °C).

Chemical analysis

Pi concentration in the beverages was determined using a phospho-molybdate spectrophotometric method (Chen et al., 1956). The concentration of Ca in the beverages was determined by atomic absorption spectroscopy (AAS; Perkin Elmer Analytical Instruments, Shelton, Conn., USA) (Vieira et al., 2005).This was performed in the presence of lanthanum (0.326%) in order to suppress phosphate interference. An air/C_2H_2 flame and a wavelength of 422.7 nm were used.
For the chemical analyses all the beverages had to be diluted with demineralised water. For the Pi analysis most of the beverages were diluted 16 times in total. The beverages with high Pi concentrations (the colas and the beers) had to be diluted 80 times. For the Ca analysis all the beverages were diluted 18.4 times.
The Ca and Pi losses from the enamel samples were determined by subtracting the Ca or Pi content of the beverages before the enamel exposure (average of 10 measurements) from the total Ca or Pi content of the solution after exposure. In addition, the ratio of the Ca dissolved to the Pi dissolved ($\Delta Ca/\Delta P$) was calculated for each exposure time.
The lesion depth was calculated from the Ca and Pi loss using the average Ca and Pi content per unit volume for bovine enamel and the exposed enamel area (Dijkman et al., 1983). A Ca concentration in enamel of 25.1%, a P concentration in enamel of 17.61% and an average enamel density of 2.93 g/cm^3 was assumed. This resulted in two depth parameters: d(Ca) and d(P), lesion depth estimated from Ca loss or Pi loss, respectively, The estimated erosion depth (µm) of the 5 samples was averaged.

Profilometric analysis

Erosion depths were measured using an optical profilometer (Proscan 2000, Scantron, Taunton, England). Before inclusion of the enamel samples in the experiment, baseline measurements were performed on each sample in order to confirm the flatness of the polished enamel surfaces.

After the demineralization procedure the PVC tape was removed. The samples were scanned over the reference and eroded surfaces. The volume lost due to erosion was calculated with the equipment's software. The erosion depth (µm) was calculated by dividing the volume loss by the exposed enamel area of the scanned surface. The erosion depths of the 5 samples were averaged. The profilometry resulted in two further depth parameters: d(prof1) and d(prof500).

pH changes and degree of saturation

The pH of the solutions after each exposure in the profilometry (1 ml) group was measured. The beverage's baseline degree of saturation with regard to hydroxyapatite and dicalcium phosphate dihydrate (DCPD) was calculated by means of a computer program (Shellis, 1988), using the baseline Ca and Pi concentrations of the beverages, together with the pH measured after degassing . To determine the possible influence of saturation of the beverages on the measurement results during the erosion process, the Ca and Pi concentrations and pH after the 30 min incubation were used to calculate the change in degree of saturation with regard to hydroxyapatite and DCPD after the erosion regime.

Statistical analysis

For investigation of the relationship between the change in Ca and Pi concentrations linear least squares regression was performed. The Pi concentration was the independent (X) variable. A one-way ANOVA followed by a Bonferroni post-hoc test in SPSS 12.01 (SPSS , Chicago IL, USA) was used to test differences between the cumulative erosive depths at 63 minutes obtained by the chemical methods (average of d(Ca) and d(P): d(CaP)), d(prof1) and the d(prof500)). The significance level for all tests was set at 0.05.

Results

The pH of the beverages ranged from 2.4 (cola) to 8.1 (bottled water) (table 1). Table 1 also shows the baseline Ca and Pi concentrations and table 2 shows the changes in ΔCa and ΔPi concentrations for all erosion times and all drinks. Pi concentration ranged from not detectable (bottled water) to 5.3 mmol/l (beer). Baseline Ca concentration ranged from 0.06 mmol/l (orange soft drink) to 1.3 mmol/l (fruit drink). For most of the drinks the ΔCa/ΔP ratio did not differ significantly from 1.6 except for some of the low exposure times (3 and 6 min), and for the cola drink, orange soft drink, and the ice tea. In table 3 the parameters for the linear least squares regression analysis of the Ca and the Pi concentrations for all beverages are presented. In most cases a high linear correlation ($r^2 > 0.8$) was found, except for the beers ($r^2 = 0.07$ and $r^2 = 0.19$), cola drink ($r^2 = 0.76$), energy drink ($r^2 = 0.63$)

and cola lemon drink (r^2 = 0.53). For this reason and because of the problems measuring the Pi concentration in drinks with a high baseline Pi concentration, d(Ca) of the beers, cola drink and cola lemon was used for the comparison with the profilometry. In table 4 and in figure 1 the cumulative results of Ca, Pi and the profilometric analyses are presented. The highest enamel loss was found for cola lemon drink in the d(prof 500) group (13.54 µm). The drinks concentrated in the middle part of the graph (dashed lines) showed lower erosive potential for the profilometry compared to the chemical analysis. The two colas (drawn lines) showed lower erosive potential in the d(prof 1) group compared to the d(prof 500) group and higher erosive potential for the d(Pi) compared to the d(Ca). In figure 1 also the rank order in which the different methods placed the drinks can be assessed. For some beverages the influence of the measurement method on its rankorder in erosiveness (1 is lowest, 11 is highest erosion) is marked, e.g., the orange soft drink is the 7th most erosive drink in d(Ca) but the 4th most erosive with d(Pi). Similarly, Ice tea 8th with d(Ca) and 4th with d(prof 500). One-way ANOVA showed a significant effect of measuring technique ($p < 0.05$) for all beverages except ice tea and the fruit drink. d(CaP) showed an enamel loss significantly higher ($p < 0.05$) than the d(prof 1) for the rum lime alcopop ($p < 0.0001$), energy drink ($p = 0.007$), vodka alcopop ($p = 0.034$), beer ($p < 0.0001$, d(Ca) only) and orange soft drink ($p < 0.0001$). When compared to the d(prof 500) the d(CaP) showed a significantly lower enamel loss for the cola lemon drink ($p = 0.004$) and a significantly higher enamel loss was found for rum lime alcopop ($p < 0.0001$), energy drink ($p = 0.022$), vodka alcopop ($p = 0.034$), beer ($p< 0.0001$), beer lemon ($p = 0.001$) and orange soft drink ($p = 0.001$). The d(prof 1) showed a significantly lower enamel loss than the d(prof 500) only for the cola drink ($p = 0.002$) and the cola lemon drink ($p = 0.003$). The results obtained for the pH measurements after each exposure in the 1 ml profilometry showed very little change in pH (-0.02 to +0.03) after the erosion process for most beverages. Only the cola and the cola lemon showed a small rise of pH (0.1) after 30 min. None of the beverages was supersaturated with respect to hydroxyapatite or DCPD after a 30 min erosive exposure in 1 ml of the samples (table 2). The highest degree of saturation for hydroxyapatite was found for the beers. The highest rise in degree of saturation after 30 min was found for the energy drink.

Table 1. Beverages used in this study, with details of their composition.

	Beverage	Producer	pH	Ca mmol/L	Pi mmol/L	DS(HA)
Cola drink	Coca Cola	Coca-Cola Enterprises Nederland B.V., Dongen, The Netherlands	2.47	0.87 ± 0.04	4.76 ± 0.15	0.005
Cola lemon drink	Coca Cola light lemon	Coca-Cola Enterprises Nederland BV, Dongen, The Netherlands	2.73	0.73 ± 0.01	4.90 ± 0.06	0.008
Orange drink	Fanta orange	Coca-Cola Enterprises Nederland B.V., Dongen, The Netherlands	3.03	0.06 ± 0.01	0.19 ± 0.01	0.001
Fruit drink	Dubbelfriss orange/pink grapefruit	Riedel Beverages, Ede, The Netherlands	3.35	1.30 ± 0.03	0.51 ± 0.03	0.018
Vodka alcopop	Smirnoff Ice	Diageo, London, UK	3.43	0.15 ± 0.005	0.004 ± 0.001	< 0.001
Energy drink	Red Bull	Red Bull, de Bilt, The Netherlands	3.43	2.40 ± 0.21	0.01 ± 0.02	< 0.001
Ice Tea	Lipton Ice tea	Unilever, Rotterdam, The Netherlands	3.80	0.12 ± 0.01	0.25 ± 0.004	0.009
Beer lemon	Grolsch beer lemon	SABMiller, London, United Kingdom	3.83	0.96 ± 0.02	3.51 ± 0.08	0.068
Rum lime alcopop	Breezer Lime	Bacardi Martini NV, Gouda, Nederlands	3.87	0.17 ± 0.01	0.02 ± 0.001	< 0.001
Beer	Bavaria beer	Bavaria NV, Lieshout, The Netherlands	4.20	0.72 ± 0.02	5.30 ± 0.14	0.125
Bottled water	Sourcy bottled water	Vrumona BV, Bunnik, The Netherlands	8.09	1.20 ± 0.03	n.m.	-

Table 2. Changes in calcium (Ca) and inorganic phosphorus (Pi) concentrations (mmol/l) of drinks after each exposure, together with the ratio of the changes in Ca and Pi (ΔCa/ΔPi). Means with SD in parenthesis. The degree of saturation with respect to hydroxyapatite after the final 30 min exposure is also given.

	3 min			6 min			9 min			15 min			30 min			
	Ca	P_i	Ca/P	Ca	P	Ca/P	Ca	P_i	Ca/P	Ca	P_i	Ca/P	Ca	P	Ca/P	DS (HA)
Cola	0.15 (0.02)	0.15 (0.05)	1.16 (0.36)	0.26 (0.02)	0.26 (0.09)	1.22 (0.84)	0.32 (0.05)	0.33 (0.26)	1.89 (1.49)	0.42 (0.05)	0.61 (0.12)	0.70 (0.13)	0.61 (0.05)	0.87 (0.06)	0.71 (0.05)	0.009
Cola lemon	0.17 (0.03)	0.26 (0.09)	0.71 (0.32)	0.25 (0.02)	0.25 (0.15)	1.81 (1.85)	0.33 (0.03)	0.20 (0.10)	1.97 (0.91)	0.47 (0.02)	0.17 (0.07)	3.16 (1.17)	0.85 (0.02)	0.74 (0.18)	1.21 (0.31)	0.001
Orange soft	0.13 (0.02)	0.05 (0.01)	2.47 (0.56)	0.14 (0.03)	0.10 (0.02)	1.37 (0.15)	0.19 (0.05)	0.15 (0.04)	1.30 (0.12)	0.26 (0.05)	0.26 (0.06)	1.03 (0.03)	0.48 (0.10)	0.53 (0.12)	0.90 (0.04)	0.007
Fruit drink	0.17 (0.04)	0.07 (0.03)	2.67 (0.59)	0.29 (0.05)	0.15 (0.03)	2.06 (0.66)	0.37 (0.04)	0.17 (0.03)	2.17 (0.28)	0.46 (0.07)	0.26 (0.04)	1.76 (0.12)	0.94 (0.15)	0.50 (0.09)	1.90 (0.32)	0.029
Vodka alcopop	0.13 (0.02)	0.06 (0.02)	2.14 (0.33)	0.21 (0.02)	0.11 (0.01)	1.97 (0.17)	0.28 (0.04)	0.15 (0.02)	1.84 (0.07)	0.36 (0.04)	0.22 (0.03)	1.66 (0.06)	0.71 (0.14)	0.46 (0.09)	1.54 (0.03)	0.017
Energy drink	-0.06 (0.05)	0.05 (0.01)	-1.18 (0.97)	0.00 (0.10)	(0.10) 0.02	-0.07 (1.25)	0.17 (0.20)	0.15 (0.04)	1.06 (1.38)	0.47 (0.15)	0.22 (0.05)	2.15 (0.69)	0.83 (0.11)	0.46 (0.09)	1.83 (0.19)	0.032
Ice tea	0.11 (0.01)	0.04 (0.001)	2.48 (0.23)	0.15 (0.01)	0.06 (0.01)	2.34 (0.29)	0.20 (0.02)	0.09 (0.01)	2.19 (0.09)	0.21 (0.03)	0.12 (0.02)	1.74 (0.09)	0.37 (0.12)	0.28 (0.02)	1.32 (0.40)	0.024
Beer lemon	0.09 (0.04)	-0.02 (0.10)	1.75 (4.12)	0.07 (0.04)	-0.14 (0.22)	2.48 (4.23)	0.11 (0.04)	-0.02 (0.10)	-0.82 (3.23)	0.12 (0.04)	-0.17 (0.17)	0.28 (2.54)	0.21 (0.04)	0.03 (0.19)	2.84 (4.08)	0.077
Rum lime alcopop	0.13 (0.02)	0.05 (0.01)	2.78 (0.32)	0.17 (0.02)	0.07 (0.02)	2.53 (0.41)	0.21 (0.06)	0.09 (0.03)	2.33 (0.33)	0.28 (0.07)	0.15 (0.03)	1.81 (0.14)	0.48 (0.10)	0.28 (0.07)	1.72 (0.10)	0.028
Beer	0.10 (0.02)	-0.09 (0.07)	-4.12 (7.07)	0.09 (0.01)	-0.08 (0.13)	-1.26 (2.42)	0.10 (0.02)	-0.02 (0.10)	1.80 (3.29)	0.05 (0.06)	-0.13 (0.07)	-0.17 (0.88)	0.11 (0.03)	0.27 (0.13)	0.51 (0.42)	0.146
Bottled water	-0.02 (0.01)	n.m.	n.m.	-0.01 (0.02)	n.m.	n.m.	-0.03 (0.02)	n.m.	n.m.	-0.01 (0.04)	n.m.	n.m.	-0.02 (0.03)	n.m.	n.m.	-

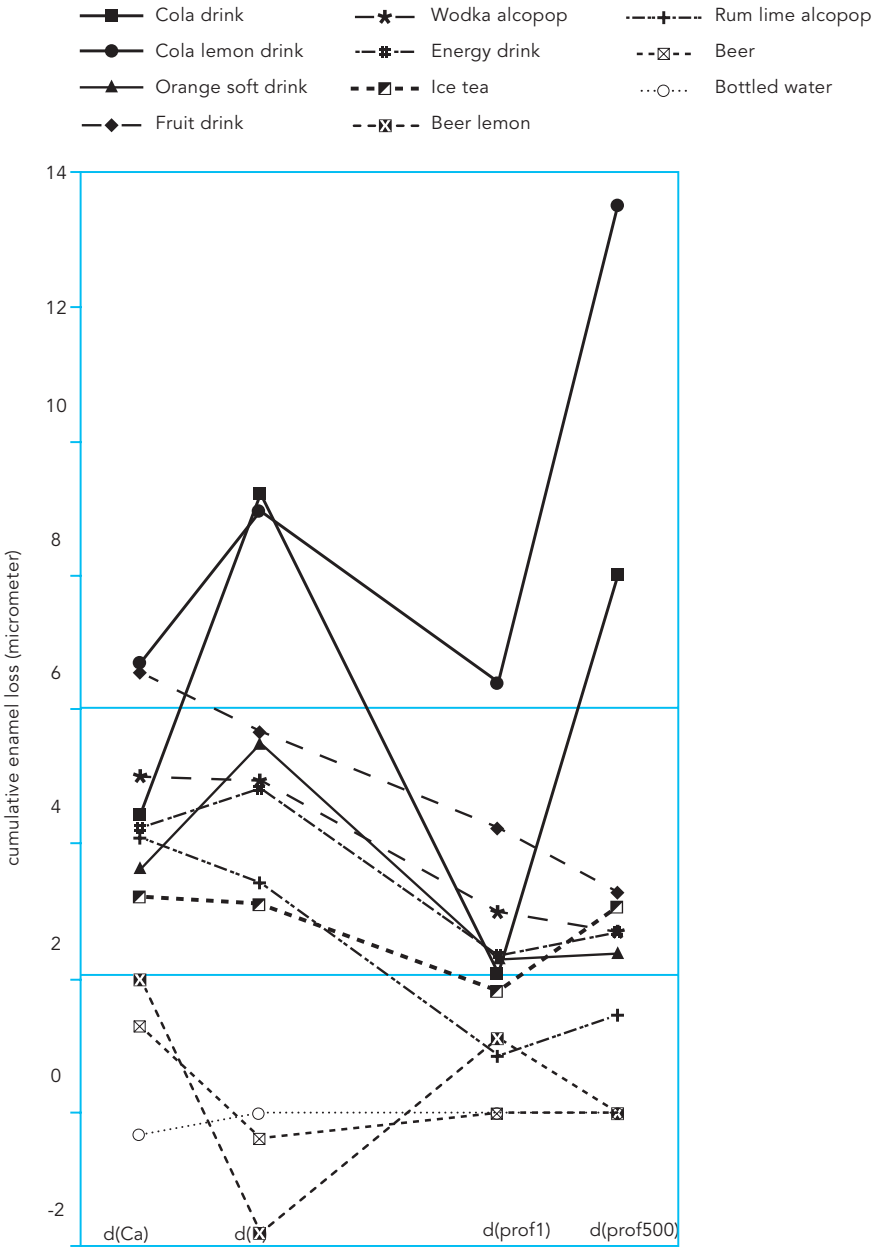
Table 3. Parameters for the linear least squares regressions of Ca concentration on Pi concentration for all beverages. The Pi concentration was the independent (x) variable.

	Slope	Y-Intercept	R^2
Cola drink	0.47	0.14	0.75
Cola drink lemon	0.75	0.17	0.53
Orange soft drink	0.76	0.07	0.98
Fruit drink	1.69	0.06	0.91
Vodka alcopop	1.45	0.05	0.99
Energy drink	2.17	0.14	0.86
Ice tea	1.07	0.08	0.80
Beer lemon	0.10	0.44	0.19
Rum lime alcopop	1.49	0.06	0.97
Beer	0.05	0.09	0.07
Bottled water	6.94	0.09	0.18

Table 4. Cumulative loss of enamel after 63-min total exposure to the beverages.

	d(Ca)	d(Pi)	d(prof 1ml)	d(prof 500 ml)
Cola drink	4.44 (0.22)	9.22 (1.25)	2.08 (0.58)	8.04 (3.62)
Cola lemon drink	6.72 (0.36)	8.97 (1.75)	6.42 (1.15)	13.54 (4.31)
Orange soft drink	3.64 (0.22)	5.50 (0.38)	2.29 (0.88)	2.37 (0.51)
Fruit drink	6.55 (0.53)	5.67 (0.72)	4.24 (2.53)	3.27 (1.17)
Vodka alcopop	5.00 (0.85)	4.94 (0.88)	2.98 (1.06)	2.69 (1.35)
Energy drink	4.25 (0.96)	4.84 (0.18)	2.34 (0.85)	2.69 (0.96)
Ice tea	3.23 (0.48)	3.11 (0.33)	1.80 (1.34)	3.08 (0.63)
Beer lemon	1.99 (0.32)	-1.79 (2.02)	1.12 (0.98)	0.00
Rum lime alcopop	4.09 (0.31)	3.43 (0.29)	0.84 (0.70)	1.47 (0.72)
Beer	1.30 (0.23)	-0.38 (1.16)	0.00	0.00
Bottled water	-0.33 (0.92)	0.01 (0.01)	0.00	0.00

Figure 1. Cumulative loss of enamel as measured by the four techniques, showing both the quantitative loss and the rank order for each beverage. To facilitate comparison between the techniques for each beverage, the points have been connected. The plot area is divided into 3 areas: little or no erosion (-2 to 2 µm); moderate erosion (2 to 6 µm); and severe erosion (6 to 14 µm).



Discussion

In this study bovine enamel was used. Most in vitro studies use bovine enamel since it has been considered a suitable substitute for human enamel (Zero, 1996). Although Meurman and Frank (1991) did not observe any difference in the progression of erosion or the surface ultrastructure of erosive lesions between bovine and human prismatic enamel, another study showed that morphological differences such as a higher porosity exist when compared to human enamel, which result in higher rates of artificial caries lesion formation (Featherstone and Mellberg, 1981). It should be considered that in this study a comparison between the methods was performed and not an extrapolation of the results to the clinical situation.

The Pi analysis of the beers and the Ca analysis of the energy drink yielded negative values. For these beverages the differences between the Ca analysis and the Pi analysis and the negative values may possibly be explained by their chemical composition. Because of the high baseline concentrations of Ca and Pi it was sometimes necessary to use high dilutions which may have increased the measurement error. A high concentration of Pi in the colas and the beers could also have interfered with the Ca measurements by calcium binding but this was prevented by adding a high concentration of lanthanum. However, the amount of Ca and Pi released from the enamel into solution by erosion was for some beverages relatively small compared to the baseline concentration Ca or Pi in the beverages, especially for the short exposure times. This resulted in small changes in mineral concentration, which did not always exceed the measurement error and made it difficult to obtain reliable measurements.

Previous studies, using standard erosive solutions as for example citric acid or lactate buffer reported almost perfect agreement between the Ca and Pi analyses (Ganss et al., 2005; Zero et al., 1990). Because of the use of standard solutions in these studies the influence of the composition of the erosive solution on the results could not be determined. In this study, for 3 of the drinks a Ca/P ratio (table 2) significant deviations from 1.6 (the Ca/P ratio of bovine enamel) was found. This suggests that re-precipitation occurred during the erosive exposure. However in none of the drinks could a supersaturation with respect to other calcium phosphates be calculated. No explanation could be found for the phenomenon which, especially for the orange drink, does seem to be systematic, looking at the decline of the Ca/P ratio with increasing exposure time. The short exposures and the low erosive drinks were found irrelevant for the calculation of the Ca/P ratio because of the low enamel losses.

Profilometric analysis showed a trend for lower enamel loss compared to Ca and Pi analysis. These results are in agreement with the findings of a previous study where Ca/Pi analysis and contact profilometry were compared (Ganss et al., 2005). The difference found between the chemical methods and the profilometry may be

explained by the fact that the erosion process does not remove only enamel layers but also causes a "softening". Profilometry does not account for the subsurface loss of the softened layer. The depth of the softened layer is unknown but may be more than 10 µm (Eisenburger et al., 2004).

Removal of reaction products and the supply of fresh acid are important for the continued formation of erosion lesions (Eisenburger and Addy, 2003). In a study investigating the relationship between enamel erosion and liquid flow rate it was concluded that the rate of erosion is dependent on liquid velocity, exposure time and the total volume of the acidic solution (Shellis et al., 2005). In this study agitation of 1 ml probably results in a different replacement of liquid at the enamel surface compared to agitation of a 500 ml reservoir, thus influencing the erosion rate. However, regarding the exposure volume of the beverage, our study found significant differences between the measured erosive potential only for the two cola drinks.

Some authors have observed that a small change in the degree of saturation resulted in a difference in the dissolution of enamel and that it is an important parameter that defines the ability of a solution to demineralise enamel (Barbour et al., 2003; Finke et al., 2000; Margolis et al., 1999; Tanaka and Kadoma, 2000). Only in a small volume of the beverages would a significant rise in saturation be expected. To ascertain whether this was the case in the present study the saturation with respect to hydroxyapatite was determined for all 1 ml volumes before and after exposure. It was found that the degree of saturation rose with increasing exposure time. However, this was seen in all beverages and the highest rise was found for the energy drink. This did not explain our findings as only the cola drinks showed a lower erosion in the 1 ml exposure.

Although pH is a parameter in the calculation in the degree of saturation it has been reported as a separate factor in erosive potential (Margolis et al., 1999; Larsen and Nyvad, 1999). A rise in pH would result in a slowing down of the erosion process. Only for the cola drink and the cola lemon drink a measurable, if low (0.1), rise in pH was found. As this corresponds to the observed reduced erosion for the 2 cola drinks in the 1 ml exposure model, we assume that pH was a determining factor.

In conclusion, the present study has shown that the composition of the beverages had a significant effect on the determination of the erosive potential with chemical analyses. This should be considered when choosing an appropriate measurement method. Optical profilometry is suggested as a beverage-independent alternative. Beverage composition also influences the effect of small vs. large exposure volumes, indicating the need for standardisation of exposure parameters such as exposure times, volumes and flow rate of the drinks during exposure to prevent differences in erosion rate due to differences in liquid velocities.

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Chapter 3

Estimated erosive potential depends on exposure time

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Abstract

Objectives

to evaluate erosive potential of beverages, using exposure times from 3 to 30 min, and to analyze the relationship between erosion and several drink parameters if possible using a multivariate approach.

Methods

pH, calcium, phosphate and fluoride concentration, saturation, titratable-acidity to pH 5.5 and the viscosity of sixteen beverages were measured or calculated. Bovine buccal enamel samples (N = 90) were serially exposed to 1 ml of the beverages for 3, 6, 9, 15, and 30 min and enamel erosion was measured as the loss of calcium to the beverage. Rate of erosion per min was calculated by linear curve fitting using all exposure times. Linear regression analysis was performed to determine the correlation between erosion and the drink parameters. A limited multivariate analysis was performed for the outcome parameter with the highest univariate correlations (erosion per minute) and 4 drink variables.

Results

A consistently negative relationship was observed for all exposure times only for pH. Only for erosion per min a significant relationship with pH and saturation was found. In a model for erosion per min using only saturation, fluoride concentration, titratable acidity and viscosity, both saturation and viscosity were shown to have a significant effect ($p = 0.01$ and $p = 0.05$, respectively).

Conclusion

This study showed that the choice of exposure time between 3 and 30 min resulted in very different estimates of erosive potential. There is no sound theoretical ground for preferring one or other exposure time / outcome as being more clinically relevant.

Clinical relevance

This study shows that effect of the choice of study methodology on the measurement of erosive potential of beverages is very large.

Introduction

Dental erosion is defined as an irreversible loss of dental hard tissue due to a chemical process without involvement of micro organisms (Imfeld, 1996). Dental erosion may be caused by either extrinsic or intrinsic factors. One of the extrinsic causes of dental erosion is excessive consumption of acidic beverages (Dugmore and Rock, 2004). The consumption of acidic beverages has risen over the last years. In the USA a 300% increase in soft drink consumption in 20 years is reported (Cavadini et al., 2000).

Research into drink erosive potential has concentrated on a number of drink parameters such as pH, titratable acidity, concentrations of calcium, phosphate and fluoride, and the degree of saturation with respect to hydroxyapatite or fluorapatite (Larssen, 1973; Lussi et al, 1993 and 1995; Larssen and Nyvad, 1999). There are more beverage characteristics, such as viscosity, that might be expected to influence the erosive potential of a drink (Busscher et al., 2000). Multivariate modelling has been proposed to predict erosive potential of beverages based on their chemical properties (Lussi et al., 1993 and 1995). This would be an attractive option, but may prove elusive, due to the number of factors that may be involved and their complex interactions (Barbour et al., 2011).

Study methodology for erosive potential of beverages varies widely (Barbour et al., 2011). Not only does this hamper comparison, the validity of different methods is not established. Most studies used single exposures, but exposure time vary, usually to fit the measurement technique used from 15 s to 2 min (Jensdottir et al., 2006; Lussi et al., 2011) up to more than 24 hr (Jensdottir et al., 2005). When multiple exposure times were used, these were analysed separately (Hara and Zero, 2008). Although it is suggested that clinical exposures are short, up to a few minutes, after which time oral conditions have returned to normal. However, drinking a normal volume of beverage (e.g. a can of 300 ml) is likely to involve a longer time. There is no current knowledge of the clinically most relevant exposure. Because erosive wear is clinically the result of cumulative exposures to acids, times of up to 30 min exposure could well be interesting.

It was the aim of this study to evaluate erosive potential of beverages, using both short and longer exposure times, and to analyze the relationship between erosion and several drink parameters, including viscosity, if possible using a multivariate approach.

Materials and Methods

Preparation of samples

A total of 90 buccal surfaces of extracted bovine incisors, stored in water, were ground flat with water-cooled siliciumoxide 220 grit grinding discs (SIA siawat P220, Frauenfeld, Switzerland) and cut into blocks of approximally 5 × 3 mm using a vertical sawing machine with a diamond saw blade (11-4243, Buehler, Düsseldorf, Germany). The blocks were embedded in acrylic resin (Autoplast polymer, Candulor AG, Wangen, Switzerland) leaving the enamel surface uncovered and subsequently the samples were polished flat (800 – 1200 grit grinding paper) and thoroughly rinsed with tap water.

Beverages

16 beverages, all available in The Netherlands, were included in this study. Six soft drinks: Sprite, Fanta Orange, Coca Cola, Coca Cola light lemon (all Coca-Cola Enterprises Nederland B.V., Dongen, The Netherlands), Lipton ice tea (Unilever, Rotterdam, The Netherlands), Schweppes Tonic (Riedel Beverages, Ede, The Netherlands). Four fruit based beverages: Appelsientje apple juice, Spa & Fruit Forest Fruit, Dubbelfriss orange / pink grapefruit and Vitamientje mixed fruit juice (all Riedel Beverages). Two sport beverages: AA-drink high energy (United Soft Drinks B.V., Utrecht, The Netherlands) and Isostar Lemon (Isostar BVBA, Erpe-Mere, Belgium). Also four alcoholic beverages: Breezer Lime (Bacardi Martini NV, Gouda, Nederlands), Smirnoff Ice (Diageo, London, UK), Grolsch beer lemon (Inbev Nederland, Breda, The Netherlands), and Bavaria beer (Bavaria NV, Lieshout, The Netherlands).

The pH of the beverages was measured 5 times using a calibrated glass pH electrode (Radiometer, PHM 84 Research meter, G202C, Copenhagen, Denmark) in 100 ml of the degassed beverages. The temperature in the laboratory was 21 °C (± 2 °C is expected). Standard buffers, pH 7.01 and 4.00 were used (measurement uncertainty: ± 0.015 units, Merck KGaA, Darmstadt, Germany). Calibration was performed with these buffers at the beginning of every experimental day.

The titratable acidity of the beverages was determined by monitoring the pH changes after serial additions of 1 ml of 0.5 M NaOH recording the volume necessary to increase the pH of the beverage up to pH 5.5 and pH 7.0 in 100 ml of each beverage.

All beverages were analyzed for phosphate concentration by a modified acid-molybdate method (Chen et al., 1956) and for calcium concentration by atomic absorption spectroscopy (Vieira et al., 2005). Calcium and phosphate concentration were expressed in mmol/l and fluoride concentration in ppm. The beverage's baseline degree of saturation with regard to hydroxyapatite (DSHA) was calculated by means of a computer program (Shellis, 1988), using the baseline pH and cal-

cium and phosphate concentrations of the beverages after degassing.
Fluoride concentration was measured using a fluoride ion-specific electrode in combination with a digital mV meter (fluoride electrode cat. no. 940900, Orion Research Inc, Cambridge MA, USA) in 5 ml of the beverage after addition of 0.5 ml TISAB III (Orion Research Inc, Cambridge MA, USA).
Viscosity was determined with 0.5 ml of beverage in a cone-plate viscometer (Brookfield DV-II + Pro Wells Brookfield cone/plate Middleboro, MA., USA) and expressed in mPas.

Erosive exposures

In order to remove the smear layer and any loosely attached material from the polished surfaces, the samples were cleaned for 3 min under agitation in a standard solution of 50 mM citric acid, 0.4 mM KH_2PO_4, 0.4 mM $CaCl_2$ and 1 mM NaN_3 (pH = 3) and subsequently rinsed with tap water before starting the demineralization procedure. The samples were partly covered with PVC tape exposing an area of approximately 3 x 3 mm in the centre of the enamel sample. Five enamel samples were individually submersed in 1 ml of each beverage (all degassed) in a test tube for exposure periods of 3, 6, 9, 15 and 30 min under constant agitation (shaking table, 100 rpm). After each exposure period the beverage was analyzed for calcium concentration and a new beverage volume was used for the next exposure period. The loss of calcium as measured by atomic absorption spectroscopy was recalculated as loss of enamel expressed in µm as described in an earlier publication (Jager et al., 2008). As erosion is expected to be linearly related to exposure time, linear regression was performed on the 5 exposure time (3, 6, 9, 15 and 30 min) results for each drink, and the slope of the fitted line was used as a measure of surface loss per minute.

Statistical analysis

Linear regression analysis was performed to determine the correlation between the 6 erosion outcome measures (5 exposure times and the surface loss per minute) and the drink parameters. A multivariate analysis was not possible for all drink parameters, due to the correlation between several parameters and the limited number of beverages. However, a limited multivariate analysis was performed for the outcome parameter with the highest univariate correlations (surface loss per minute) and 4 drink variables.

Results

The baseline pH, titratable acidity to pH 5.5, calcium concentration, phosphate concentration, fluoride concentration, saturation with respect to hydroxyapatite (DSHA) and viscosity of the beveraged are presented in table 1. For all outcome measures from the chemical analysis the surface loss in µm as estimated from the measured calcium loss are presented in table 2: 3, 6, 9, 15, and 30 min exposure and surface loss per minute.

Table 3 summarizes all the correlation coefficients of enamel loss with the beverage parameters. Only the relationship with pH is consistently negative, and it shows a monotonic relationship with erosive challenge time. For all single chemical measurement outcomes the correlations are quite low and variable. Only when they are combined into the loss per minute outcome variable, do correlations become substantial, although still only the relation with pH and saturation are significant.

Although most beverages show a linear relationship between erosion and exposure time (figure 1), excluding the beers which show only negligible erosion, two beverages, though causing erosion, show no relationship of erosion with exposure time at all: Vitamientje and Isostar. This is reflected in table 4, where they rank among the highest eroders in the 3 min exposure, but among the lowest in the 30 min exposure. Also, the regression lines of several beverages do not cross the Y-axis at or near the 0-level, indicating relatively high erosion during the first few minutes, with Sprite as the most extreme example.

Multivariate analysis was not possible using all drink parameters, as there was substantial correlation between many of them and the data set was limited. However, in a model for erosion per minute, and using only saturation (assuming that pH, calcium and phosphate were represented in this variable), fluoride concentration, titratable acidity and viscosity, both saturation and viscosity were shown to have a significant effect ($p = 0.01$ and $p = 0.05$, respectively). However, the strength of the model was limited (adjusted $r^2 = 0.37$), and the plot of erosion per minute by saturation (figure 2) shows that the assumption of a linear relationship does not hold.

Table 1. Drinks used in this study with their composition variables, results are average of 2-5 (pH, Ca) measurements. ⟶

Table 2. Enamel loss results for the different drinks, for all exposures / measurements separately (n = 5 for each measurement). Loss after 3, 6, 9, 15 and 30 min exposures chemically measured as calcium loss. The slope of a linear curve fitting is presented as estimated loss per minute. The drinks are arranged in order of decreasing surface loss per minute (last column). All results are presented as µm, calculated from the calcium loss for chemical measurements. ⟶

Table 1.

Drink	pH	TA to pH = 5.5	Ca mmol/l	Pi mmol/l	Fluoride ppm	Saturation (HAP)	Viscosity mPas
Sprite	2.81	6.80	0.07	0.00	0.16	0.0000	1.32
Fanta orange	3.03	11.80	0.06	0.19	0.11	0.0014	1.55
Coca Cola	2.47	1.60	0.87	4.80	0.00	0.0054	1.49
Coca Cola light lemon	2.73	8.90	0.73	4.90	0.60	0.0085	0.99
Lipton Ice tea	3.8	12.40	0.12	0.25	0.46	0.0095	1.19
Schweppes	2.95	4.20	0.00	0.01	0.07	0.0000	1.27
Appelsientje	3.46	14.20	2.61	2.20	0.03	0.0489	1.47
Spa & Fruit	3.19	6.10	0.61	0.70	0.09	0.0101	1.24
Dubbelfriss	3.35	17.10	1.30	0.51	0.05	0.0177	1.29
Vitamientje	3.63	26.00	2.62	3.59	0.16	0.0785	2.32
AA-drink	2.76	10.70	1.12	0.03	0.09	0.0021	1.58
Isostar Lemon	3.9	14.50	7.69	5.43	0.07	0.2324	1.20
Breezer Lime	3.87	14.50	0.17	0.02	0.04	0.0056	1.63
Smirnoff Ice	3.43	19.20	0.15	0.00	0.13	0.0000	1.47
Grolsch beer lemon	3.83	6.60	0.96	3.51	0.11	0.0679	1.24
Bavaria beer	4.2	3.60	0.72	5.30	0.09	0.1254	1.44

Table 2.

Drink	3 min	6 min	9 min	15 min	30 min	Loss per minute
Sprite	3.74	3.88	4.04	4.41	5.34	0.060
Apple Juice	1.06	0.93	1.28	2.04	3.81	0.110
AA-drink	1.53	1.30	1.34	1.74	2.74	0.052
Coca Cola light lemon	0.37	0.33	0.47	0.76	1.56	0.083
Spa & Fruit	0.52	0.81	1.07	1.54	2.78	0.047
Dubbelfriss	0.51	0.85	1.10	1.35	2.75	0.080
Isostar Lemon	1.59	1.14	1.52	2.13	1.05	-0.011
Vitamientje	1.55	0.86	0.81	1.84	1.24	0.006
Smirnoff Ice	0.80	1.01	1.23	1.09	2.12	0.045
Schweppes	0.46	0.39	0.75	1.02	1.81	0.053
Fanta Orange	0.40	0.42	0.58	0.80	1.44	0.040
Coca Cola	0.38	0.65	0.80	1.04	1.53	0.040
Lipton ice tea	0.34	0.46	0.61	0.64	1.18	0.029
Beer lemon	0.29	0.25	0.37	0.39	0.70	0.016
Breezer Lime	0.43	0.55	0.68	0.89	1.55	0.041
Bavaria beer	0.29	0.28	0.28	0.15	0.31	0.001

Estimated erosive potential depends on exposure time

Table 3. Pearson's correlation of measured loss with drink parameters for all outcome measures. A star indicates a significant correlation.

	3 min	6 min	9 min	15 min	30 min	Loss per minute
pH	-0.23	-0.34	-0.35	-0.36	-0.54*	-0.53*
TA 5.5	0.15	0.03	0.02	0.16	0.03	-0.03
Calcium	0.23	0.04	0.09	0.24	-0.14	-0.35
Phosphate	-0.13	-0.21	-0.21	-0.12	-0.38	-0.39
Fluoride	-0.04	0.03	0.03	0.02	0.07	-0.26
Saturation	0.09	-0.09	-0.07	0.01	-0.40	-0.62*
Viscosity	0.17	-0.01	-0.09	0.06	-0.13	-0.26

Table 4. Ranking of the beverages in erosive potential, using selected outcome measures. While some drinks have a fairly stable position (for example, Sprite and Apple Juice in the high range, and the beers and Lipton ice tea in the low range), for some drinks, notably Vitamientje and Isostar, their ranking is highly dependent on the selected outcome measure.

3 min	30 min	Loss per minute
Sprite	Sprite	Apple Juice
Isostar	Apple Juice	Coca Cola light lemon
Vitamientje	Coca Cola light lemon	Dubbelfriss
AA-drink	Dubbelfriss	Sprite
Apple Juice	AA-drink	AA-drink
Smirnoff Ice	Smirnoff Ice	Spa & Fruit
Coca Cola light lemon	Schweppes	Smirnoff Ice
Dubbelfriss	Spa & Fruit	Schweppes
Schweppes	Breezer Lime	Fanta Orange
Breezer Lime	Coca Cola	Coca Cola
Fanta Orange	Fanta Orange	Breezer Lime
Coca Cola	Vitamientje	Lipton ice tea
Spa & Fruit	Lipton ice tea	Grolsch beer lemon
Lipton ice tea	Isostar	Vitamientje
Grolsch beer lemon	Grolsch beer lemon	Bavaria beer
Bavaria beer	Bavaria beer	Isostar

Figure 1. Results of the chemical measurement of erosion at the 5 different exposure times for all beverages, with linear curve fitting. On the Y-axis surface loss (in mm) is shown, on the X-axis exposure time (always up to 30 mins).

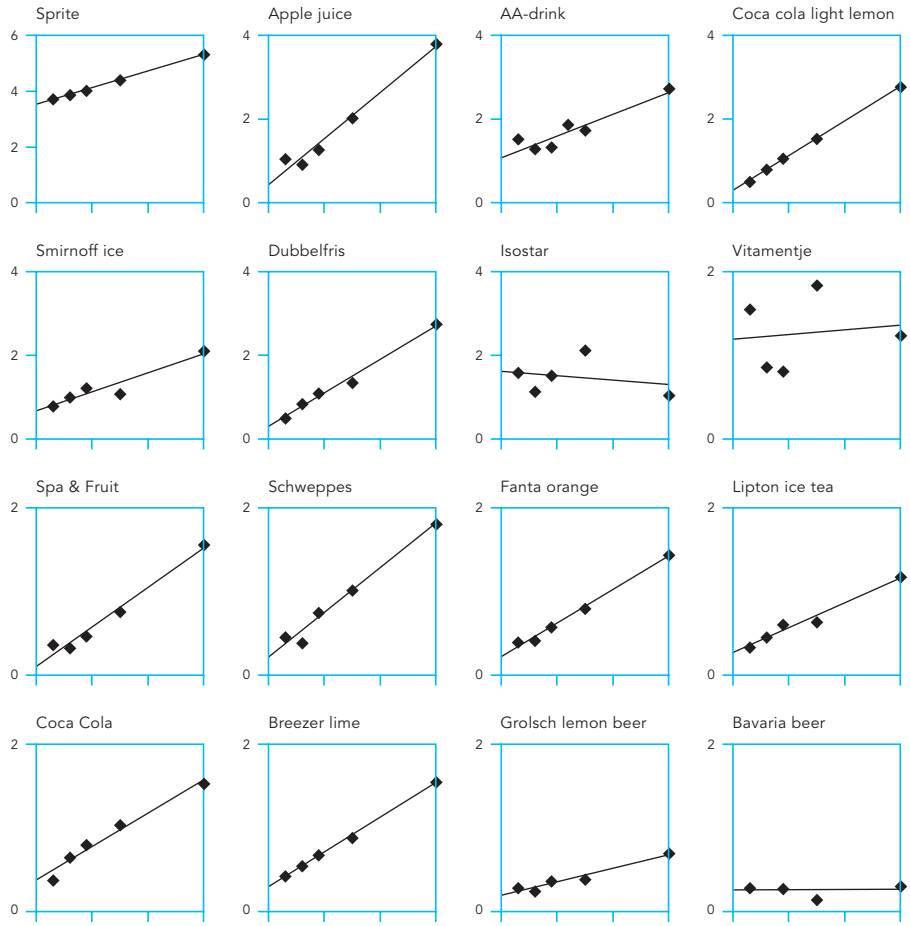
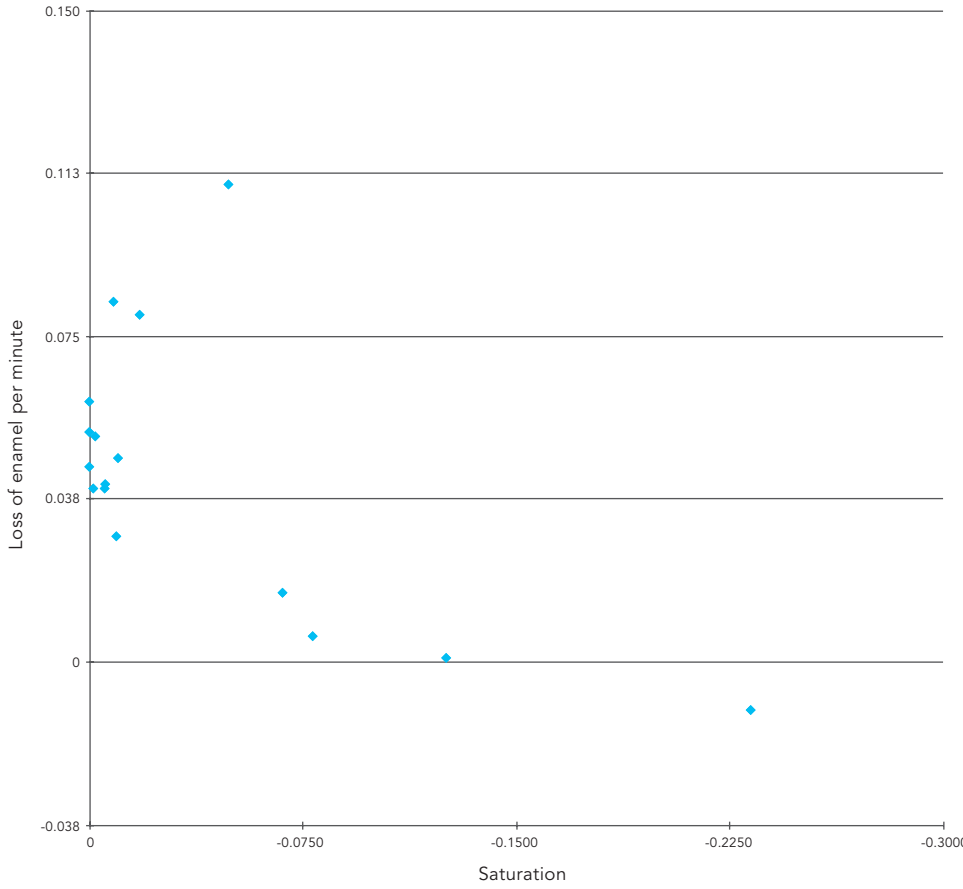


Figure 2. Relationship between saturation and enamel loss per minute.



Discussion

In this study it was confirmed that main parameters involved in erosive potential of beverages are pH and saturation. The only consistent parameter across the different outcomes, even if only significant for 3 of them, was pH, confirming previous reports (Grobler and van der Horst, 1982; Larsen and Nyvad, 1999; Larsen and Richards 2002). In our study the enamel loss decreased linearly with a rise in pH between pH = 2 and 4, again in accordance with previous reports (Larsen and Nyvad, 1999; Barbour et al., 2003). Also the apparent limitation of erosion at about pH = 5.0 fits with other publications (Barbour et al., 2011).

It is well recognized that degree of saturation is the basic thermodynamic driving force for dissolution. However, the value of this parameter in predicting levels of erosive potential has been questioned, especially below levels of about 0.005 (Barbour et al., 2011). It was expected that most beverages would show lower saturations levels. However, in our study, only 5 out of 16 beverages fell below this level. Overall, the relationship between saturation and one of the outcome measures, loss per minute, was strong, if not linear (figure 2).

Calcium and phosphate have been identified as factors in erosive potential many times with calcium being the more important factor (Barbour et al., 2011). This was not confirmed in our study. Possibly, the range of concentrations represented in the study was not high enough. In a study with beverages with added calcium, a significant effect of calcium was found, but for generally higher concentration (≥ 3.2 mmol/l, Hara and Zero, 2008). However, when calcium and phosphate are added the pH also usually rises, and the effects are hard to separate (Barbour et al., 2011).

The limitations of the above mentioned variables to predict erosive potential could be seen when two beverages are compared: Apple Juice and Vitamientje fruit drink. Quite similar in pH, calcium, phosphate concentration and degree of saturation, they still have completely different erosive behaviour (figure 1). It must be concluded that there are important variables yet unknown and unmeasured, which influence this behaviour.

Titratable acidity did not emerge as an important parameter. In our model we only included titratable acidity to pH = 5.5 and not to pH = 7 as has been used before (Lussi et al., 1993). In many studies, as well as in this study, erosion is minimal from a pH of about 5.0 (in our study even pH = 4) or higher (Barbour et al., 2011). It could therefore be assumed that a titratable acidity above pH = 5.0 is not relevant anymore.

Fluoride concentration was not confirmed as a significant factor in this study. Earlier, Lussi and co-workers (1993 and 1995) found a significant effect using 20 min exposures, whereas others found no effect using 48-72 hrs exposures (Larsen and Nyvad, 1999; Larsen and Richards, 2002). Overall, it is unlikely that the fluoride levels in the beverages, all well below 1 ppm, would have an erosion reducing effect (Larsen, 2001).

The factor that was not studied before, viscosity, was only found to be significant in a multivariate model using loss per minute as the outcome variable. It was hypothesized that viscosity would contribute to the effect of a so-called Nernst layer, a thin layer of solution closest to the enamel surface, which is relatively stable. By slowing down replacement of the solution at the surface, viscosity could slow down erosion. This phenomenon could also be related to the penetration coefficient of liquids. The viscosity of a drink, together with contact angle and surface tension, determines its penetration coefficient (Perdok et al., 1990), a measure of the ability of a liquid to penetrate into a capillary space, such as pores. According to this theory a beverage with a low viscosity will have a high penetration coefficient and this results in a higher erosive potential. This phenomenon would depend on the formation of a porous, softened layer. The direction of the effect found agreed with this hypothesis, however, the evidence is for now too weak to conclude that drink viscosity is a relevant factor.

Our study used both of short and long exposure times, in order to evaluate whether this aspect of study methodology would have a large effect on results regarding erosive potential. The results show that this effect is very large, and for some beverages the estimated erosive potential is relatively high for short exposures and low for long exposure (table 4). The lack of linear relationship between exposure time and erosion (figure 1, Vitamientje and Isostar) and the relatively high erosion values for some beverages at the shortest exposure time (figure 1, Sprite, AA-drink and Apple juice) are two features, which hamper conclusions about relative erosive potential of beverages from a single exposure measurement. Table 4 shows how different conclusions about some beverages may be, depending on the chosen outcome variable.

This study showed that the choice of exposure time between 3 and 30 min resulted in very different estimates of erosive potential. There is no sound theoretical ground for preferring one or other outcome variable as being more clinically relevant and clinical studies comparing the erosive effect of different beverages are needed to be able to determine the validity of in vitro experiments. For ethical reasons, such studies will be difficult to perform.

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Chapter 4

The effect of saliva factors on the susceptibility of hydroxyapatite to early erosion

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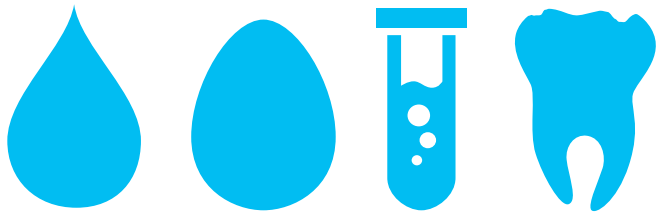

Abstract

Objective

Salivary pellicle is known to reduce the erosion of enamel and differences in level of protection exist between individual saliva sources, but which parameters or components are important is not known. The focus of this study was to investigate the relationship between saliva parameters and early erosion of hydroxyapatite (HAp) with an in situ grown saliva film.

Methods

Twenty-eight volunteers carried two hydroxyapatite and one porcelain discs in their buccal sulcus for 1.5hr. Next, the discs covered with pellicle and attached saliva film were exposed extraorally to 50 mM (pH = 3) citric acid for 2 min and unstimulated and stimulated saliva was collected. Calcium loss from HAp after erosive challenge was measured, corrected for calcium loss from pellicle on porcelain discs and averaged. Several salivary parameters were analysed. Pearson's linear correlation and multiple regression analysis was used to study the relation between saliva parameters and HAp-erosion.

Results

Significant correlations were found between HAp-erosion and the concentration of phosphorus in unstimulated saliva ($r = 0.40$, $p = 0.03$) and between HAp-erosion and the concentration of sodium ($r = -0.40$, $p = 0.03$), chloride ($r = -0.47$, $p = 0.01$), phosphorus ($r = 0.45$, $p = 0.01$) and flow ($r = -0.39$, $p = 0.04$) of stimulated saliva. Multivariate-analysis revealed a significant role in the HAp-erosion for sodium, urea, total protein, albumin, pH and flow of unstimulated saliva and sodium, potassium, urea, and phosphorus of stimulated saliva.

Conclusion

Several salivary parameters are associated with the susceptibility of hydroxyapatite to erosion.

Introduction

A wide variation between individuals has been found regarding their susceptibility to develop dental erosion (O'Sullivan and Curzon, 2000; Vieira et al., 2007). Also in in vitro research it was found that saliva from different donors affords different levels of protection against erosion (Wetton et al., 2007) and in an in situ study it was found that the variation between high and low eroders can reach up to ten-fold differences (Hughes et al., 1999b). Moreover, results of in vitro studies investigating the erosive potential of soft drinks showed losses of enamel many orders of magnitude greater than recorded on specimens in situ (West et al., 1998; Hughes et al., 1999a). In all these phenomena saliva may play an important role (Hall et al., 1999).

Saliva can theoretically protect against erosion in several ways, but it is unclear how effective its protective capacity is. Saliva can act as a diluting agent for acids and salivary clearance removes the acid gradually via the swallowing process. In addition saliva contains phosphate, protein and bicarbonate buffers and saliva is supersaturated with respect to tooth minerals, such as calcium and phosphate. Moreover, saliva contains a wide array of proteins and some of them might have protective properties. Finally, proteins can protect the teeth by the formation of a salivary pellicle when teeth are exposed to saliva (Siqueira et al., 2007). This pellicle may act as a barrier for acids (Dawes, 2008). In hyposalivation, carious destruction and erosive wear are phenomena that occur simultaneously (Jansma et al., 1989; Lajer et al., 2009). With respect to the development of caries it was found that the salivary pellicle derived from whole saliva plays a preventive role (Featherstone et al., 1993).

It is still unclear which salivary parameters are most important in explaining the differences in susceptibility to erosive wear. We hypothesize that salivary parameters can, at least in part, explain variability in susceptibility to erosion. Therefore the aim of this study was to investigate the inter-individual variation in early erosion of hydroxyapatite covered with and in situ grown pellicle/salivary film and to relate these to salivary parameters.

Materials and Methods

Subjects, substrate and growth of pellicle

Twenty-eight volunteers with no relevant medical or pharmacotherapy histories (16 females, 12 males) in the age range of 19 to 59 years were recruited from advertisements displayed in the UMCG. The sample size was based on an earlier performed pilot study (UMCG IRB #2007170). Of the measured saliva parameters a correlation was found between the concentration phosphorus in stimulated saliva and the loss of calcium from HAp. With the data from this pilot study an estimation of the sample size was calculated with the software PS-power and sample size (Dupont WD and Plummer WD., 1990). The σ was calculated with the standard deviation of the independent variable (phosphorus concentration in stimulated saliva; 0.32), the slope of the regression curve (λ ,0.108) and the standard deviation of the dependent variable (loss of calcium from HAp; 1.02). The sample size was calculated with an α of 0.05, a power of 0.8 and a slope of the regression curve of 1. This resulted in an estimated sample size of 24. Only participants with a healthy oral environment (i.e. Dutch Periodontal Screening Index (van der Velden, 2009)) score of 1 or lower, no recent caries activity, no erosive wear and no hypo salivation) and with no relevant medical or pharmacotherapy history (American Society of Anaesthesiologists score 1, (Owens et al., 1978)) were allowed to participate. Informed written consent was obtained from all the subjects. The study design was reviewed and approved by the University Medical Center Groningen Investigators Research Board (UMCG IRB #2008109).

In every volunteer, two sintered hydroxyapatite (HAp) discs (Himed medical applications Inc, Old Bethpage, NY, USA) and one porcelain disc (IPS Emax press, IvoclarVivadent, Schaan, Principality of Liechtenstein) were placed in the buccal sulcus of the lower jaw in close proximity to the first molar of every volunteer at 9.00 a.m. The discs had a diameter of 8 mm and a thickness of 2 mm. All the HAp discs came from the same batch (batch no: 100406). Before placing the discs into the mouth, the discs were submersed in 15 mL of a standard solution of 50 mM citric acid (pH = 3) for 1 hr and rinsed with water to remove any loosely attached or more soluble material. After this exposure the discs are clean and dissolve all in a very homogenous way (Hemingway et al., 2008).

Eating, drinking, brushing and smoking were not allowed from 1 hr before insertion until removal of the samples from the mouth (both the HAp and porcelain samples were 90 minutes in situ)

Exposure to citric acid

The HAp discs covered with pellicle and attached saliva film were removed from the oral cavity and without rinsing immediately exposed for 2 minutes to 2 mL of an erosive solution (50 mM citric acid, pH = 3) under agitation (100 rpm) and

rinsed with 2 mL of demineralised water. The loss of calcium was determined by atomic absorption spectroscopy as described in a previous publication (Jager et al., 2008). The porcelain discs were exposed to the erosive solution in a similar fashion to determine calcium loss from pellicle and salivary film only. The loss of calcium from the two HAp discs was averaged and the loss of calcium from the pellicle from the porcelain disc was deducted from this value to correct for the extra calcium measured coming from the pellicle or the salivary film. The thus corrected amount of calcium loss was used as a measure of HAp-erosion.

Collection of saliva, storage and analysis

Twenty minutes after removal of the HAp and porcelain discs from the mouth unstimulated and stimulated whole saliva were collected for a series of analyses. Unstimulated saliva was collected by the draining method in a preweighed plastic cup (Navazesh and Christensen, 1982). Stimulated saliva was collected by chewing on a piece of parafilm (Parafilm M, Pechiney Plastic Packaging Company, Chicago, IL, USA) at a chewing frequency of 70 chews per minute during collection. After each collection period the plastic cup was reweighed and the salivary flow rate (mL/min) was estimated by dividing the volume of the saliva sample (1 g of saliva equals 1 mL) by the collection time (min) (Navazesh and Christensen, 1982). Immediately after collection the salivary pH of both unstimulated and stimulated saliva was measured using a calibrated glass pH electrode (Radiometer, PHM 84 Research Meter, G202C, Copenhagen, Denmark). Calibration was performed daily using standard buffers, pH 7.01 and 4.00 (measurement uncertainty for both ± 0.015 units) (Merck KGaA, Darmstadt, Germany). The buffering capacity was measured by adding 0.5 mL of 5 mM HCI to the saliva used for the pH measurement. The end pH after addition of HCI was regarded as an indication for the buffering capacity of the saliva. The remaining saliva was transferred to Eppendorf tubes (Eppendorf AG, Hamburg Germany) and centrifuged for 5 min at 10000g at 4°C (Silletti et al., 2007). After centrifuging, the saliva supernatant was decanted and frozen in liquid nitrogen and stored at -80°C in plastic containers (Cryogenic Vials Nalgene tubes, Nalgene Nunc, Rochester, NY, USA).

The unstimulated and stimulated whole saliva were analysed for electrolytes (calcium, phosphorus, sodium, chloride) and urea concentration, total protein concentration and albumin. Sodium and chloride concentrations were measured after appropriate dilution using an ion-selective electrode. Total calcium was determined by a colorimetric assay based on the reaction of calcium with 0-Cresolphthalein Complexeone (Sigma-Aldrich, St. Louis, MO, USA) in alkaline solution (Gindler and King, 1972). Phosphorus concentration was measured by a modified acid-molybdate method (Chen et al., 1956). A kinetic UV assay based on the Talke and Schubert's method (Talke and Schubert, 1965) was used to measure the urea

concentration. The total protein concentration was determined turbidimetrically. For this method the saliva sample was preincubated in an alkaline solution containing EDTA, which denatured the protein and eliminated interferences from ions. Benzethonium chloride was added to produce turbidity, which was measured at a wavelength of 505 nm (Luxton et al., 1989). The albumin concentration was determined with an immunoturbidimetric assay. Anti-albumin antibodies were added to the saliva sample to form antigen/antibody complexes which, following agglutination, were measured turbidimetrically (Hubbuch, 1991). All the above-mentioned analyses were performed on a Roche/Hitachi 911 analyser and a COBAS Integra Chemistry Platform (Roche Diagnostics, Indianapolis, IN, USA).

Statistical methods

Pearson's correlation coefficient analysis was used to study the association between the HAp-erosion and the various salivary parameters. A p-value of 0.05 or lower was considered statistically significant. Furthermore, a multiple regression analysis with backward elimination was performed to determine the contribution of every saliva parameter to the HAp-erosion. This information was used to design a model containing all the variables of interest by a step-wise removal of the variable with the smallest F-statistic (cut-off level for p to remain in the model: 0.1). For the explanation of the variation in HAp-erosion by the model the adjusted r^2 was determined because it adjusts for the number of explanatory terms in a model. This was performed by R statistical software (version 2.10.1, R Development Core Team 2009).

Results

Calcium loss from the HAp (uncorrected) and from the pellicle plus salivary film are depicted in figure 1, showing the individual variation. When relating the measured HAp-erosion with the various salivary parameters, significant correlations were found between the HAp-erosion and the concentration of phosphorus in unstimulated saliva ($r = 0.40$, $p = 0.03$). For stimulated saliva a significant correlation was found between HAp-erosion and the concentration of sodium ($r = -0.40$, $p = 0.03$), chloride ($r = -0.47$, $p = 0.01$), phosphorus ($r = 0.45$, $p = 0.01$) and flow rate ($r = -0.39$, $p = 0.04$). All the correlation coefficients and the corresponding confidence intervals are presented in table 1. The results of the multiple regression analysis with backward elimination revealed that a significant role in the HAp-erosion was found for sodium, urea, total protein, albumin, pH and flow of unstimulated saliva and sodium, potassium, urea, and phosphorus of stimulated saliva. From this data a model predicting the HAp-erosion was suggested as shown in table 2. Using these parameters 72% (adjusted $r^2 = 0.72$) of the variation in HAp-erosion could be explained.

Figure 1. *Loss of calcium from HAp and pellicle for every volunteer.*

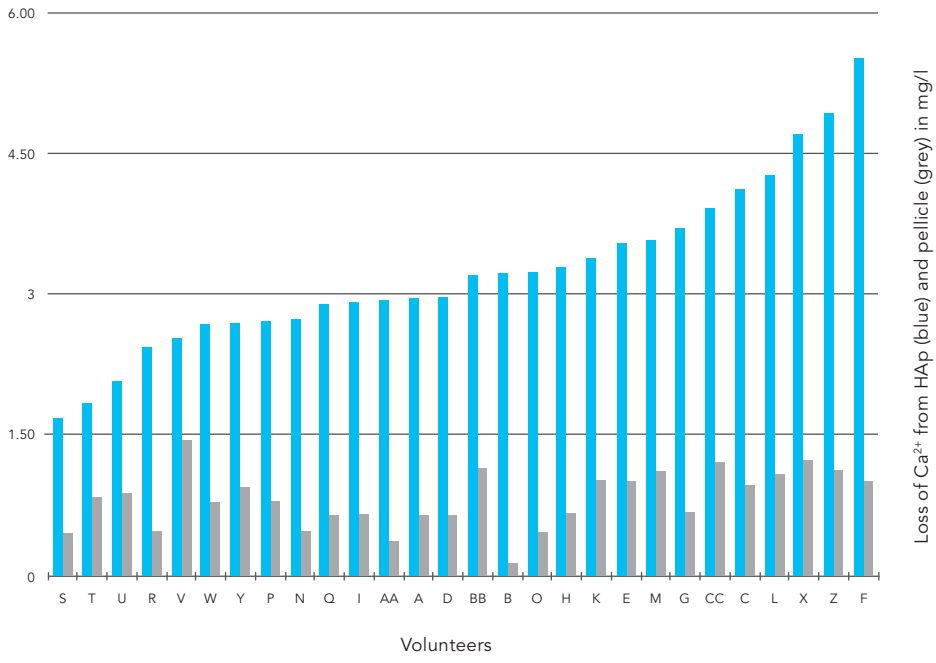

Table 1. Results of the analyses of (un)stimulated saliva, Pearson's correlation coefficients between HAP-erosion and salivary parameters and 95% CI. P values ≤ .05 are marked with an asterisk. [1]pH after addition of 0.5 ml 5 mM hydrochloric acid to 0.5 ml saliva.

	UNSTIMULATED SALIVA					STIMULATED SALIVA				
Salivary parameter	Mean	SD	Pearson's correlation coefficient	p-value	95% CI	Mean	SD	Pearson's correlation coefficient	p-value	95% CI
Sodium mmol/L	4.00	1.79	-0.338	0.073	[-0.63...0.03]	11.47	7.72	-0.402	0.031*	[-0.67 ... -0.04]
Potassium mmol/L	23.61	5.20	-0.026	0.893	[-039...0.34]	21.05	4,20	-0.053	0.785	[0.41 ... 0.32]
Chloride mmol/L	19.79	5.65	-0.020	0.916	[-0.38...0.35]	20.79	5.46	-0.472	0.010*	[-0.72 ... -0.13]
Urea mmol/L	6.89	2.60	0.154	0.424	[-0.23...0.49]	4.32	1.20	0.161	0.404	[-0.22 ... 0.50]
Calcium mmol/L	1.36	0.33	0.224	0.243	[-0.16...0.55]	1.05	0.20	0.082	0.672	[-0.29 ... 0.44]
Phosphate mmol/L	6.75	1.94	0.399	0.032*	[0.04...0.67]	4.50	1.25	0.450	0.014*	[0.10 ... 0.70]
Total Protein g/L	0.34	0.22	-0.076	0.694	[-0.43...0.30]	0.29	0.17	-0.191	0.320	[-0.52 ... 0.19]
Albumin mg/L	30.33	19.30	0.268	0.160	[-0.11...0.58]	21.12	14.26	0.250	0.190	[-0.13 ... 0.57]
pH	7.08	0.36	-0.118	0.543	[-0.46...0.26]	7.42	0.25	-0.056	0.773	[-0.41 ... 0.32]
Buffer Capacity[1]	1.10	0.44	-0.214	0.265	[-0.54...0.17]	0.90	0.25	-0.002	0.990	[-0.37 ... 0.36]
Flow mL/min	0.45	0.24	0.124	0.521	[-0.25...0.47]	1.93	1.01	-0.388	0.037*	[-0.66 ... -0.03]

Table 2. Results from the multiple regression analysis with backward elimination (cut-off level ≤ 0.1).

Variable	Type of saliva	Effect	p	95% CI
Constant		-11.40	0.002	[-17.79 ... -5.00]
Sodium	Unstimulated	-0.17	0.008	[-0.29 ... -0.05]
Sodium	Stimulated	0.07	0.010	[0.02 ... 0.12]
Potassium	Stimulated	-0.10	0.025	[-0.19 ... -0.01]
Urea	Unstimulated	-0.18	0.052	[-0.36 ... 0.00]
Urea	Stimulated	0.35	0.024	[0.05 ... 0.65]
Phosphorus	Stimulated	0.90	0.000	[0.61 ... 1.19]
Total Protein	Unstimulated	4.68	0.004	[1.67 ... 7.69]
Total Protein	Stimulated	-4.56	0.002	[-7.15 ... -1.97]
Albumin	Unstimulated	0.03	0.000	[0.02 ... 0.04]
pH	Unstimulated	1.39	0.003	[0.54 ... 2.23]
Flow	Unstimulated	1.62	0.000	[0.84 ... 2.40]

Discussion

This study investigated the relationship between whole salivary parameters and erosion of HAp. Analysis of the results revealed that several salivary parameters were related to the extent of erosion.

A higher flow rate of stimulated saliva was associated with a suppression of HAp-erosion. This observation corresponded with earlier reports in which also an inverse relationship between stimulated salivary flow rate and erosion was shown (Jensdottir et al., 2005). It was demonstrated that a high salivary flow rate resulted in higher concentrations of specific ions (such as sodium, calcium, chloride and bicarbonate) and proteins and was associated with a higher salivary buffer capacity (Larsen and Pearce, 2003; Dawes and Kubieniec, 2004). Moreover, a high salivary flow rate resulted in a better clearance of acids from the teeth surfaces (Jarvinen et al., 1991; Bashir et al., 1995). In our model, clearance of acids from HAp surfaces could not have played an important role as the HAp samples were extra-orally exposed to acids.

The concentration of chloride and sodium in stimulated saliva was found to be associated with the suppression of HAp dissolution as well. Earlier research has shown, however, that HAp dissolution is not inhibited by the incorporation of Cl^- ions into HAp through either ion exchange or adsorption in an ambient aqueous solution (Sugiyama et al., 1999). Therefore, it was suggested that the inhibition of

dissolution of HAp by Na^+ and Cl^- could be the result of a competition for HAp surface protonation sites between Na^+ and H^+ ions (Kwon et al., 2009). In addition, it should be noted that Na^+ and Cl^- ions account for more than 60% of the ionic strength of saliva (Schneyer et al., 1972) and therefore possibly may significantly contribute to the dissolution of HAp surfaces. Furthermore, the observed correlations could also be the result of an indirect effect as a rise in salivary flow rate is accompanied by a rise in sodium and chloride concentration.

Another electrolyte influencing the dissolution of HAp in our model was phosphorus in unstimulated and in stimulated saliva. It is suggested in earlier research that an increase of salivary phosphate concentration may result in desorption of salivary proteins from HAp (McGaughey and Stowell, 1974). This effect could be important in our study in which the HAp was exposed to an acidic challenge in the presence of only the salivary pellicle. Higher phosphate concentrations in saliva result in desorption of proteins form HAp, which in turn result in a reduction of the protective strength of the salivary pellicle to an acidic challenge, increasing the HAp-erosion. Futhermore, it is shown that the phosphate concentration in saliva is inversely related to flow rate (Dawes and Kubienic, 2004). We showed that a high flow of saliva is associated with lower HAp-erosion. Therefore, the role of phosphate in erosion of HAp could be an indirect effect of the flow. Some studies have shown that a high susceptibility to erosion is associated with a low buffering capacity of saliva (Meurman et al., 1994; Lussi and Schaffner, 2000). This was not confirmed in our study. In the extra-oral erosion model the effect of salivary buffer capacity on the loss of hydroxyapatite was probably limited due to the small amount of saliva present on the HAp during the acidic challenge. Moreover, our method of collection of saliva and the determination of its pH and buffer capacity could have influenced the results. During collection of the saliva and determination of pH and buffer capacity, the saliva is exposed to the atmosphere causing a loss of CO^2. This loss of CO^2 causes a pH change in the alkaline direction influencing the buffer capacity measurements (Bardow et al., 2000).

The experimental model with the extra-oral challenge concentrated on effects of the pellicle and adhering saliva film, and therefore does not incorporate the full potential of saliva in erosion protection, as mentioned before for flow and buffering capacity. Therefore it is surprising that flow rate was a significant factor in our study. Possibly, flow rate is related to a compositional factor we did not measure yet.

For this study we used synthetically prepared HAp discs, which is a close analogue of human enamel mineral. The discs have greater porosity and their structure, particle size and shape differ from human enamel (Hemingway et al., 2008). Due to the greater porosity of the HAp discs the absorption of proteins and especially peptides may be higher compared to human enamel. HAp has used in many in vitro and in situ studies (Vacca Smith and Bowen, 2000; Barbour et al., 2008; He-

mingway et al., 2008; Zaman et al., 2010). The composition of HAp discs derived from the same batch is stable. This reduces variation in sample composition making inter-individual comparisons of the saliva/pellicle effect more straightforward. The pellicle's protective effect is lost during within 10 min of exposure with a citric acid solution at pH = 2.3 (Nekrashevych and Stösser, 2003). This model aimed at studying early erosion, using a shorter and milder erosive challenge, simulating a short period of drinking a citric acid based drink with an intermediate pH (Jager et al., 2008). Using a porcelain disc control made it possible to correct the calcium loss measured for the HAp, for calcium lost from the pellicle / saliva film. Figure 1 shows that calcium loss from the pellicle / saliva film was significant and also showed considerable inter-individual variation.

The multivariate model should be interpreted with considerable caution, as the balance between studied variables and the volume of data is not ideal. It has been included only to give an indication of the variable most likely to be involved in the complex process. As factors included are now corrected for all other included factors, a complex picture emerges, where saliva components appear to have opposing effects, depending on their source from stimulated or unstimulated saliva. The exact effect sizes are of little consequence. However, the model gives information about which salivary parameters, in addition to the ones appearing in the univariate analysis, could be of potential interest for further research.

Within the limits of this preliminary in vitro study it can be concluded that there are associations between several investigated salivary parameters and loss of pellicle / saliva covered hydroxyapatite due to an erosive challenge. Direct investigation of the pellicle itself, and its composition in relation to early erosion is needed to further clarify the protective role of various factors. Additionally, clinical research is needed to investigate whether or not these factors can be shown to play a role in clinical erosion and erosive wear.

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Chapter 5

Association between carbonic anhydrase 6 and erosion of hydroxyapatite

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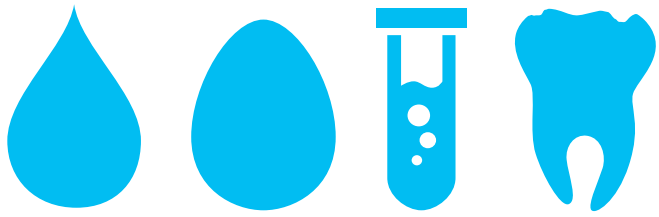

Abstract

Objective

To investigate the relationship between concentration of carbonic anhydrase 6 (CA-6), statherin and total protein in saliva and salivary film/pellicle (SFP) formed on hydroxyapatite (HAp) and susceptibility of HAp to acid erosion.

Methods

Twenty-one volunteers carried three HAp discs in their buccal sulcus for 1.5hr. Two SFP-coated discs were exposed to citric acid (pH= 3) for 2 min and loss of calcium was measured. Unstimulated (UWS) and stimulated (SWS) whole mouth saliva was collected. Protein was eluted from the surface of the third HAp disc for analysis. Composition of proteins in SFP, UWS and SWS were analysed by SDSPAGE and assayed for total protein (BCA method), whilst the CA-6 and statherin content of SFP was determined using Western Blotting. CA-6 concentration in UWS and SWS was measured using an immunoassay (ELISA) and statherin concentration was determined by Western Blotting.

Results

Pearson's correlation analysis showed significant associations between loss of calcium from HAp and concentration of CA-6 in SWS ($r = -0.49$, $p = 0.025$), in UWS ($r = -0.43$, $p = 0.05$) and in SFP ($r = -0.62$, $p = 0.003$) and between loss of calcium from HAp and concentration of statherin in SWS ($r = -0.45$, $p = 0.042$).

Conclusions

The concentration of CA-6 in UWS, SWS and SFP is inversely correlated with erosive demineralisation of HAp.

Clinical relevance

This paper increases our understanding of the effects of potentially protective salivary proteins against acid erosion.

Introduction

A wide variation between individuals has been reported regarding their susceptibility to dental erosion (O'Sullivan and Curzon, 2000; Vieira et al., 2007). In addition, in vitro research has shown that saliva from different donors exerts different levels of protection against erosion (Wetton et al., 2007; Bruvo et al., 2009). Thus saliva may play a crucial role in a subject's susceptibility to erosive wear. Saliva contains a wide array of proteins and many of these could be involved in the protection against erosion. Patients suffering from hyposalivation are more susceptible to erosive wear (Lajer et al., 2009).

Saliva forms a thin, slow moving, mucin containing layer over the hard and soft tissues of the mouth (Dawes, 2008; Pramanik et al., 2010) and the amount of saliva on surfaces differs according to position (Disabato-Mordarski and Kleinberg., 1996; Osailan et al., 2011). Exposure of surfaces to saliva results in the rapid formation of a pellicle of adsorbed salivary proteins that might act as a diffusion barrier or a selective permeable membrane, reducing direct contact between acids and tooth surface (Hannig and Balz, 1999; Ameachi et al., 1999) and thus reducing demineralization of this surface (O'Sullivan and Curzon, 2000; Hannig and Balz, 2001). The proteins that form the pellicle affect its functions such as ion transport potential, regulation of calcium phosphate crystallization and bacterial adherence (Hannig and Joiner, 2006; Cheaib and Lussi, 2011).

Siqueira et al (Siqueira et al, 2007) studied the composition of the pellicle and divided the pellicle proteins in three groups. The first group consists of calcium binding proteins. These proteins, such as statherin and PRPs, can interact with calcium ions on the enamel surface and are considered pellicle precursor proteins. The second group consists of phosphate binding proteins that are binding to the phosphate ions on the enamel surface. Proteins showing interactions with other proteins are the third group. These proteins, such as MUC 5b are involved in the formation of protein layers (Siqueira et al., 2007). Pellicle proteins can also be grouped according to function such as buffer capacity and remineralization. It is found that there is a partial overlap in proteins that are involved in remineralization processes and those that have a high affinity to enamel surfaces (Siqueira et al., 2007). Based on this information, numerous salivary pellicle proteins could be involved in the protection of teeth against erosion. Hannig et al. (Hannig et al., 2005) suggested that carbonic anhydrase-6 (CA-6) is a potential factor in the development of dental erosion. Carbonic anhydrases are a class of enzymes that have the function of maintaining the pH homeostatis by catalyzing the reversible hydration of carbon dioxide and the dehydration of bicarbonate in the reaction $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$ (Lindskog and Coleman, 1973). CA-6 is also known as gustin (Thatcher et al., 1998) and is present in the acquired enamel pellicle (Leinonen et al., 1999; Siqueira et al., 2007), saliva from submandibular

and parotid glands (Parkkila et al., 1990) and in milk (Karhumaa et al., 2001). It has been shown that CA-6 plays a role in the pH homeostasis of the oesophagus (Helm et al., 1984) and it is suggested that in saliva, CA-6 facilitates acid neutralization by bicarbonate (Kimoto et al., 2006). In earlier research it was suggested that CA-6 plays a role in regulating the pH or buffer capacity of saliva (Feldstein and Silverman, 1984), whilst other studies indicate that the pH and buffer capacity of saliva are not directly associated with CA-6 concentration in saliva (Kivela et al., 1997; Parkkila et al., 1993). The presence of CA-6 in the enamel pellicle implies that it may function as a local pH regulator since it has been shown to be active on the enamel surface. Also, it was confirmed that in in vivo and in vitro formed enamel pellicle the bound CA-6 enzyme retains its enzymatic activity (Leinonen et al., 1999). Furthermore, a low concentration of CA-6 in saliva has been shown to be associated with the prevalence of caries (Kivela et al., 1999).

Salivary statherin is also of interest in the control of enamel mineralization and demineralization. It is a 6kD salivary protein that prevents primary precipitation of apatite in saliva and interacts with enamel and hydroxyapatite (HAp) surfaces (Hay et al., 1984). It is suggested that the presence of a statherin/calcium-enriched layer on the surface of teeth may provide a zone of high calcium concentration that possibly facilitates the remineralization of teeth (Proctor et al., 2005). Furthermore it was shown that a statherin fragment (the first 21 residues, StN21) reduced the rate of mineral loss from HAp due to a demineralising solution (Kosoric et al., 2011).

Since CA-6 and statherin in pellicle and saliva are potentially important in regulating tooth mineralization, the present study investigated the possible relationship between susceptibility of HAp to erosive demineralisation and the concentrations of statherin and CA-6 in unstimulated whole saliva (UWS), stimulated whole saliva (SWS) and the combined salivary film and pellicle (SFP). Although the salivary film and pellicle on enamel are different entities, in the mouth they are present together on the tooth surface and collectively influence events. Additionally, the relationship between the total protein concentration in UWS, SWS and SFP and the susceptibility of HAp to erosive demineralisation was studied.

Materials and methods

Subjects and substrate

Twenty-one volunteers (12 females, 9 males) in the age range of 19 to 59 years participated, after giving informed consent. 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, no erosive wear and no subnormal flow rates (data not shown)) and with no medical or pharmacotherapy history (American Society of Anaesthesiologists score 1, (Owens et al., 1978)) were allowed to participate. The study design was reviewed and approved by the University Medical Center Groningen Investigators Research Board (UMCG IRB #2008109).
On every test day only one volunteer participated because of logistical reasons. Three sintered HAp discs (Himed, Old Bethesda, NY, USA; batch #100406) and one porcelain disc (IPS Emax, IvoclarVivadent, Schaan, Liechtenstein) were placed in the buccal sulcus of the lower jaw in close proximity to the first molar (2 left side and 2 right side) of the volunteer at 9.00 a.m. The porcelain disc served as a non-dissolving control sample. When placing the discs in the buccal sulcus, we observed that the discs did not translocate to other positions in the mouth during the 90 minutes experimental period. In other words there was no need to mount the discs in a special device. Before placing the discs (Ø 12.7 mm, thickness 2 mm, surface area: 333.15 mm^2) into the mouth, the discs were submersed in 15 mL of a standard solution of 50 mM citric acid (pH = 3) for 1 hr to remove any loosely attached or more soluble material, which is present on the discs after production (Hemingway et al., 2008). Next the discs were rinsed thoroughly with deionised water and stored in water until use. After this preconditioning the discs are clean and dissolve all in a very homogenous way (data not shown) (Hemingway et al., 2008; Shellis et al., 2010). Eating, drinking, brushing and smoking were not allowed from 1hr before insertion until removal of the samples from the mouth and both the HAp and porcelain samples were held for 90 minutes in situ.

Exposure to citric acid

The three HAp discs and the porcelain disc were removed after 90 minutes from the oral cavity without rinsing (thus covered with pellicle and attached saliva film, SFP). In this way we mimicked the intra oral situation where on top of the pellicle always a salivary film is present. Furthermore, this salivary film functions as a reservoir of bicarbonate facilitating CA-6 regulated dehydration of bicarbonate during the extra oral exposure to the citric acid. Two discs were immediately exposed for 2 min to 2 mL of an erosive solution (50 mM citric acid, pH = 3) under agitation (test tubes were placed on shacking table at 100 rpm) and rinsed with 2 mL of demineralised water. Calcium loss into the acid solution was measured using atomic absorption spectroscopy (AAS) as described in a previous publication (Jager et al., 2008).

The measurement from the two HAp discs was averaged and the loss of calcium from the exposed porcelain disc was deducted from this value to correct for calcium originating from the SFP. The corrected calcium loss was used as a measure of erosive demineralisation of HAp: HAp-erosion.
The HAp disc that was not exposed to the erosive solution was stored at -80°C immediately after removal from the oral cavity for further analyses of the SFP.

Collection, storage and analysis of saliva and SFP

Twenty minutes after removal of the discs from the mouth, UWS and SWS were collected. The waiting period was established to rule out a possible stimulating effect of the discs on the saliva production. UWS and SWS were collected in a plastic cup placed in crushed ice to prevent proteolysis (McDonald et al., 2011). The volunteers were instructed to swallow to begin collection and the unstimulated saliva was collected behind closed lips and expectorated every 30 sec in the cup (37). SWS was collected by chewing on a piece of parafilm (ParafilmM, Pechiney, Chicago, IL, USA) at a chewing frequency of 70 strokes per minute during collection (Navazesh and Christensen, 1982) The collected saliva was centrifuged immediately after collection for 5 min at 10000g at 4°C (Silletti et al., 2007). The saliva supernatant was decanted and frozen in liquid nitrogen and stored at -80°C (Schipper et al., 2007) in plastic containers (Cryogenic Vials, Nalgene Nunc, Rochester, NY, USA).
The HAp discs that were not exposed to the erosive solution were placed in 300 µl of a solution containing double purified water, 0.1% sodium-dodecyl-sulphate (SDS) and 0.01% ethylenediaminetetraacetic acid (EDTA) to remove the SFP from the HAp surface. In order to completely remove the SFP from the HAp surface the solution was heated to 100°C in an open system. Heating of the solution resulted in a reduction of the volume of 25 to 30 µl. UWS, SWS and SFP were analysed for total protein-, statherin- and CA-6- concentration. Each assay was performed in a blinded fashion.

Total protein concentration

The total protein concentration in UWS, SWS and SFP was measured using bicinchoninic acid (BCA) method. 100 µl of sample (diluted 1:10) or 100 µl BCA standard (bovine serum albumin) was applied to the wells of a 96-wells Elisa plate. Next, working reagent (200 µl BCA reagent, diluted 1:50) was added and the plates were incubated for 25 min at 65°C. After this period the absorbance was measured with a microplate absorbance reader at 595 nm (Model 168-1130XTU, BioRad Lab., Hemel Hempstead, UK).

CA-6 in UWS and SWS

The concentration CA-6 in UWS and SWS was determined using an enzyme-linked immunosorbent assay (ELISA). Sheep anti-rabbit CA-6 antibody, diluted 1:10000, was coated overnight on 96-wells ELISA plates in pH 9.6 carbonate buffer at 4 °C. After this incubation period the plate was washed three times with PBS-Tween 20 buffer solution (0.1M PBS, 0.5% Tween 20, pH 7.2 diluted 1:10). To obtain human CA-6 standards, CA-6 was purified from the parotid saliva of 4 donors by inhibitor affinity chromatography (Murakami and Sly, 1987). The stimulated parotid saliva was collected with Lashley cups while the donors sucked on sugar free candy.
The saliva samples and the purified CA-6 standards (both diluted 1:50 in PBS-T 20) were added to the wells (100 µL of diluted standard or sample) and diluted 4 times down the plate. The mixture was incubated for 2h at 37°C and after incubation the plate was washed again three times and a second antibody (100 µL of 1:1000 goat-anti human CA-6 in PBS-T 20) was added to each well. After another 2h incubation at 37°C the plates were washed three times with PBS-T and then incubated with horseradish peroxidase labelled anti-goat (100 µL) diluted 1:2000 in PBS-T 20, for a further hour at 37°C. After three more washes with PBS-T the substrate was added. This consisted of 0.5 ml of tetramethylbenzidene stock solution (3 mg/ml in DMSO) and 5 µL of 3% hydrogen peroxide in 20 ml of sodium acetate buffer (100 mM, pH 5.5). The reaction was stopped after 3 min by the addition of 50 µl of 2M sulphuric acid and the absorbance was read at 450 nm in a microplate absorbance reader (Model 168-1130XTU, BioRad Lab., Hemel Hempstead, UK). Before starting the measurements the reproducibility of the ELISA was tested by repeatedly analysing saliva samples from 3 different donors and CA-6 samples (both diluted 1:50 in PBS-T 20) with a known amount of CA-6 (data not shown).

CA-6 and statherin concentration in SFP and statherin concentration in UWS and SWS

To examine the composition of proteins and glycoproteins in saliva and on HAp surfaces Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS PAGE) with a Coomassie and periodic acid Schiff (PAS) staining was used. Pre-cast 4-12% Bis-Tris gels (Nupage Novex, Invitrogen, Paisley, UK) were run at 125V. The gels were stained with 0.2% Coomassie Brilliant Blue R250 (Sigma Aldrich, Dorset, UK) followed by a PAS.
The presence of CA-6 in SFP and statherin in UWS and SWS was determined by Western blotting. For the determination of CA-6, a goat-anti human CA-6 antibody diluted 1:5000 in tris buffered saline (TTBS), pH 7.6 was used as primary and anti-goat horseradish peroxide (diluted 1:5000 in TTBS) as second antibody.
A sheep anti-human statherin primary antibody diluted 1:2000 in TTBS and a horseradish peroxidase rabbit anti-sheep secondary antibody (diluted 1:2000 in

TTBS) were used to detect statherin (Proctor et al., 2005). Bound secondary antibody was detected using a chemiluminscent substrate (3% H_2O_2, TBS pH 7, 90 mM coumaric acid, 250 mM luminol) and detection with photographic film. Band densitometry analysis was performed with Image J software (National Institutes of Health, Bethesda, MA, USA) to evaluate the quantity of the proteins.
Purified preparations of either full-length synthetic statherin or recombinant CA-6 (R&D Systems, Minneapolis, MN, USA) or CA-6 purified on a p-aminomethyl benzenesulphonamide affinity matrix as previously described (Murakami and Sly., 1987), were used to prepare standard curves for quantification of statherin or CA-6.

Statistical methods

Pearson's correlation coefficient analysis was used to study the association between the HAp-erosion and the statherin, CA-6 concentration and the total protein concentration in UWS, SWS and SFP. A p-value of less than 0.05 was considered statistically significant. This was performed by SPSS-software (SPSS 16.0, SPSS Inc., Chicago, IL., USA).

Results

The calcium loss from HAp discs ranged between 0.83 and 4.5 mg/L with an average loss of 2.36 mg/L and was normally distributed. The results of the UWS, SWS and SFP analyses (total protein, CA-6 and statherin) are summarized in table 1. Correlation analysis of saliva parameters with loss of calcium from HAp showed a negative association between loss of calcium and concentration of CA-6 in SWS ($r = -0.49$, $p = 0.025$) and UWS ($r = -0.43$, $p = 0.05$). A typical western blot for the presence of statherin in saliva is shown in figure 1b and concentration of statherin in SWS showed a significant negative association with loss of calcium from HAp ($r = -0.45$, $p = 0.042$). Figures 1a and 2a show typical SDS-PAGE profiles of proteins and glycoproteins present in saliva and SFP as demonstrated by Coomassie Blue and PAS staining. There appear to be similarities in composition, for example SFP contains PAS positive bands that correspond in mobility to MUC5B and MUC7 in saliva. However, there are also clear differences in protein band patterns particularly in the 3-14kD (mw marker protein) range at the bottom of the SDS gels. Typical Western blots for statherin and CA-6 in SFP are shown in figure 2b and 2c respectively.
The CA-6, statherin and total protein results for SFP, expressed per mm2 of HAp surface, are shown in table 2. There was a significant negative correlation between concentration of CA-6 in SFP and calcium loss from HAp ($r = -0.62$, $p = 0.003$). Although the content of statherin in HAp elutes was almost double that of CA-6, statherin concentration in SFP did not show a statistically significant association with calcium loss from HAp.

Table 1. Results of the analyses of UWS and SWS, Pearson's correlation coefficients between HAp-erosion and UWS/SWS parameters and corresponding 95% confidence intervals.

UWS parameter	Mean	SD	Pearson's correlation coefficient	p-value	95% CI
Total Protein mg/mL	1.67	0.66	-0.17	ns	- 0.560 … 0.284
CA-6 (ng/mm²)	22.20	10.75	-0.43	0.05	- 0.725 … 0.005
Statherin (ng/mm²)	14.3	6.6	-0.23	ns	- 0.220 … 0.605
SWS parameter	**Mean**	**SD**	**Pearson's correlation coefficient**	**p-value**	**95% CI**
Total Protein mg/mL	1.16	0.41	-0.07	ns	- 0.487 … 0.377
CA-6 (ng/mm²)	18.14	9.42	-0.49	0.025	- 0.759 … - 0.071
Statherin (ng/mm²)	13.2	6.8	-0.45	0.042	- 0.737 … - 0.020

Table 2. Results of the analyses of SFP, Pearson's correlation coefficients between HAp-erosion and SFP parameters and corresponding 95% confidence intervals.

Salivary parameter	Mean	SD	Pearson's correlation coefficient	p-value	95% CI
Total Protein (ng/mm²)	79.00	19.4	0.09	ns	- 0.351 … 0.506
CA-6 (ng/mm²)	6.1	3.5	-0.26	0.003	- 0.830 … - 0.258
Statherin (ng/mm²)	11.5	5.9	0.005	ns	- 0.368 … 0.492

Figure 1a.

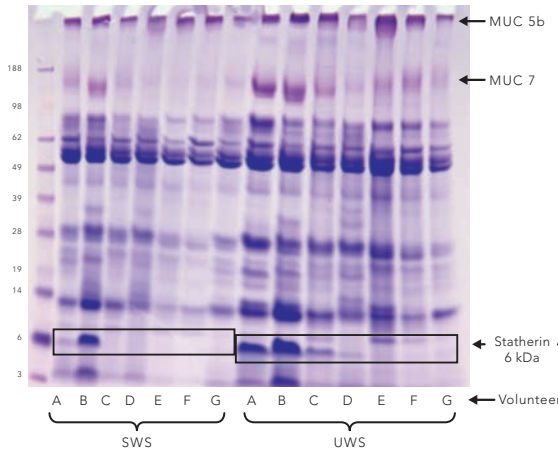

Figure 1b.

Figure 2a.

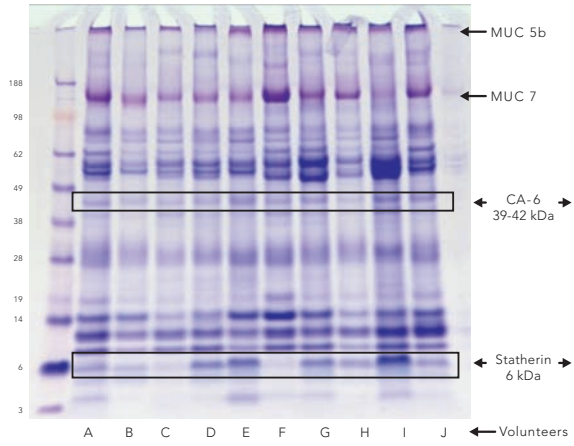

Figure 2b.

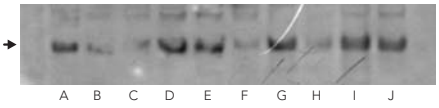

Figure 2c.

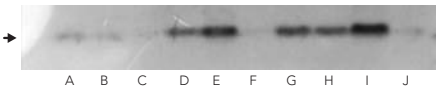

Figure 1. SDS PAGE and western blot analysis of UWS and SWS protein composition.

a) The electrophoresis gel in (a) has been stained with Coomassie Brilliant Blue for proteins followed by periodic acid Schiff (PAS) reagent for glycoproteins. Molecular weight standards ranging from 188 - 3 kD are also shown. High molecular weight PAS stained bands corresponding to MUC5B and MUC7 are indicated (arrows).

b) The immunoblot of UWS and SWS shows statherin and the intensity of the bands in different samples is similar to the relative intensity of the proteinstained band shown in the gel in (a) (outlined in box).

Figure 2. SDS PAGE and western blot analysis of SFP protein composition.

a) Saliva film/pellicles eluted from HAp discs of subjects A to G have been electrophoresed and stained with Coomassie Brilliant Blue for proteins followed by periodic acid Schiff (PAS) reagent for glycoproteins. Molecular weight standards ranging from 188 - 3 kD are also shown. High molecular weight PAS stained bands corresponding to MUC5B and MUC7 are indicated (arrows).

b) The immunoblot of saliva films/pellicles showing statherin and the intensity of the bands in different samples is similar to the relative intensity of the proteinstained band shown in the gel in (a) (lower box) running at approximately 6kD.

c) Immunoblot of saliva films/ pellicles showing carbonic anhydrase 6. The intensity of staining corresponds to a stained band in (a) (upper box) running at approximately 39-42kD.

Discussion

Low concentrations of CA-6 in saliva have been shown to be associated with the prevalence of caries (Kivella et al., 1999). However, an association with erosion has not been reported previously.

When the mean CA-6 content of the SFP samples from the HAp surface was expressed in relation to mean BCA assayed total protein a value of 77 ng/µg total protein was obtained. The equivalent value for UWS was 13.3 ng/µg and for SWS 15.6 ng/µg total salivary protein, indicating that CA-6 in SFP on the HAp surface is enriched approximately 5 fold higher. This suggests that it may have a significant function at the enamel surface, particularly since previous enzyme histochemical studies have found CA-6 to be active on the enamel surface (Leinonen et al., 1999). In the present study, HAp discs were not rinsed with water after removal from the oral cavity in order to preserve the SFP. We have chosen for a design mimicking the mouth where on top of the pellicle always a salivary film is present. In addition to being a more clinically relevant design, the presence of a salivary film can be an important source of bicarbonate facilitating CA-6 regulated neutralization of acid during the extra oral exposure to the citric acid in our study design. To investigate the amount of SFP present on the HAp, 6 discs were placed in the mouth of 1 volunteer. The SFP was measured as an average weight increase of the discs of 5.1 mg, indicating that a SFP is present after 90 min in the oral cavity. Moreover, rinsing of SFP covered discs with water resulted in a strong decrease of the stained band intensity on SDSPAGE gels of the proteins present on the discs (data not shown). These observations suggest that saliva is present on the non-rinsed discs and therefore could be a source of the bicarbonate for CA-6 during the exposure to citric acid.

To detect CA-6 in SFP, a Western blot analysis was used. Unfortunately it was impossible to use the described CA-6 Elisa for the SFP samples because of the effects of SDS and EDTA, used to collect the samples, on the binding of the antibodies in the ELISA. Nevertheless, when CA-6 values for UWS and SWS obtained using ELISA were compared with values obtained using the Western blotting technique, there was a significant association ($p<0.001$) and an r-value of 0.7.

As mentioned above, a previous study showed that truncated statherin peptide (SN21) could be involved in reducing mineral loss due to an acidic challenge of HAp in vitro (Kosoric et al., 2011). These results could only be confirmed for the concentration of statherin in SWS in our in situ study. The difference between the two studies could perhaps be explained by the presence of other proteins on the HAp surface in our study. Because we studied an almost mature pellicle (90 min) and statherin is one of the first proteins to adsorb to the HAp surface, the effect of statherin on the loss of calcium could be small in our study design. Furthermore, the previous study (Kosoric et al., 2011) found no dependence of demineralization

on statherin peptide concentration (4-150 µg/ml) and in the present study over 80% of the saliva samples had statherin concentrations greater than 6 µg/ml (see table 1 mean and SD values). Furthermore, no significant correlation could be found between the concentration of statherin in SFP and UWS or SWS.

It might be expected that a higher total protein concentration would result in better protection against erosive wear because of the formation of a thicker barrier to acids. We did not find a relationship between the total protein concentration in UWS or SFP and the loss of calcium from HAp. These results confirm the results of a previous study (Jager et al., 2011) even though the total protein concentration was measured using a different method. In a further study of the relationship between total protein concentration and the prevalence of erosion it was even found that a higher concentration of proteins in SWS resulted in more erosion (Piangbrach et al., 2009). Therefore it could be suggested that the protective effect of the SFP is determined by specific proteins or other SFP components and not by the total protein concentration.

We decided to collect and analyse SWS and UWS. Different opinions exist regarding the role of UWS and SWS in the development of dental erosion. Some studies suggest that different characteristics of the two are related to dental erosion (O'Sullivan and Curzon, 2000; Piangbragh et al., 2009) but others have stated that UWS is of more importance because it is the basal rate of saliva flow (Zero and Lussi, 2005). We collected and analysed both UWS and SWS because there is a possibility that the introduction of the discs in the oral cavity resulted in stimulation and therefore exposure of the discs to UWS and SWS. Due to the contribution of the parotid glands, CA-6 and statherin concentrations may differ between SWS and UWS. Furthermore, levels of components in UWS or SWS may not reflect levels entering the mouth in saliva since components such as statherin are subject to degradation by proteases and appear to bind to surfaces (Thomadaki et al., 2011). The amount on enamel may reflect the amounts entering the mouth in saliva.

For the present study we used synthetically prepared sintered HAp discs, which is a close analogue of human enamel mineral with a comparable density. This is an approach previously adopted in many studies (Vitorino et al., 2004; Barbour et al., 2008; Hemmingway et al., 2008; Zaman et al., 2010; Kosoric et al., 2010; Jager et al., 2011). The discs have greater porosity, larger particle size and their structure, and shape differ from human enamel (Hemmingway et al., 2008, Shellis et al., 2010). There are a few but relatively large pores in the discs and the HAp dissolves at a slower, but constant, rate compared to human enamel (Shellis et al., 2010). Due to the pores in the HAp discs the absorption of proteins and especially peptides could be higher compared to human enamel. However, in our study the discs were rinsed with water and stored in water after the cleaning procedure resulting in water filled pores. The main benefit of HAp is that the composition of

the discs derived from the same batch is stable which is definitely an advantage for the present study design since it eliminates the variation associated with varying of tooth enamel sample composition.
As mentioned in the introduction numerous other SFP and salivary proteins could be involved in demineralisation or remineralisation. A group of proteins not included in our study but interesting for further research are the mucins. In an earlier study, van Nieuw Amerongen et al. (van Nieuw Amerongen et al., 1987) showed that exposure of enamel to UWS for 6 days resulted in a reduction of demineralisation of 45%. Exposure of enamel to submandibular/sublingual saliva for 60 min resulted in complete prevention of erosion. These authors suggested that this prevention of erosion was due to salivary mucins.
Based on the results and within the limits of this study it can be concluded that CA-6, present in UWS and SFP, may play a role in reducing the erosive demineralisation of HAp. Further clinical research is needed to investigate whether or not this factor can be shown to play a role in clinical erosion on human enamel.

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Conflict of interest

The authors declare that they have no conflict of interest. The authors' institutions funded this research.

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Chapter 6

Reduction of erosion by protein containing toothpastes

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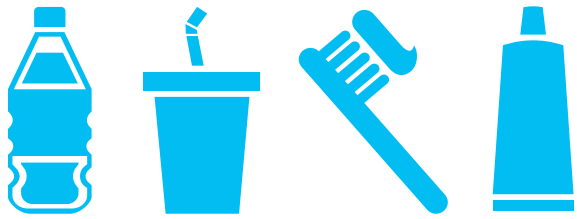


Abstract

Objective

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 (extra-oral water or toothpaste brushing) and cross over (type of toothpaste) set-up was used. Two protein containing (high/low concentrations of colostrum) and one non-protein (placebo) toothpaste were investigated. Sixteen volunteers wore intra-oral appliances containing 2 human enamel samples on 3 afternoons for pellicle growth during 90 min. One enamel sample was brushed for 5 sec 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 (extra-orally). Loss of calcium and inorganic phosphate were determined. The same sequence of exposures was applied to 16 enamel samples in an in vitro set up without pellicle.

Results

With in situ formed pellicle, all toothpastes significantly reduced calcium loss as 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 non-protein toothpaste. Overall there were only slight differences between the toothpastes. Toothpaste effects were less clear in the vitro experiment.

Conclusions

Addition of proteins to toothpaste shows some promise for the prevention of erosion. Further research is needed to investigate the performances of the protein containing toothpastes in longer in situ studies considering erosive wear.

Introduction

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, next to 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 to dental erosion could be provided. A number of studies has been performed investigating different toothpaste modifications (Newby et al., 2006; Rees et al., 2007; Hooper et al., 2007; Lussi et al., 2008; Kato et al., 2010). Examples of these modifications are higher fluoride concentrations and exclusion of sodium lauryl sulphate (SLS) from the toothpaste. SLS is able to remove the pellicle and a smear layer present on dentin (Moore and Addy, 2005). Toothpaste formulations without SLS could be favourable 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 to 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 to reduce the loss of enamel, could be the addition of proteins to toothpaste such as present in colostrum. For casein, one of the components of colostrum, this has been recently confirmed in an in vitro study investigating the erosion inhibiting effect on enamel of the casein protein with and without fluoride compared to water and fluoride solution (White et al., 2010). This erosion inhibiting effect of casein is ascribed to the adsorbtion 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 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 non-protein (placebo) toothpaste.

Method and materials

Three toothpastes formulations were investigated. All the pastes did not contain sodium lauryl sulphate (SLS). The toothpastes were coded as follows:

P-: Zendium Acid Defence without proteins (SaraLee Household and Bodycare b.v., Amersfoort, The Netherlands) (free Ca^{2+} : 0.026 mg/g; free Pi: 3 mg/g; 1450 ppm NaF, pH 6.0 ± 0.1). A specially prepared placebo toothpaste. Not commercially available.

P+: Zendium Acid Defence, commercially available toothpaste with 0.21% w/w protein. This paste contains: amyloglucosidase, glucose oxidase, lactoperoxidase, lysozyme, lactoferrin, IgG and casein (free Ca^{2+} : 0.029 mg/g; free inorganic phosphate (Pi): 3 mg/g; 1450 ppm NaF, pH 6.0 ± 0.1).

P++: Experimental Zendium Acid Defence: same proteins as toothpaste P+ in higher concentrations (0.57% w/w protein, i.e. 2.7 times as much more proteins compared to the P+ paste) (free Ca^{2+} : 0.041 mg/g; free Pi: 3 mg/g; 1450 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 mould that produced blocks of 5 × 9 × 3 mm with an oblique side that was used for 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, Kopenhagen, Denmark). Sterilization of the samples was performed with ethylene oxide according to the protocol of the Department of Microbiology of the UMCG.

Volunteers

Ethical approval was granted by the UMCG Institutional Review Board (UMCG IRB: #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 Anaesthesiologists 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, 1998). Because of possible dropouts a sample size of 16 volunteers was chosen.

Study Design for the in Situ Study

The trial was a single centre, double-blind split-mouth (extra-oral water or toothpaste brushing), cross-over (type of toothpaste) design. All volunteers wore intra-oral 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 intra-oral 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 30 minutes earlier in the appliance than the sample on the left side. After 60 min in the oral cavity this sample was extra-orally brushed with water and incubated for 2 min in water, thoroughly rinsed for 30 seconds with running tap water and returned to the oral cavity for another 30 min. After 30 min both samples were removed, the water brushed sample permanently, for acid exposure, the toothpaste sample for brushing with one of the toothpastes.

Brushing was performed by the investigator for 5 sec with an electric toothbrush with the toothpaste (Oral B Professional-care 7500 DLX, Braun, Germany) and the sample was subsequently incubated for 2 min in a slurry (1:2 weight ratio toothpaste to water) of the corresponding toothpaste on a shaking table (100 rpm). It was chosen to first brush the samples with the pastes for 5 sec and than 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 that first the pellicle was disturbed by brushing where after proteins present in the toothpaste were introduced into the pellicle. Furthermore, the handheld 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 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 extra-orally on enamel samples that were not exposed to the oral cavity, thus without the presence of saliva or pellicle. For this, sixteen enamel samples were randomly brushed with toothpastes or water and exposed to the toothpastes slurry or water as described above and subsequently rinsed with water and exposed to the citric acid. Instead of the placement in the oral cavity these samples were placed in deionised water (22°C) for the same time period. In between treatments the samples were briefly polished and cleaned to remove the eroded surface layer 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 inorganic phosphate 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 in enamel of 17.61% and an average enamel density of 2.93 g/cm^3 were assumed. The calcium concentration was determined by atomic absorption spectroscopy as described in a previous publication (Jager 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, MA, USA) on the basis of the number of pixels. The loss of calcium and inorganic phosphate was expressed in mmol/mm^2.

Statistical Analysis

The effect of the toothpaste compared to water brushing within one person (in situ) or one sample (in vitro) and the differences between the three toothpastes were analysed using paired t-tests. The above-mentioned analyses were all performed using SPSS software (SPSS 16.0, SPSS Inc., Chicago, IL., 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/cm^2, water 2.29 mmol/cm^2, $p < 0.001$), followed by P- (1.83 mmol/cm^2, water 2.28 mmol/cm^2, $p < 0.001$) and by P++ (1.85 mmol/cm^2, water 2.50 mmol/cm^2, $p < 0.001$). Toothpaste P++ showed the lowest loss of phosphate (0.85 mmol/cm^2, water 1.94 mmol/cm^2, $p = 0.025$), followed by P- (1.04 mmol/cm^2, water 1.46 mmol/cm^2, $p > 0.05$) and by P+ (1.38 mmol/cm^2, water 2.12 mmol/cm^2, $p > 0.05$). Figure 1 shows the results of the (paired) comparison of the toothpaste results to the water 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++ when 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 (figure 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 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/cm^2), followed by P+ (2.61 mmol/cm^2) and by P- (2.63 mmol/cm^2). Toothpaste P+ showed the lowest loss of phosphate (2.82 mmol/cm^2), followed by P++ (3.40 mmol/cm^2) and by P- (4.42 mmol/cm^2). 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++ (figure 2).

All three toothpastes reduced erosion compared to water (loss of calcium: 2.37 mmol/cm^2; loss of phosphate: 6.24 mmol/cm^2) for the phosphate measurement ($p = 0.04$ to 0.001).

Figure 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 the exposure/brushing with toothpaste from the loss of calcium/phosphate after the exposure/brushing with water. Furthermore, the three pastes are mutually compared.

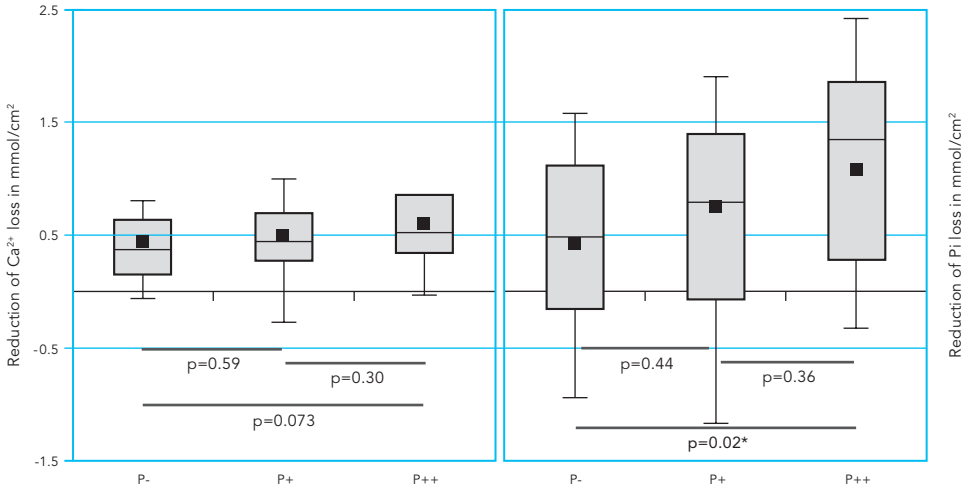
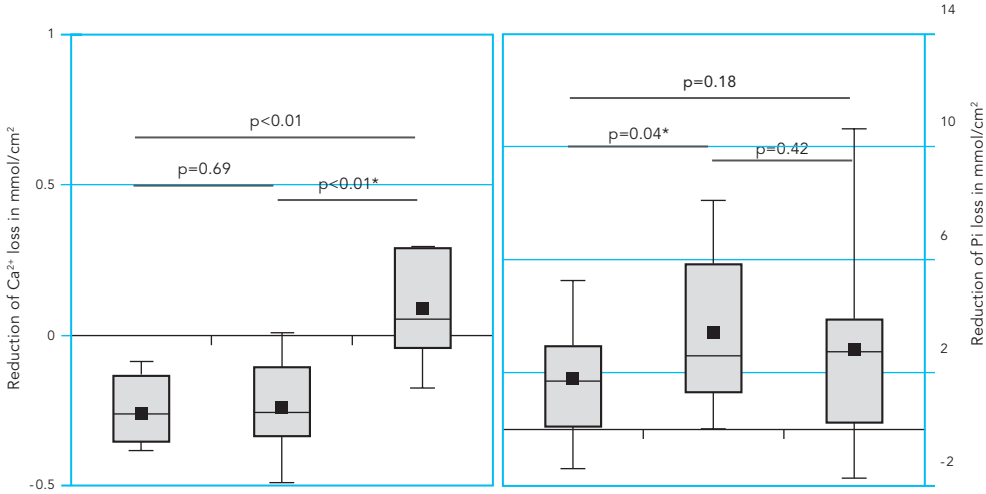


Figure 2. In vitro (no pellicle)

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 the exposure/brushing with toothpaste from the loss of calcium/phosphate after the exposure/brushing with water. Furthermore, the three pastes are mutually compared.



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 an possible effect of the 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 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., 1994; Tenuvuo, 2002; Pedersen et al., 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 immobilisation of enzymes in an in situ pellicle indeed can 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 firmly adsorbing 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 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 extra-oral experiments compared to the intra-oral 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). Exposures 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+/P- paste. This contribution is considered small because the samples were exposed to the toothpaste slurry and brushing extra-orally, rinsed with water and than replaced in the oral-cavity for 30 minutes. 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, 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 as 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 dissolution of enamel under caries like conditions (Weiss and Bibby, 1966). Recent work by White et al (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 potentials of soft drinks (West et al., 1998) and the protective effect of fluoride varnish (Vieira et al., 2008). The main benefit of this system is that the enamel samples are placed in the oral environment with natural pellicle development and the samples 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 extra-oral 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 performances of the protein containing toothpastes in longer in situ studies, considering 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 subject of further investigation.

Acknowledgements and conflict of interest statement

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

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Chapter 7

Reduction of erosive wear in situ by stannous fluoride containing toothpaste

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Abstract

The aim of this single centre, randomized, double blind, in situ study was to evaluate the effect of toothpastes with stannous fluoride in the prevention of erosive enamel wear. Twelve volunteers wore palatal appliances containing human enamel samples. Three toothpastes were used, in randomized order: two toothpastes containing stannous fluoride (coded M and PE) and one toothpaste containing only sodium fluoride (coded C). On day 1 of each run the appliances were worn for pellicle formation. On days 2 to 5 the samples were also brushed twice using a brushing machine with a toothpaste-water slurry or only water (control). Erosion took place on days 2 to 5 extra-orally 3 times a day (5 min) in a citric acid solution (pH 2.3). Enamel wear depth was quantified by optical profilometry. The effect of toothpastes and differences between toothpastes were tested using General Linear Modeling. Average erosive wear depth of control samples was 23 µm. Both stannous fluoride toothpastes significantly reduced erosive wear: M by 34% (SD 39%) and PE by 26% (SD 25%). The control toothpaste reduced erosive wear non-significantly by 7% (SD 20%). Both stannous fluoride containing toothpastes significantly reduced erosive wear compared to the sodium fluoride toothpaste.

Introduction

Increasing scientific effort is invested in identifying optimal fluoride formulations for the prevention of erosive tooth wear. Common formulations used in caries prevention, neutral solutions of sodium fluoride (NaF) at 250 – 500 mg/kg F and NaF toothpastes at 1000 – 1500 mg/kg F have been shown to have limited effect (Attin et al., 1998; Lussi et al., 2004; Lussi et al., 2008). For toothpastes the added concern is that the brushing action with toothpaste on erosion-softened enamel increases abrasion (Attin et al., 2000).

Acidic solutions, such as native solutions of stannous fluoride (SnF_2), titaniumtetrafluoride (TiF_4) en hydrofluoric acid (HF) have been shown to be effective in vitro and in situ (Hove et al., 2008; Hjortsjö et al., 2010). Of the above, SnF_2 is recently considered the most promising, as both TiF_4 and HF are probably too acidic for clinical use (pH < 2), and pH-adjustment of TiF_4 reduces its protective effect (Wiegand et al., 2009). SnF_2 is already being used in toothpastes and mouthrinses, and its effect on plaque and gingivitis is well recognized (Paraskevas and van der Weijden, 2006). Recently, most research has focused on solutions containing different concentrations of SnF2 or combinations of different fluorides with $SnCl_2$. In an in vivo model, a 1 min exposure to a 0.78% w/v SnF_2-solution reduced enamel dissolution during a 1 min exposure to citric acid by 67% (Hjortsjö et al., 2009a). However, the effect did not last for more than one day (Hjorstjö et al., 2009b). A novel approach to tin-containing solutions has been studied extensively, using $SnCl_2$ as the source of tin with amine fluoride and/or NaF as the source of fluoride. In an in vitro erosive cycling model, such solutions reduced tissue loss significantly, even when using a severe erosion regime (Schlueter et al., 2009a and 2009b). In an in situ study, an experimental mouthrinse containing 1900 mg/kg stannous (from $SnCl_2$) and 1000 mg/kg F (from NaF and amine fluoride) used once a day, reduced erosive wear of enamel and dentine by 73% and 50%, respectively (Schlueter et al., 2009c). The stannous is thought to work through uptake into the surface enamel and/or the formation of a tin–containing surface layer on top of the enamel (Schlueter et al., 2009d; Yu et al., 2010).

Less is known about the erosion preventive effect of SnF_2 in toothpastes. The concentration of SnF_2 in the toothpastes is usually lower than those used in the solutions, and the abrasive effect of the toothpaste may interfere with the protective effect. Immersion in toothpaste slurries reduced microhardness loss during erosion in vitro, but no significantly better effect of a SnF_2 toothpaste was observed, compared to NaF toothpastes (Lussi et al., 2008). In the in vivo model mentioned above as used by Hjorstjö, enamel dissolution was significantly reduced by 4 min application with a soft brush of a SnF_2 toothpaste (Young et al., 2006). It is unclear whether this method mirrored real tooth brushing. Moreover, tissue loss due to the brushing was not measured. In vitro (Attin et al., 2000) and in situ studies (Jaeggi and Lussi,

1999) have shown that eroded enamel and dentine are susceptible to toothbrush abrasion. The presence of toothpaste during brushing is more important than the brushing action by itself (Voronets et al., 2008; Voronets and Lussi, 2010), and fluoride in toothpaste reduces the abrasion (Ganss et al., 2007). Although increasing the time period between erosion and brushing reduces the abrasion, it still occurs at least up to 2 hrs after erosion (Attin et al., 2000; Ganss et al., 2007).

We hypothesized that in an in situ set up with palatal sample placement, brushing samples twice a day with a stannous fluoride containing toothpaste would not increase erosive wear, compared to brushing with water only and would reduce erosive wear compared to brushing with a sodium fluoride toothpaste.

Materials and methods

This study was a single centre, randomized, double blind, in situ study. Ethical approval was obtained from the regional accredited Medical Research Ethics Committee (MREC code: NL28303.091.09). Twelve healthy volunteers from the staff and students of the Radboud University Nijmegen Medical Centre provided written informed consent to participate.

The participants wore acrylic palatal appliances, each containing 2 acrylic blocks with 2 imbedded human enamel samples (Vieira et al., 2007). The samples were prepared from recently extracted human (pre)molars that were obtained from patients with verbal informed consent, as approved by the regional MREC. The samples were prepared from the facial or lingual surface of the teeth (approximate dimensions: 3 × 3 × 2 mm) and were embedded in groups of 2 in acrylic resin (De Trey, Self-cure Acrylic, England) using a mould that produced blocks of 5 × 9 × 3 mm. Subsequently the embedded enamel samples were ground flat on a rotating polishing machine under water cooling (Phoenix Beta grinder/polisher, Buehler, Germany; Buehler SiC grinding paper P1200). The samples were sterilized with ethylene oxide (WIMAC Kliniekdiensten B.V., Rotterdam, The Netherlands, ISO 9001:2000 and EN 13485:2003). Before insertion in the appliance, the blocks were partially covered with PVC tape leaving an exposed enamel window of each sample of about 1 mm wide, and protecting enamel reference areas for measurement of surface loss. For each subject the enamel samples of one of the blocks were brushed with toothpaste slurry twice a day. The samples of the other block were brushed with water and served as control.

Three toothpastes were used in the study. Two of these contained stannous fluoride: Meridol (coded M; GABA Benelux, Weesp, The Netherlands) containing 1050 ppm stannous fluoride and 350 ppm amine fluoride, and Oral B Pro-Expert Enamel Protection (coded PE; Procter & Gamble, Weybridge, UK) containing 1100 ppm stannous fluoride and 350 ppm sodium fluoride. The third toothpaste was a sodium fluoride control: Oral B 123 (Coded C; Procter & Gamble) containing 1450 ppm sodium fluoride.

Study design

The participants wore the appliances for 3 experimental runs of 5 working days from 9.00 AM until 5.00 PM (both times ± 30 mins). During the entire study period, starting 1 week before the first run, the subjects were instructed to use the NaF-toothpaste (C) for home brushing. With the appliances in situ the participants were instructed not to eat and were only allowed to drink coffee or tea without sugar. From 12.00 till 1.00 PM (lunch break) and from 5.00 PM till 9.00 AM the next day, as well as during the weekends, the appliances were stored in saline at room

temperature. On day 1 of every run, in order to allow pellicle formation on the enamel surfaces, the appliances were worn and no erosive challenges took place. From day 2 till day 5 the experimental procedure was as follows (all times ± 15 min): 8.15 AM samples brushed with toothpaste or water (controls); at 10.30 AM, 1.00 PM and 3.00 PM samples exposed to erosion challenge, 5.30 PM samples brushed as before. Between the runs, a wash out period of at least 2 days was observed. During this time the sample blocks were polished to remove the top layer of enamel of 100 ± 20 µm, as controlled by digital calliper, thereby providing a fresh surface for the next experimental run.

The erosion challenge consisted of immersing the appliance with the samples for 5 min in 100 mL of a 0.05M citric acid solution (pH = 2.3), with no agitation and at room temperature. For every exposure a fresh volume was used. After the exposure, the appliances were rinsed for 10 sec under running tap water and immediately reinserted. For brushing, the sample blocks were removed from the appliance and inserted into a brushing machine. Toothpaste slurries (1:3 toothpaste to demi-water ratio) were freshly prepared and poured into the individual well for each sample block. For control samples, the wells were filled with demi-water. Samples were exposed to the slurry / water for 2 min, and with that time period, were brushed for 10 strokes (150 g). After 2 min the samples were rinsed under running tap water for 10 sec and replaced in the saline storage containers until further use.

Wear measurement

Enamel surface loss was measured using light profilometry (Proscan 2100, Scantron, England). Before PVC tape application, baseline measurements were performed on each sample in order to evaluate the flatness of the polished enamel surfaces. If baseline curvature was higher than 1 µm the samples were polished again. After each run, the PVC tape was removed and scans of were made over the exposed surfaces and reference surfaces (step size 10 mm). The function "3 point step height" of the equipment's software package was used to determine surface height loss for each sample. Two areas of approximately 0.25×2 mm were selected on the scan, at the edges of the two reference surfaces. A third area of about 0.7x2 mm was selected in the centre of the exposed surface. The enamel surface loss was calculated as the difference between the average height of the reference surfaces and that of the exposed surface. The results for the 2 samples in each block were averaged before further statistical analysis.

Scans were analyzed twice, with a time interval of 2 weeks, in order to evaluate measurement reproducibility, showing a Limits of Agreement (95% CI of repeated measurement) of +/- 0.5 µm.

SEM

After the last run, a sample from each condition (control and toothpastes) was prepared for Scanning Electron Microscopy. Organic deposits were removed using immersion in 1 M sodium hydroxide for 18.5 hrs (of which 30 min with ultrasonication). Subsequently, samples were dehydrated using ethylalcohol, dried in an incubator at 37°C, fractured and gold-sputtered.

Statistical analysis

General Linear Modeling was used to statistically analyze the data (GLM, SAS 9.2). Control samples were first analyzed separately, to check for a run effect or a cross-over effect of the toothpaste on the water brushed controls. Subsequently, the effect of the toothpaste compared to water brushing was analyzed, and the effects of the toothpastes mutually compared. A significance level of $p = 0.05$ was used.

Results

Twelve healthy volunteers were included, 11 female and 1 male, aged between 20 and 50 yrs, all with normal salivary flow rates. All subjects completed the study without problems. For 1 appliance during 1 run, accidentally an exposure of 17 min occurred. This was partly compensated for by omitting the subsequent exposure, and the results for this run was included in the effects analyses. One scan was lost and could not be replaced (toothpaste C, run 2).

Average erosive wear depth of control samples in the three runs was 22.3, 23.4, and 24.7 µm, respectively, showing a small but significant run effect (effect 1.1 µm; $p = 0.01$). No cross-over effect of the toothpaste on the surface loss of the control samples could be observed.

The results grouped by toothpaste, each with their own control results, can be seen in figure 1. Both stannous fluoride toothpastes significantly ($p \leq 0.01$) reduced erosive wear: M by 34% (SD 39%) and PE by 26% (SD 25%) compared to the water brushed controls. The sodium fluoride toothpaste reduced erosive wear by 7% (SD 20%), but this was not statistically significant. Mutually comparing the toothpastes showed a significant difference ($p < 0.05$) between group C and both M and PE, who were not significantly different from each other.

SEM images (figure 2) show a typical honeycomb structure of etched prisms for the control sample. A similar, though slightly less distinct, appearance is seen for the sample from group C. Samples from groups M and PE both show a mixed appearance, with areas of etched prisms combined with areas where a surface layer appears to cover the enamel.

Figure 1. *Boxplot of the erosive wear results for the 3 toothpastes and their respective water brushed controls.*

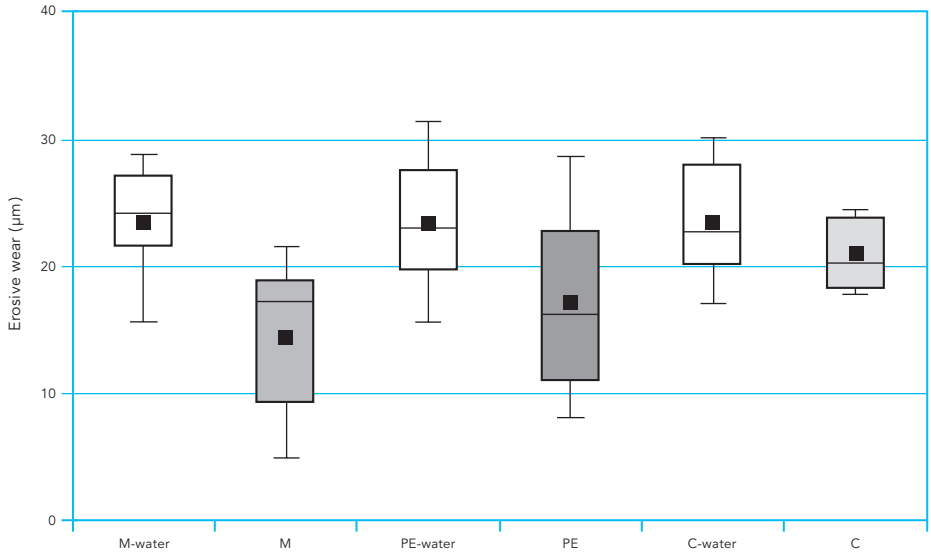
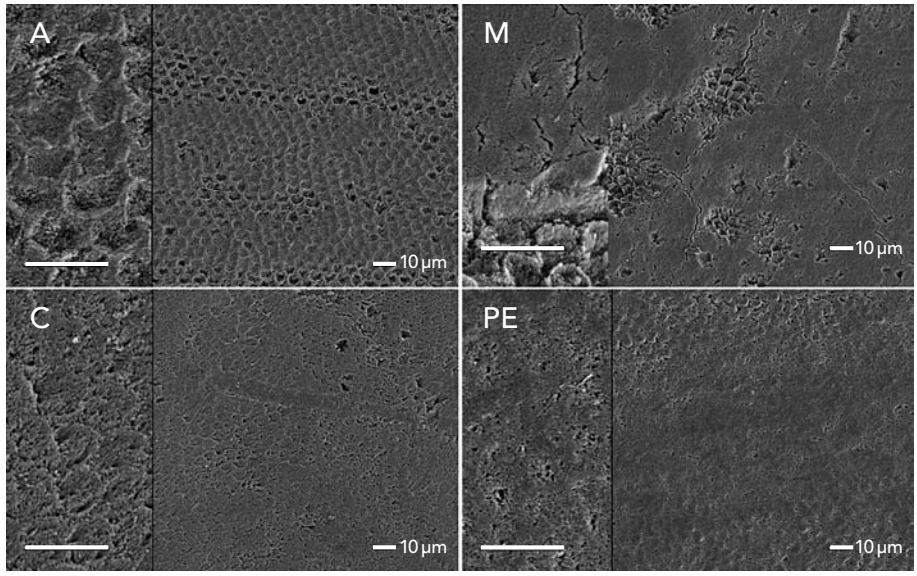


Figure 2. *Scanning Electron Micrographs (each a composition of 2 magnification level images) of the surface of a water brushed sample (A) and a sample from each of the toothpaste groups M, PE and C.*



Discussion

In this in situ study we showed that brushing twice daily with SnF_2-containing toothpastes had a preventive effect on the development of erosive wear. The erosive cycling model was rather severe, as can be seen from the tissue loss in the control group: on average 23 µm in 4 days. It is noteworthy that a basic home care product like toothpaste may influence reduce even such severe wear.

The model was designed to include maximum pairing of data: a split-mouth design was used for the water brushed control, and samples were re-used after each run, so different toothpaste results were obtained using the same samples. This design carried with it the risks of contamination: toothpaste effects carried over from one side of the mouth to the other side, and of a run effect: samples changing from one run to the next. A cross-over effect could not be shown, nor could a run-effect be shown when the complete sample group was analyzed. Only for the separate analysis of the control samples could a significant run effect be observed: for each new run, an extra 1.1 µm of wear was seen. The most likely explanation for this is the combined tissue removal during erosion and polishing of about 125 µm in each run. This exposes deeper layers of enamel, expected to have a lower degree of mineralization and higher solubility. However, this effect was not relevant for the analysis, as the run order was randomized.

The range of individual erosive wear results for control samples was between 13 and 33 µm. This is more uniform than reported before (Wetton et al., 2007; Vieira et al., 2007). However, the range in preventive effects of the toothpastes was great: for toothpaste M the effect ranged from a 69% increase to an 86% decrease in wear. The individual with the lowest wear of water brushed samples showed increased wear for all toothpastes. Neither the factors involved in individual susceptibility to erosive wear, nor those involved in the individual response to preventive agents have as yet been identified. This aspect of erosion (prevention) urgently needs more research.

In this study we found no added wear from toothbrushing with toothpaste. The palatally placed samples were exposed to tongue friction after each erosive challenge, and it has been shown that this removes a softened enamel layer (Gregg et al., 2004, Vieira et al., 2007).

One study reporting the effect of SnF_2-toothpaste on erosion failed to find a significant effect, or difference with NaF toothpastes (Lussi et al., 2008), though elsewhere, calcium loss was reduced immediately after a 4 min application of a SnF_2 toothpaste (Young et al., 2006). Such single erosion challenge studies may not adequately model the complex situation leading to erosive tooth wear, where abrasion is a significant factor. Also, a cycling of both erosion and SnF_2 exposure may be important, as Schlueter and coworkers (2009d) showed that the incorpo-

ration of tin in the enamel is related to the preventive effect. The SEM-images support the theory that stannous fluoride, like titanium fluoride, may work through the formation of a protective surface layer, limiting or delaying the direct contact of the acid with the enamel mineral (Schlueter et al., 2009d).

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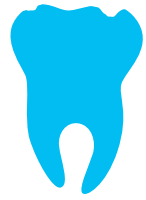
Chapter 8

General discussion and future perspectives



8.1 Introduction

Dental erosion is the irreversible loss of dental hard tissue due to a chemical process without involvement of micro organisms (Imfeld, 1996). Unfortunately, the development of dental erosion is still not fully understood. The general aim of this PhD research was to obtain insight into the effect of beverage parameters, saliva, salivary film/pellicle (SFP) and protein containing toothpaste in the development of dental erosion. Additionally, the effect of beverage composition on measurement techniques for wear quantification was studied. The presented work can be seen as a step on the road to a better understanding of the processes behind the phenomenon of dental erosion.

8.2 Beverage parameters

Dental erosion may be caused by extrinsic and intrinsic factors. A major extrinsic cause of dental erosion is thought to be the consumption of acidic beverages (Dugmore and Rock, 2004). The consumption of acidic beverages has risen over the last years. In the USA a 300% increase in soft drink consumption in a period of 20 years has been reported (Cavadini et al., 2000), an increase that probably has not been reduced as the consumption of soft drinks has not reduced since then.
Recently, a summary of the outcomes of the Workshop on Methodology in Erosion Research has been published (Shellis et al., 2011). Different aspects to which erosion models should comply are suggested such as the use of bovine or human enamel, the use of standardised polishing procedures and storage of samples, the use of an erosive agent resembling an acidic beverage, the use of a clinically relevant pH of the applied erosive agent, agitation during exposure, temperature and duration of erosive challenge. In the studies described in chapter 2 and 3 we already complied partly with the workshop suggestions although these studies were performed far before the outcomes of the workshop were published. For example, we used in these studies bovine enamel as a substrate. The use of bovine enamel is considered acceptable because of its relatively close resemblance to its human counterpart (Zero, 1996). It should be noted, however, that morphological differences exist between bovine and human enamel, such as a higher porosity of bovine enamel (Featherstone and Mellberg, 1981). Fortunately, Meurman and Frank (1991) did not observe any difference in the progression of erosion or the surface ultrastructure of erosive lesions between bovine and human enamel, making bovine enamel a sound alternative to human enamel, thereby surpassing the inherent ethical and other problems that arise when using human material. Furthermore, it should be noted that in our studies with bovine enamel, a comparison between the measurement methods and beverages was performed and not an extrapolation of the results to the clinical situation.

A major limitation of the studies described in chapters 2 and 3 was the absence of saliva during the erosive challenge. The presence of saliva allows for dilution and buffering of the acids. Moreover, the presence of a salivary pellicle can reduce acid diffusion to the enamel (O'Sullivan and Curzon, 2000; Hannig and Balz, 1999). This limitation raises questions with respect to the external validity of the performed experiments. It can be expected that in the clinical situation the loss of enamel will probably be less than the extent of hard tissue loss that was reported in chapters 2 and 3 as usually saliva is present in the mouth of an individual. A possible solution for this caveat could be the use of (artificial) saliva prior to applying the erosive challenge.

In chapter 3 of this thesis we have described that the pH of a beverage is more closely related to the loss of enamel after exposure to an acidic beverage than any of the other studied parameters (calcium and phosphorus concentration, saturation (DSHA), titratable acidity (TA) to pH 5.5, fluoride concentration, viscosity of the beverage and the interaction terms between pH and calcium concentration and titratable acidity and pH). The results of the experiments reported in chapter 3 confirm the results of other studies, viz. that the pH of an acidic beverage is the main indication for their erosive potential (Grobler and van der Horst, 1982; Larsen and Nyvad, 1999; Larsen and Richards, 2001).

In a number of studies it has been suggested to modify acidic beverages chemically to reduce the erosive potential (Grenby, 1996, Hughes et al., 1999; Jensdottir et al., 2005). Modifications of drinks by addition of calcium have been shown to reduce its erosive potential (Barbour et al., 2011). A drawback of the addition of calcium is the relatively high concentration (5 - 10 mmol/l) that is needed to reduce the erosive potential (Barbour et al., 2011). The addition of high concentrations of calcium is accompanied by an unpleasant metallic taste (Jensdottir et al., 2005). Moreover, the effect of the addition of calcium on reducing the erosive potential of a beverage was shown to be higher in drinks with pH values above 4.0 (Jensdottir et al., 2005). As such, the modification of very acidic beverages requires high concentrations of calcium with a negative effect on its taste. In literature, it is also suggested that the addition of phosphate to a beverage might reduce its erosive potential (McDonald and Stookey, 1973; Attin et al., 2003). However, in contrast to the latter studies, Hemingway et al (2006) and Barbour et al (2011) failed to show a relationship between erosive potential and phosphorus concentration. Furthermore, it has been suggested that calcium / phosphate ratios may influence the erosive potential of an agent independent of saturation levels (Barbour et al., 2003). Our volume of data did not allow for assessing this potential relationship, therefore we were not able to evaluate an interaction between phosphate, calcium and pH or between calcium and phosphate. Further research should take this interaction into account.

In a recent published longitudinal study assessing factors associated to erosive wear, no positive significant association was found between erosive wear and consumption of acidic beverages (El Aidi et al., 2011). A comparable result was also reported in some other studies (Bartlett et al., 1998; van Rijkom et al., 2002; Milosevic et al., 2004). In contrast with the above-mentioned results, numerous other studies have reported an association between erosive wear and the consumption of acidic beverages (e.g. O'Sulllivan and Curzon, 2000; Lussi and Schaffner, 2000; Dugmore and Rock, 2004; Bardolia et al., 2010). The difference between the longitudinal study by Bardolia et al. (2010), where a positive association was found between beverage consumption and erosion, and the study by El Aidi et al (2011) could be explained by the much higher average consumption of beverages in the study by Bardolia et al (2010). This somewhat contradictory information illustrates that soft-drinks and acidic beverages are only one factor in the development of erosion and not the explanation whether erosion will occur or not. In other words, in the susceptibility of individuals to dental erosion a variety of individual biological and behavioural factors are involved, as was also mentioned in chapter 1. Therefore, it should be suggested that the biological factors and behavioural factors play an important role, next to the beverage parameters, in explaining and preventing the susceptibility of a particular subject to dental erosion. Especially behavioural factors such as drinking habits may play an important role whether dental erosion will occur in individuals, as well as to which extent. Unusual eating and drinking methods, as well as swallowing habits that increase the direct contact time of acidic foods and beverages with the teeth, are factors that will increase the risk of dental erosion. In addition, the manner that dietary acids are introduced in the mouth (gulping, sipping, use of a straw) will affect the clearance pattern by saliva and which teeth are in contact with the erosive product (Zero and Lussi, 2006). The use of the measurement of erosive potential (chapter 3) has its value, but the results obtained should be interpreted with caution and should be considered as a limited source of information about the erosive potential of soft drinks and beverages. In vivo these in vitro obtained results will be modified by behavioural and biological factors. Therefore, for future research it is recommended to test the erosive potential of drinks in vivo. To facilitate such research, special dentures for edentulous volunteers containing enamel samples could be designed. In this way it will become possible to expose enamel samples to the acidic beverages in vivo without causing unwanted or unexpected damage to the natural dentition of the volunteer similarly to studies assessing the result of the effect of exposure of enamel to a dry oral environment. A model used by Jansma et al (1988) to investigate xerostomia-related dental caries could be modified for use in erosion research. By inclusion of biological factors such as saliva and salivary pellicle in the above mentioned model it becomes interesting to know which individual components of saliva and salivary pellicle influence the development of erosion.

8.3 Saliva and salivary film/pellicle

In chapters 4 and 5 the role of individual components of saliva and the salivary pellicle in the development of dental erosion is described. In earlier research the role of the pellicle as a barrier to acids has been investigated (Hannig and Balz, 2001; Wetton et al., 2006). In our studies we focused on specific individual components of the salivary film/pellicle and saliva. Therefore, a number of potentially interesting salivary and pellicle proteins, minerals and other parameters such as salivary buffer capacity, total protein concentration and flow were studied in the various studies performed in this PhD research.

In chapter 4 it was shown that several salivary parameters were related to the susceptibility of hydroxyapatite (HAp) to erosion. Based on earlier research it was expected that the buffer capacity of saliva was one of the associated factors (Meurman et al., 1994; Lussi and Schaffner, 2000; Lussi and Jaeggi, 2008). This was not confirmed in our study. A reason for this could be that the effect of the salivary buffer capacity on the loss of HAp was limited in our study due to the small amount of saliva present during the extra oral acidic challenge. Furthermore, the measurement of the buffer capacity of saliva is complicated. We used the Ericsson's laboratory method (Ericsson, 1959), which is a titration method. Currently, there is no other method than acid/base titration of saliva to determine its buffer values. Also strip-type tests are actually measuring the titratable acidity and not the buffer capacity (Cheaib et al., 2011a). A drawback of the used method is that during collection of saliva and determination of its buffer capacity, CO^2 could be lost due to exposure to the atmosphere. This can cause a pH change in the alkaline direction, influencing the measured buffer capacity. However, the saliva samples were consistently analysed as soon as possible after sample collection (within 1 min) in order to reduce the effects of variable CO^2 loss in the open system.

In chapter 4 and 5 also the role of the total protein concentration in UWS, SWS and SFP on the susceptibility of HAp to dental erosion was investigated. As mentioned earlier it might be expected that a higher total protein concentration would result in better protection against erosive wear because of the formation of a thicker barrier to acids or by the salivary protein buffer system. The buffer capacity of saliva involves three buffer systems, namely the carbonate, phosphate and protein buffers (Bardow et al., 2000; Lenander-Lumikari and Loimaranta, 2000). It is suggested in earlier research that the buffering below pH 5 is mainly based on the protein system (Bardow et al., 2000). In the study described in chapter 4 we could not find a relationship between the total protein concentration in UWS, SWS and the loss of calcium from HAp. In chapter 5 we measured again the total protein concentration in UWS, SWS and the SFP. Despite using a different method for measuring the total protein concentration we again could not find a relationship with the loss of calcium from HAp. Furthermore, we could also not find a associ-

ation between the total protein concentration in UWS and SWS and the corresponding buffer capacity values measured in chapter 4 and 5 (data not shown). In a study investigating the relationship between the total protein concentration and the prevalence of erosion it was even found that a higher concentration of proteins in SWS was associated with more erosion (Piangprach et al., 2009). Therefore it could be suggested that the protective effect of the SFP is determined by specific proteins or other SFP components and not by the total protein concentration.

In the studies presented in chapters 4 and 5, HAp discs, instead of human or bovine enamel, were used to determine the susceptibility of the volunteers to erosive wear and to collect pellicle. HAp is a close analogue of human enamel mineral. It has been used in many in vitro and in situ studies (e.g. Vittorino et al., 2004; Barbour et al., 2008; Hemingway et al., 2008; Zaman et al., 2010). HAp discs have greater porosity and their structure, particle size and shape differ from human enamel (Hemingway et al., 2008). Due to the greater porosity of the HAp discs, the absorption of the salivary pellicle may be higher compared to human enamel, but a major advantage is that the composition of HAp discs derived from the same batch is stable. This reduces variation in sample composition making inter-individual comparisons of the saliva/pellicle effect more straightforward. Another advantage is that due to the large surface of the samples, a large quantity of the salivary film/pellicle can be harvested by the harvesting method described in chapter 5. Furthermore, HAp discs are commercially available in large quantities. Despite the mentioned benefits of HAp, the participants of the Workshop on Methodology in Erosion Research (Shellis et al., 2011) decided that HAp should be used for exploratory in vitro studies only. Therefore, in future research the results from chapters 4 and 5 should be replicated with human enamel. For this, the in situ model mentioned in paragraph 8.1 could be used.

In chapter 5 it is suggested that, amongst other factors, a high concentration of carbonic anhydrase-6 (CA-6) in salivary film/pellicle is associated with a low susceptibility of a subject to dental erosion. To the best of our knowledge, an association between the concentration of CA-6 in saliva or pellicle/salivary film and the susceptibility to erosion has not yet been reported in the literature, although in earlier research it has been suggested that the presence of CA-6 in enamel pellicle could imply that CA-6 might function as a local pH regulator on the enamel surface (Kivelä et al., 1997; Leinonen et al., 1999). Furthermore, it was suggested that CA-6 might play a role in regulating the pH or buffer capacity of saliva (Feldstein and Silverman, 1984), whilst other studies indicated that latter variables were not directly associated with CA-6 concentration in saliva (Parkkila et al., 1993; Kivelä et al., 1997). Also, a role for CA-6 in the neutralization of acid by bicarbonate in dental plaque has been suggested (Kimoto et al., 2006). In our in vitro study, HAp discs were covered with pellicle and salivary film when being exposed to citric acid

because we did not rinse the discs after removal from the oral cavity. By using this combination of pellicle and salivary film (SFP), we modelled the in vivo situation where bicarbonate is present in saliva to facilitate the transition of H^+ ions to H^2O regulated by CA-6.

To quantify the amount of carbonic anhydrase 6 (CA-6) present in saliva we developed an Enzyme Linked ImmunoSorbent Assay (ELISA) technique (chapter 5). However, it appeared to be difficult to use this technique for analysis of salivary films/pellicles collected with a rinsing solution containing EDTA and SDS. Therefore, we used a Western Blot technique to detect CA-6 in pellicle in addition. This limitation of the technique we developed formed a draw-back of our assay because the Western Blot technique is a semi-quantitative technique and therefore possibly less accurate. Nevertheless, when values for salivary CA-6 measured with our ELISA assay were compared with those obtained using the Western blotting technique, there was a significant, moderately strong correlation ($r = 0.7$, $p<0.001$).

Another important factor that needs further investigation is the enzymatic activity of CA-6. We measured the concentration of this protein but it is also interesting to know whether CA-6 retains its activity in the salivary flim/pellicle on our HAp samples because the pH regulating properties of this protein depend on its activity. Based on an earlier publication where it was confirmed by histochemical staining of in vitro formed enamel pellicle that CA-6 remained its activity (Leinonen et al., 1999), it can be expected that CA-6 will also remain its activity in our study design but this should be subject of further research.

It should be noted that the parameters mentioned in chapters 4 and 5 are just a selection of salivary and pellicle components that may play a role in the susceptibility of a subject to dental erosion. For example, we did not study the role of mucins in the development of erosion. In a study by van Nieuw Amerongen et al. (1987) the role of mucins in protection against erosion was discussed. Because exposure of enamel to submandibular/sublingual saliva for 60 min resulted in complete prevention of erosion and this saliva contains a high concentration of mucins, it was suggested that these proteins might play an important role in prevention to erosion. Mucin concentrations and activity should therefore be subject of further research. In chapter 4 we combined the studied saliva and salivary film/pellicle factors in a multivariate analysis to develop a model to predict susceptibility to dental erosion of individuals based on their saliva composition. To test such a model in situ/in vivo and to confirm the results from our studies, a large case-control study has to be set up. In such a study also the activity of CA-6 and the concentrations and activity of mucins (for ex MUC5b and MUC7) should be assessed.

8.4. Erosion reducing toothpastes

Until now, a variety of toothpastes has been introduced to the market claiming to reduce dental erosion (Lussi et al., 2008). In this thesis we focused on the possible reduction of erosion by toothpastes containing specific proteins or by stannous fluoride. In the study described in chapter 6, the effect of toothpastes containing colostrum on erosive wear was tested with an in situ formed pellicle and in vitro without pellicle. It was shown that this product reduced erosive wear compared to brushing with water. Also for pellicle-covered enamel, a high concentration of protein in toothpaste reduced erosion compared to a protein-free control, when phosphate loss was considered.

The toothpaste studied in chapter 6 is the only yet commercial available one that uses proteins (colostrum) to reduce dental erosion. Colostrum used in this study contained amyloglucosidase, glucose oxidase, lactoperoxidase, lysozyme, casein and lactoferrin. Colostrum and other proteins have been included in toothpastes before to reduce the susceptibility to caries and to maintain overall oral health (Lenander-Lumikari et al 1993; Kirstila et al., 1994; Tenuvuo, 2002; Pedersen et al., 2002). The addition of proteins to toothpaste is still somewhat 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, a recent study on the efficacy of enzymatic toothpastes showed that immobilisation of enzymes in an in situ pellicle indeed can be achieved by using toothpaste (Hannig et al., 2010). One of the proteins present in colostrum is casein. In studies this protein was shown to provide some protection against erosion (Barbour et al., 2008; Hemingway et al., 2010) and it is known that this protein can be incorporated in the pellicle (Guggenheim et al., 1994). It has been suggested that the reduction in dissolution rate observed by adding colostrum is related to the firm adsorption of casein onto the HAp surface, which stabilizes the crystal surface and inhibits ion detachment (Barbour et al., 2008). Furthermore, it was shown that the efficacy of casein as a barrier to acids in the presence of pellicle is enhanced (Hemingway et al., 2010).

In chapter 7 the effect of stannous fluoride in two commercially available toothpastes on erosive wear was compared to water and a sodium fluoride containing toothpaste. Recently stannous fluoride has been considered a promising agent to reduce erosive wear (Wiegand et al., 2009). In our study it was shown that SnF_2 containing toothpastes reduced erosive wear compared to sodium fluoride toothpaste. It was suggested that the stannous is incorporated in the enamel, which causes the preventive effect (Schlueter et al., 2009). One of the toothpastes investigated in chapter 7 contained next to the stannous fluoride amine fluoride, which is known to have a strong tendency to adsorb to enamel and to result in a thicker adsorbed layer (Busscher et al., 1988). This could explain the largest reduction of

erosive wear by the paste containing both amin- and stannous fluoride. Moreover this paste was also slightly more acidic compared to the other pastes. It has been shown before that acidic fluoride applications reduce erosive wear more effectively than neutral ones (Hove et al., 2008; Wiegand et al., 2008).

For further research different other biomimetic approaches in oral care products such as the use of mucins or nanomaterials could be interesting. In a recent in vitro study the use of combinations of mucins and casein on the salivary pellicle showed an improvement of the erosion inhibiting properties of the salivary pellicle (Cheaib et al., 2011b). Therefore, it was suggested that the synergetic use of different proteins, rather than the use of a single protein, increases the protective function of a salivary pellicle. Carbonic anhydrase-6 could be one of the proteins of interest for such research. Another interesting development is the use of bioinspired nanomaterials such as casein phosphopeptide stabilized amorphous calcium phosphate (CPP-ACP). These are proteinaceous products such as casein phosphopeptides in combination with apatite nanoparticles. CPP-ACP has been suggested to promote remineralization of initial enamel erosive lesions and to prevent demineralization (Hannig et al., 2010). The latter has only been shown in vitro in a limited number of studies and it is therefore interesting to use in an in situ or in vivo setup to test whether this product is able to reduce erosive wear.

It could be questioned whether modifications of acidic beverages, such as mentioned in paragraph 8.1, or preventive measures like the development of special toothpastes are more effective in reduction of erosive wear. A major drawback of the modification of beverages by for example calcium is, as mentioned before, the (unpleasant) change in taste. Until now a couple of special developed non-erosive beverages are available on the market (Huysmans et al., 2006; West et al., 2003). Unfortunately these products are not very popular compared to the traditional acidic beverages. Therefore, it could be suggested that better opportunities are available for preventive oral care products in combination with education of patients about the consequence of the consumption of acidic beverages. In particular, proteinaceous products with the ability to remineralize erosive lesions meanwhile preventing demineralization are promising and should be subject of further research. Furthermore, the effect of addition of proteins like carbonic anhydrase-6 to oral care products could be subject of further research.

Concluding remarks

The general aim of this PhD research was to obtain insight in the effects of beverage parameters, saliva, salivary film/pellicle and toothpaste on the development of dental erosion. By investigating different factors influencing the susceptibility to dental erosion a better insight is obtained in the development of erosive wear. Factors on the "attack side" (acidic beverages) and on the "defence side" (saliva, pellicle and toothpaste components) are important factors whether and to which extent erosive wear occurs. Overall, it can be suggested that no erosion occurs unless the acidic challenge (strength, frequency, drinking methods) exceeds a certain threshold or when the host response (biological factors) is not adequate enough. Different interventions, such as proteins and fluorides in toothpastes, may have a role to play in the reduction in the susceptibility to erosive wear.

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Chapter 9

Summary

Dental erosion, the progressive loss of tooth substance by chemical processes that does not involve bacterial action, seems to play a major role in the development of dental wear. In *chapter 1*, an overview of different forms of wear is presented. As mentioned in this chapter, the development of dental erosion is still not fully understood. Therefore, the general aim of this PhD research was to obtain insight in the role of beverage parameters, saliva, salivary film/pellicle and toothpaste in the development of dental erosion. Additionally, the effect of beverage composition on measurement techniques for erosive potential quantification was studied.

The influence of beverage composition on the measurement of their erosive potential by chemical methods (measurement of loss of calcium and loss of phosphate from enamel) and optical profilometry was investigated in the study described in *chapter 2*. Also the influence of exposure of enamel to acidic drinks in small and large volumes was assessed in that study. Eleven beverages were included: water (control), 3 alcopops, 2 beers and 5 softdrinks. For assessing the erosive potential of each beverage, per beverage 15 bovine enamel samples were used: 5 for chemical and 10 for profilometric analysis. After exposure to the beverages the resulting solutions were analyzed for calcium and phosphate content. For optical profilometry, the samples were submersed sequentially in 500 ml or in 1 ml of the drinks for 3, 6, 9, 15 and 30 min (total 63 min). Some beverages had high baseline concentrations of calcium (energy drink) or phosphate (cola drink, cola lemon drink, beer, beer lemon). Some of the beverages showed a good correlation between the chemical methods. One-way statistical analysis of the results showed a significant effect of measuring technique (ANOVA; $p < 0.05$) for all beverages except ice tea and the fruit drink. Profilometry showed generally lower enamel losses than the chemical methods. When comparing exposures to large versus small volumes of the beverages it was found that there were no differences except for the cola drinks. The cola drinks showed lower enamel losses for the 1 ml profilometry compared to the 500 ml profilometry. It can be concluded that the composition of the beverages had a significant effect on the determination of the erosive potential with chemical analyses. Drink composition also influenced the effect of small versus large exposure volumes indicating the need for standardization of exposure parameters.

The aim of *chapter 3* was to investigate the erosive potential of beverages, using exposure times from 3 to 30 min, and to analyze the relationship between erosion and several drink parameters if possible using a multivariate approach. For this the pH, calcium, phosphate and fluoride concentration, saturation, titratable-acidity to pH 5.5 and the viscosity of sixteen beverages were measured or calculated. Bovine buccal enamel samples (N = 90) were serially exposed to 1 ml of the beverages for 3, 6, 9, 15, and 30 min and enamel erosion was measured as the loss of calcium to

the beverage. The rate of erosion per min was calculated by linear curve fitting using all exposure times. Linear regression analysis was performed to determine the correlation between erosion and the drink parameters. A limited multivariate analysis was performed for the outcome parameter with the highest univariate correlations (erosion per minute) and 4 drink variables.
A consistently negative relationship was observed for all exposure times only for pH. Only for erosion per min a significant relationship with pH and saturation was found. In a model for erosion per min using only saturation, fluoride concentration, titratable acidity and viscosity, both saturation and viscosity were shown to have a significant effect ($p = 0.01$ and $p = 0.05$, respectively).
This study showed that the choice of exposure time between 3 and 30 min resulted in very different estimates of erosive potential. There is no sound theoretical ground for preferring one or other exposure time / outcome as being more clinically relevant.
The second part of this thesis focused on the investigation of the role of saliva and pellicle in the development of erosion. Saliva and the salivary film/pellicle are known to reduce the erosion of enamel and differences in level of protection exist between individual saliva sources, but which parameters or components are important was not yet known.

In *chapter 4* a study on the role of different salivary parameters in the development of erosion is described. The focus of that study was to investigate the relationship between saliva parameters and early erosion of hydroxyapatite (HAp) with an in situ grown saliva film and pellicle (SFP). For this twenty-eight volunteers carried two HAp and one porcelain discs in their buccal sulcus for 1.5hr. Next, the two discs covered with pellicle and attached saliva film were exposed extraorally to 50 mM (pH = 3) citric acid for 2 min and unstimulated and stimulated whole mouth saliva (UWS and SWS) was collected. Calcium loss from HAp after erosive challenge was measured, corrected for calcium loss from pellicle on the porcelain discs, and averaged. Several salivary parameters were analysed, viz. calcium, phosphorus, sodium, chloride, urea, total protein and albumin concentration. Furthermore, the pH, flow and buffer-capacity were analyzed. Pearson's linear correlation and multiple regression analysis were used to study the relation between saliva parameters and HAp-erosion. This analysis showed significant associations between HAp-erosion and the concentration of phosphorus in UWS ($r = 0.40$, $p = 0.03$), and between HAP-erosion and the concentration of sodium ($r = -0.40$, $p = 0.03$), chloride ($r = -0.47$, $p = 0.01$), phosphorus ($r = 0.45$, $p = 0.01$) and flow ($r = -0.39$, $p = 0.04$) of SWS. Multivariate-analysis revealed a significant role in the HAp-erosion for sodium, urea, total protein, albumin, pH and flow of UWS and sodium, potassium, urea, and phosphorus of SWS. Thus, it is likely that salivary parameters are associated with the susceptibility of HAp to erosion.

In *chapter 5* a study is described that assessed the role of a small selection of parameters of the salivary pellicle and saliva in the development of erosion. The objective of this study was to investigate the relationship between concentration of carbonic-anhydrase 6 (CA-6), statherin and total protein in saliva and salivary film/pellicle (SFP) formed on HAp and susceptibility of HAp to acid erosion. To investigate this relationship, twenty-one volunteers carried three HAp discs in their buccal sulcus for 1.5hr. Two SFP-coated discs were exposed to citric acid (pH=3) and loss of calcium was measured. Unstimulated and stimulated whole mouth saliva (UWS and SWS) were collected. Protein was eluted from the surface of the third HAp disc for analysis. Composition of proteins in SFP, UWS and SWS were analysed by SDSPAGE and assayed for total protein (BCA method), whilst the CA-6 and statherin content of SFP was determined using Western Blotting. CA-6 concentration in UWS and SWS was measured using an immunoassay (ELISA), the statherin concentration was determined by Western Blotting. Pearson's correlation analysis showed only significant associations between loss of calcium from HAp and concentration of CA-6 in SWS ($r = -0.49$, $p = 0.025$), in UWS ($r = -0.43$, $p = 0.05$) and in SFP ($r = -0.62$, $p = 0.003$) and between loss of calcium from HAp and concentration of statherin in SWS ($r = -0.45$, $p = 0.042$). From these results it was concluded that the concentration of CA-6 in UWS, SWS and SFP is inversely correlated with erosive demineralisation of HAp.

In the third part of this thesis we were interested in different options to enhance the natural protection against dental erosion Therefore, we studied whether it is possible that the introduction of proteins into the oral cavity by toothpaste could give a reduction in the susceptibility of enamel to erosion (*chapter 6*). Moreover, the erosion reducing effect of stannous fluoride in toothpastes was tested in *chapter 7*.

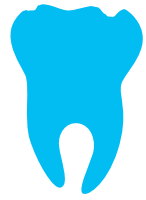
First, we studied whether proteins added to toothpaste could reduce the dental erosion (*chapter 6*). For this a combined split-mouth (extra-oral water or toothpaste brushing) and cross over (type of toothpaste) set-up were used. Two protein containing (high/low concentrations of colostrum) and one non-protein (placebo) toothpaste were investigated. Sixteen volunteers wore intra-oral appliances containing 2 human enamel samples for pellicle growth during 90 min during 3 afternoons. One enamel sample was brushed for 5 sec with one of the three toothpastes and subsequently exposed to slurry of the corresponding toothpaste for 2 min. The other sample was exposed to water. Both samples were subsequently exposed to citric acid (extra-orally). Loss of calcium and inorganic phosphate were determined. The same sequence of exposures was applied to 16 enamel samples in an in vitro set up without pellicle. With in situ formed pellicle, all toothpastes significantly reduced calcium loss as compared to water brushing. For the loss of phosphate, a significant reduction could be found with the use of the high-protein toothpaste compared to

the non-protein toothpaste. The calcium loss showed a similar trend. Overall, there were only slight differences between the toothpastes. Toothpaste effects were less clear in the vitro experiment. Brushing with toothpaste of pellicle-covered enamel resulted in lower erosion compared to brushing with water. Also for pellicle-covered enamel a high concentration of protein in toothpaste reduced erosion compared to a protein-free control, when phosphate loss was considered.
Next, to investigate the effect of stannous fluoride on dental erosion, a combined split-mouth (extra-oral water or toothpaste brushing) and crossover (type of toothpaste) set-up was used (*chapter 7*). Twelve volunteers wore palatal appliances containing human enamel samples. Three toothpastes were used, in three consecutive runs and in randomized order: two toothpastes containing stannous fluoride (SnF_2) (coded M and PE) and one toothpaste containing only sodium fluoride (NaF) (coded C). On day 1 of each run the appliances were worn for pellicle formation. On days 2-5 the samples were also brushed twice with a toothpaste-water slurry or only water (control). Erosion took place on days 2-5 extra-orally 3 times a day (5 min) in a citric acid solution (pH 2.3). Enamel wear depth was quantified by optical profilometry. The effect of toothpastes was tested using General Linear Modelling. The average erosive wear depth of control samples was 23 µm. Both SnF_2 toothpastes significantly reduced erosive wear: M by 34% (SD 39%) and PE by 26% (SD 25%). The control toothpaste reduced erosive wear non-significantly by 7% (SD 20%). Both SnF_2-containing toothpastes significantly better reduced erosive wear than the sodium fluoride toothpaste. From this information it was concluded that SnF_2-containing toothpastes are able to reduce erosive tooth wear in situ.

Finally in *chapter 8* the critical points of the methodology and the main research outcomes are discussed. Especially attention was paid to the outcomes of the Workshop on Methodology in Erosion Research (Shellis et al., 2011). The setup of the studies and the used substrates were compared to these recommendations. The role of beverage parameters in the erosive potential of a beverage is discussed. Furthermore, the possibilities to reduce the erosive potential of beverage are mentioned. Concerning the role of saliva and salivary film it is suggested that, amongst other factors, CA-6 could play a role in the susceptibly of individuals to dental erosion.
Also some suggestions for future research are given. Because the experiments with regard to saliva and salivary film were performed with HAp as a substrate, these results should be repeated in future research with human enamel and in an in situ setting. Next to the already investigated salivary and salivary film parameters, also the role of other saliva/salivary film components in erosive wear, such as the mucin concentration, should be topic of future research. Concerning the prevention of erosion it was suggested that further research should focus on biomimetic approaches such as the use of proteinaceous products or nanomaterials.

Chapter 10

Samenvatting



Tanderosie, het oplossen van glazuur en dentine onder invloed van zuren die niet door bacteriën zijn geproduceerd, lijkt een grote rol te spelen in het ontstaan van gebitsslijtage. In *hoofdstuk 1* wordt een overzicht gepresenteerd van de verschillende vormen en oorzaken van slijtage. Zoals genoemd in *hoofdstuk 1*, is nog steeds niet volledig bekend hoe tanderosie ontstaat. Daarom was het doel van dit promotie onderzoek het verkrijgen van inzicht in de rol van (fris)dranken, speeksel, speekselfilm/pellikel en tandpasta op het ontstaan van tanderosie. Daarnaast is het effect van de samenstelling van frisdranken op de meetmethoden voor erosieve potentie bestudeerd.

De invloed van de samenstelling van dranken op het bepalen van de erosieve potentie met behulp van chemische methoden (het meten van het verlies van calcium en fosfaat van glazuur) en een optische methode (profilometrie) is onderzocht in *hoofdstuk 2*. Tevens is de invloed van het blootstellen van glazuur aan zure dranken in kleine en grote volumes bestudeerd. Elf frisdranken zijn geïncludeerd: water (controle), 3 alcholische dranken ("alcopops"), 2 bieren en 5 frisdranken. Om de erosieve potentie van elke frisdrank te bepalen werden per frisdrank 15 koeglazuurpreparaten blootgesteld aan de dranken: 5 voor chemische analyse en 10 voor profilometrische analyse. Voorafgaand en na het blootstellen van het koeglazuur aan de dranken werden de dranken geanalyseerd op hun calcium- en fosfaatinhoud. Voor optische profilometrie werden vijf van de tien glazuur preparaten geplaatst in 500 ml of 1 ml van de dranken gedurende 3, 6, 9, 15 en 30 min (cumulatief 63 min).
Een aantal dranken had, ten opzichte van de overige dranken, een erg hoge basisconcentratie calcium (energiedrank) of fosfaat (cola, cola lemon, bier, bier lemon). Een aantal dranken liet een goede correlatie zien tussen de chemische methoden. One-way Anova analyse resulteerde in een significant effect voor de parameter meetmethode ($p < 0.05$) voor alle dranken met uitzondering van ijsthee en de fruitdrink. Profilometrie liet over het algemeen lagere glazuurverliezen zien dan de chemische methoden. Nagenoeg geen van de onderzochte dranken verschilde in glazuurverlies na blootstelling aan grote versus kleine volumes. Een uitzondering vormden de cola's. Deze lieten lagere glazuurverliezen zien voor de blootstelling aan 1 ml van de frisdrank vergeleken met blootstelling aan een volume van 500 ml. Er kan geconcludeerd worden dat de samenstelling van de dranken een significant effect lijkt te hebben op het bepalen van de erosieve potentie met chemische methoden. De samenstelling van de dranken beïnvloedde ook het effect van kleine en grote volumes. Dit leidt tot de aanbeveling om de blootstellingsparameters te standaardiseren in het verdere onderzoek naar de erosieve potentie van frisdranken.

Het doel van *hoofdstuk 3* was het onderzoeken van de erosieve potentie van dranken met blootstellingstijden van 3 tot 30 minuten. Tevens werd de relatie tussen het optreden van tanderosie en verschillende parameters van dranken, indien mogelijke via een multivariate benadering, bestudeerd. Hiervoor werden de pH, calcium-, fosfaat-, en fluorideconcentratie, saturatie, de titreerbare zuurgraad tot pH 5,5 en de viscositeit van 16 dranken gemeten. Koeglazuurpreparaten (N = 90) werden blootgesteld aan 1 ml van elke drank gedurende 3, 6, 9, 15 en 30 min. Het oplossen van glazuur werd bepaald als het verlies van calcium van het glazuur aan de drank. Het gemiddelde verlies aan calcium werd omgerekend naar het verlies van glazuur in mm. De hoeveelheid erosie per minuut werd berekend door middel van "linear curve fitting" met gebruikmaking van alle blootstellingstijden. Lineaire regressieanalyse werd gebruikt om de correlatie tussen het calciumverlies en de verschillende drank parameters te bepalen. De correlatieanalyse van calciumverlies met de drankparameters liet alleen voor pH een consistente negatieve relatie zien. In een model voor erosie per minuut, waarbij alleen de parameters saturatie, fluorideconcentratie, titreerbare zuurgraad en viscositeit werden gebruikt, werd zowel voor saturatie als viscositeit een significant effect gevonden (respectievelijk $p = 0.01$ and $p = 0.05$).
Deze studie laat zien dat de keuze voor lange of korte blootstellingstijden of een geconstrueerde uitkomstvariabele waarbij een combinatie wordt gebruikt, resulteert in erg uiteenlopende schattingen van de erosieve potentie. Een goede theoretische onderbouwing ontbreekt om de ene uitkomstvariabele als meer klinisch relevant te verkiezen boven de andere. Klinische studies waarbij de erosieve potentie van dranken wordt vergeleken zijn nodig om de validiteit van in vitro experimenten te bepalen.

In het tweede deel van dit proefschrift ligt de nadruk op de rol van speeksel en pellikel in het ontstaan van tanderosie. Speeksel en de op de elementen aanwezige speekselfilm/pellikel zouden bescherming kunnen bieden tegen tanderosie. Verder lijken er persoonsgebonden verschillen te bestaan in de bescherming die speeksel biedt. Welke parameters of onderdelen van het speeksel hiervoor verantwoordelijk zijn is tot nu toe onbekend.

Hoofdstuk 4 beschrijft het onderzoek naar de rol van verschillende speekselparameters in het ontstaan van tanderosie. De studie focust zich op de relatie tussen een selectie van speekselparameters en het ontstaan van erosie van hydroxyapatiet (HAp) waarop een speekselfilm en pellikel aanwezig waren. Daartoe droegen 28 vrijwilligers twee HAp- en één porseleinpreparaat in de buccale omslagplooi van de mandibula gedurende 90 min. Vervolgens werden de twee HAp-preparaten 2 min extra-oraal blootgesteld aan 50 mM (pH = 3) citroenzuur. Ook werd

ongestimuleerd en gestimuleerd totaalspeeksel verzameld. Het calciumverlies van de twee HAp-preparaten werd gemeten na de erosieve blootstelling. Tegelijkertijd werd ook het calciumverlies gemeten uit de pellikel door het op dezelfde wijze blootstellen aan citroenzuur van het porselein. Het calciumverlies uit het HAp werd gecorrigeerd met deze waarde en vervolgens gemiddeld. Het verzamelde totaalspeeksel werd geanalyseerd op concentratie van calcium, fosfaat, natrium, chloride, kalium, ureum, totaaleiwit en albumine. Daarnaast werden ook de pH, vloed en de buffercapaciteit van het speeksel bepaald. Pearson's lineaire regressieanalyse en multivariate regressieanalyse werden gebruikt om de relatie tussen speekselparameters en HAp-erosie te bestuderen. Deze analyse liet zien dat er significante associaties bestonden tussen erosie van HAp en de concentratie fosfaat in ongestimuleerd speeksel ($r = 0.40$, $p = 0.03$) en tussen erosie van HAp en de concentratie natrium ($r = -0.40$, $p = 0.03$), chloride ($r = -0.47$, $p = 0.01$), fosfaat ($r = 0.45$, $p = 0.01$) en vloed ($r = -0.39$, $p = 0.04$) van gestimuleerd speeksel. Multivariate regressieanalyse liet een significante ($p \leq 0.1$) rol zien voor natrium, ureum, totaaleiwit, albumine, pH en vloed van ongestimuleerd speeksel en voor natrium, kalium, ureum en fosfaat van gestimuleerd speeksel. Hieruit kan geconcludeerd worden dat bepaalde speekselparameters geassocieerd zijn met erosie van HAp.

Hoofdstuk 5 beschrijft een studie naar de rol van een kleine selectie van speeksel en pellikelparameters in het ontstaan van tanderosie. Het doel van deze studie was het onderzoeken van de relatie tussen de concentratie koolzuuranhydrase-6 (CA-6), statherine en de totaaleiwitconcentratie in speeksel en de speekselfilm/pellikel en de gevoeligheid van HAp voor erosie. Daartoe droegen 21 vrijwilligers 3 HAp-preparaten gedurende 90 min in de buccale omslagplooi van de mandibula. Twee met een speekselfilm/pellikel bedekte preparaten, werden na 90 min verwijderd uit de mond en extra-oraal blootgesteld aan citroenzuur (50 mM, pH = 3). Vervolgens werd het verlies van calcium uit HAp bepaald. Daarnaast werd ongestimuleerd en gestimuleerd speeksel verzameld. Het derde HAp preparaat werd gebruikt om de speekselfilm/pellikel te oogsten. De eiwitsamenstelling van het speeksel en de speekselfilm/pellikel werden geanalyseerd met behulp van SDSPAGE en een BCA methode bepaalde de totaaleiwitconcentratie. De concentratie CA-6 en statherine in de speekselfilm/pellikel werden gemeten door middel van Western Blotting. De CA-6 concentratie in speeksel werd bepaald door middel van een ELISA techniek en een Western Blot techniek bepaalde de statherineconcentratie.
Pearson's correlatie analyse liet een significante associatie zien tussen het verlies van calcium uit HAp en de concentratie CA-6 in gestimuleerd speeksel ($r = -0.49$, $p = 0.025$), in ongestimuleerd speeksel ($r = -0.43$, $p = 0.05$) en in de speekselfilm/pellikel ($r = -0.62$, $p = 0.003$). Daarnaast werd een significante associatie tussen

de concentratie statherine in gestimuleerd speeksel en het verlies van calcium van HAp gevonden (r = -0.45, p = 0.042). Uit deze resultaten kan geconcludeerd worden dat de concentraties CA-6 in ongestimuleerd en gestimuleerd speeksel en de speekselfilm/pellikel negatief geassocieerd zouden kunnen zijn met erosieve demineralisatie van HAp.

In het derde deel van dit proefschrift werden verschillende opties bestudeerd om de natuurlijke bescherming tegen tanderosie te verbeteren. De vraag daarbij was of eiwitten die in de mond geïntroduceerd zijn door een tandpasta, een reductie kunnen geven in de gevoeligheid voor tanderosie (*hoofdstuk 6*). Daarnaast werd in *hoofdstuk 7* onderzocht of tandpasta's die tinfluoride bevatten in staat zijn om de gevoeligheid voor tanderosie te verminderen.

Om te beginnen werd onderzocht of eiwitten toegevoegd aan tandpasta in staat zijn om de vatbaarheid voor tanderosie te verminderen (*hoofdstuk 6*). Hiertoe werd een gecombineerd "split-mouth" (poetsen met water of poetsen met tandpasta) en "cross-over" (soort tandpasta) onderzoek gebruikt. Twee eiwitbevattende pasta's (hoge en lage concentratie colostrum) en één eiwitvrije pasta werden onderzocht. Zestien vrijwilligers droegen intra-orale beugels waarin twee preparaten humaan glazuur ingebouwd waren. Deze beugels werden 90 minuten gedragen gedurende drie middagen. Het ene glazuurpreparaat werd eerst gedurende 5 seconden gepoetst met één van de drie pasta's en aansluitend 2 minuten blootgesteld aan een oplossing van de corresponderende pasta en water. Het andere preparaat werd blootgesteld aan water. Beide preparaten werden vervolgens extra-oraal blootgesteld aan citroenzuur (pH = 2.3) en het verlies van calcium en fosfaat uit het glazuur werd bepaald. Dezelfde volgorde van blootstellingen werd toegepast op zestien glazuurpreparaten in een in vitro experiment zonder de aanwezigheid van een pellikel.
In de aanwezigheid van een in situ gevormde pellikel lieten alle tandpasta's een significante ($p \leq 0.05$) reductie in het verlies van calcium zien vergeleken met het poetsen met water. Voor fosfaatverlies werd een significante reductie gevonden na het gebruik van de tandpasta met een hoge concentratie eiwitten vergeleken met de pasta zonder eiwitten. Het calciumverlies na het gebruik van de pasta's of water liet een vergelijkbare trend zien. Echter, over het geheel genomen waren er weinig verschillen tussen de pasta's onderling. Het effect van de pasta's was minder duidelijk in de in vitro experimenten. Het poetsen met de pasta's van glazuur bedekt met een pellikel resulteerde in minder erosie vergeleken met het poetsen met water. Daarnaast werd gevonden dat een hoge concentratie eiwitten resulteert in een lager fosfaatverlies vergeleken met een eiwitvrije tandpasta.

Om het effect van tinfluoride op tanderosie te bestuderen werd eveneens een gecombineerd "split-mouth" (poetsen met water versus poetsen met tandpasta) en "cross-over" (verschillende tandpasta's) onderzoek gebruikt (*hoofdstuk 7*). Twaalf vrijwilligers droegen een beugel met daarin 2 stukjes humaan glazuur. Drie tandpasta's werden gebruikt in drie opeenvolgende sessies en in een gerandomiseerde volgorde: twee pasta's met tinfluoride (SnF_2) (gecodeerd M en PE) en één natriumfluoride bevattende pasta (NaF) (gecodeerd C). Op dag 1 van elke sessie werden de beugels gedragen om een pellikel te kweken op het glazuur. Op dagen 2 tot 5 werden de preparaten 2 maal gepoetst met een pasta-water oplossing of alleen water (controle). Gedurende dagen 2 - 5 werd het glazuur driemaal per dag blootgesteld aan een erosieve citroenzuuroplossing (pH 2,3). Vervolgens werd het verlies van glazuur vastgesteld door middel van optische profilometrie. Het effect van de pasta's werd geanalyseerd door middel van General Linear Modelling. De gemiddelde diepte van de erosieve laesies was 23 µm. Beide SnF_2 bevattende tandpasta's verminderden significant de erosieve slijtage: M met 34% (SD 39%) en PE met 26% (SD 25%). De controletandpasta verminderde de erosieve slijtage niet significant met 7% (SD 20%). Beide SnF_2 bevattende tandpasta's verminderden de erosieve slijtage significant beter dan de natriumfluoride tandpasta. Op basis van deze informatie werd geconcludeerd dat SnF_2-bevattende tandpasta's in staat zijn om tanderosie in situ te verminderen.

In *hoofdstuk 8* werden de kritische punten van de methodologie en de onderzoeksresultaten besproken. Met name is er aandacht besteed aan de uitkomsten van de "Workshop on Methodology in Erosion Research" (Shellis et al., 2010). De aanbevelingen van Shellis et al werden vergeleken met de methodologie van de studies. De rol van drankeigenschappen in de erosieve potentie van die drank is besproken. Daarnaast werden de mogelijkheden benoemd om de erosieve potentie van een drank te verminderen. Met betrekking tot de rol van speeksel en de speekselfilm/pellikel wordt gesuggereerd dat, naast andere factoren, koolzuuranhydrase 6 een rol zou kunnen spelen in de gevoeligheid voor tanderosie van individuen. Daarnaast wordt een aantal suggesties gedaan voor vervolgonderzoek. Aangezien de experimenten met speeksel en de speekselfilm/pellikel uitgevoerd zijn met hydroxyapatiet zouden deze experimenten in toekomstig onderzoek herhaald moeten worden met menselijk glazuur in een in situ setting. Naast de reeds onderzochte speeksel- en speekselfilm/pellikel parameters wordt gesuggereerd dat andere speekselparameters, zoals de mucineconcentratie, onderwerp van toekomstig onderzoek kunnen zijn. Voor wat betreft de preventie van tanderosie zou toekomstig onderzoek zich kunnen richten op een biomimetische benadering zoals het gebruik van eiwitbevattende producten of nanomaterialen.

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U bent nu aangekomen bij het dankwoord van het proefschrift. U heeft het proefschrift bijna uit en als u goed gelezen heeft weet u nu meer over de rol van (fris) dranken, speeksel, eiwitten en tandpasta's in het ontstaan van tanderosie. Mogelijk zijn vele vragen met betrekking tot dit onderwerp beantwoord en/of zijn er juist nieuwe vragen of verbazing bij u opgeroepen.
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Curriculum Vitae

Derk-Jan Jager was born on april 27th 1979 in Apeldoorn, the Netherlands. After finishing the gymnasium secondary school in 1997 at the Stedelijk Gymnasium Apeldoorn he studied Environmental and Infrastructure Planning and Social Geography at the University of Groningen. After obtaining his propaedeutics, he switched to Dentistry. During this study he was secretary of the board of the dental student faculty association Archigenes. He obtained his qualification as a dentist in 2005. Subsequently he started his PhD research project and combined this with his work as a dentist at Tandartspraktijk Heino. Furthermore he worked as a lecturer and dentist in prosthodontics at the UMCG Center for Dentistry and Oral Care. Since 2010 he works at the Center for Special Dental Care (in Dutch: Centrum voor Bijzondere Tandheelkunde) and the Refer Practice for Oral Rehabilitation at the Martini Hospital Groningen.