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Impact of Acquired Enamel Pellicle Modification on Initial Dental Erosion

Z. Cheaib A. Lussi

Department of Preventive, Restorative and Paediatric Dentistry, University of Bern, Bern, Switzerland

Key Words
Dental erosion · Microhardness · Pellicle

Abstract
The acquired enamel pellicle that forms on the tooth surface serves as a natural protective barrier against dental erosion. Numerous proteins composing the pellicle serve different functions within this thin layer. Our study examined the effect of incorporated mucin and casein on the erosion-inhibiting potential of the acquired enamel pellicle. Cyclic acidic conditions were applied to mimic the erosive environment present at the human enamel interface during the consumption of soft drinks. One hundred enamel specimens were prepared for microhardness tests and distributed randomly into 5 groups (n = 20) that received the following treatment: deionized water, humidity chamber, mucin, casein, or a combination of mucin and casein. Each group was exposed to 3 cycles of a 2-hour incubation in human saliva, followed by a 2-hour treatment in the testing solution and a 1-min exposure to citric acid. The microhardness analysis demonstrated that the mixture of casein and mucin significantly improved the erosion-inhibiting properties of the human pellicle layer. The addition of individual proteins did not statistically impact the function of the pellicle. These data suggest that protein-protein interactions may play an important role in the effectiveness of the pellicle to prevent erosion.

Dental erosion is defined as the loss of dental hard tissue by a chemical process without the impact of bacteria. It is a major problem in dental health and appears to be a growing problem, affecting 4–82% of adults and 6–50% of children [Jaeggi and Lussi, 2006].

Although fluoride-containing dental care products have been used to prevent erosive wear [Lussi, 2009], it has been shown that moderate or low fluoride concentrations are unable to provide a significant preventive effect against erosion [Larsen and Richards, 2002]. Nevertheless, fluoride may have a protective effect under conditions in which the erosive factors are not excessive [Lussi and Jaeggi, 2006]. Additionally, it has been suggested that the use of fluoride to protect against erosion would not be feasible from a toxicological point of view [ten Cate, 1999]. On the other hand, human saliva, which is naturally present in the mouth, has the ability to protect enamel against erosive challenges through its clearing and buffering effects and the formation of a acquired enamel pellicle on the tooth surface [Lendenmann et al., 2000; Sreebny, 2000]. The pellicle layer, in turn, also possesses buffering capacity and functions as a diffusion barrier that prevents direct contact between acids and the tooth surface. It is an organic film formed by the continuous selective adsorption of salivary proteins and glycoproteins onto the enamel surface. Moreover, constant exchange of the proteins occurs during the formation of the layer. Therefore, high molecular weight, large salivary

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Zeinab Cheaib, MSc
Department of Preventive, Restorative and Paediatric Dentistry, University of Bern
Freiburgstrasse 7
CH–3010 Bern (Switzerland)
Tel. +41 31 632 8603, E-Mail zeinab.cheaib@zmk.unibe.ch

proteins, such as mucin, replace the adsorbed smaller proteins, such as statherin, and acidic proline-rich proteins [Svendsen et al., 2008] to incorporate into the pellicle layer. This dynamic adsorption-desorption process takes up to 2 h [Hannig and Joiner, 2006].

The protein composition of the pellicle significantly affects its function, including its ion transport potential, the regulation of calcium phosphate crystallization, and bacterial adherence [Hannig and Joiner, 2006]. The clinical relevance of the human acquired enamel pellicle is exemplified by the medical conditions present in xerostomia patients with a decreased salivary pellicle thickness [Dawes, 2004].

It is well known that different host substances have the ability to incorporate into the pellicle layer, and the incorporation of these compounds affects the function of the pellicle, including its erosion-inhibiting potential [Dickinson and Mann, 2005]. For example, casein – the predominant phosphoprotein (αS1, αS2, β, κ) present in bovine milk [Fox and McSweeney, 1998] – combines with salivary compounds within the pellicle and affects pellicle formation in vivo [Vacca Smith and Bowen, 2000; Barbour et al., 2008]. Different single proteins have been reported to protect enamel against demineralization [Kielbassa et al., 2005]. However, it is not yet clear whether the interaction of a single protein with the pellicle significantly affects its anti-erosion function. It has been previously reported that 'heterotopic complexation' enhances the development of the human pellicle. These interactions are considered a precursor of the micelle-like globules that constitute a major component of the acquired enamel pellicle [Hannig, 1999]. It has been suggested that these globules are formed by the binding of proline-rich proteins, amylase, histatins, lysozyme and lipids to high molecular weight mucin. Therefore, mucin is considered a carrier that can concentrate molecules at the enamel surface. This micelle-like structure is more relevant in a mature pellicle, which demonstrates greater anti-erosion capabilities than the incomplete pellicle coating [Hannig and Joiner, 2006; Hara et al., 2006]. Therefore, it is reasonable to postulate that the synergic supply of proteins, rather than the adsorption of single proteins, increases the protective functions of the pellicle. A previous study has demonstrated that casein can adsorb onto a mucin film [Malone et al., 2003]. We hypothesized that this adsorption can lead to the generation of a strong pellicle layer when a mixture of casein and mucin is applied to the human pellicle. Therefore, the aim of the present in vitro study was to investigate modification of the human acquired enamel pellicle by casein, mucin, or a mixture of casein and mucin. The hypothesis tested was that the protein mixture would lead to a significantly more protective pellicle as compared to pellicles formed following treatment with either protein alone.

Materials and Methods

Preparation of Enamel Specimens

One hundred cavity-free human premolars were selected from a pool of extracted teeth and viewed under a stereomicroscope (Zoom 2000, Leica, USA; ×25 magnification) to check for damage to the buccal surface. Only those with sound surfaces were selected. After brushing the teeth with deionized water, the crowns were separated from the roots, and the buccal sides were cut out using a low-speed saw (Isomet, 11-1,180 Low Speed Saw, Buehler, USA). Each slab was then embedded in resin (Paladur, Bad Homburg, Germany) in 2 planar parallel molds. After removing the thinner mold (200-μm thick), the slab embedded in the thicker mold (7-mm thick) was serially polished on a Kuntch-Rotor polishing machine with carbide paper (30-, 18- and 6-μm grain size) with water cooling. The embedded enamels were taken out of the molds before final polishing with 3- and 1-μm diamond abrasivse on a Buehler polishing cloth (DP-U2 and Lubricant Blue, Struers, Copenhagen, Denmark). Between the two polishing steps and after the final polishing, all samples were sonicated for 5 min in deionized water and rinsed again for 3 min under running tap water. All of the specimens were then stored in a saturated mineral solution (1.5 mmol/l $CaCl_2$, 1.0 mmol/l KH_2PO_4, 50 mmol/l NaCl, pH 7.0) [Zero et al., 1990]. Prior to the experimental procedures, the specimens were further polished with a 1-μm diamond abrasive for 1 min in order to remove remnants which might have been occurred due to storage.

Salivary Collection

Paraffin-stimulated saliva samples from healthy donors of both sexes (aged 26–53 years) were collected into ice-chilled vials and pooled. Human subject recruitment, obtaining informed consent and saliva collection were performed in accordance with the protocol approved by the University of Bern. Immediately after collection, the whole saliva was centrifuged for 20 min at 4°C and 3,000 *g*. The supernatants were divided into 13-ml aliquots and stored at –80°C [Schipper et al., 2007].

Artificial Protein Solutions

The solutions were prepared as follows: Mucin 0.27%: 2.7 g gastric mucin from pig (Merck for analysis, Dietikon, Switzerland) was dissolved in 1,000 ml deionized water. Casein 0.5%: 5 g casein from bovine milk (Merck) was dissolved in 1,000 ml deionized water. Mucin 0.27% + casein 0.5%: 2.7 g pig gastric mucin (Merck) and 5 g casein from bovine milk (Merck) were dissolved in 1,000 ml deionized water. All solutions were freshly prepared prior to the experiment, and the pH was adjusted to 7.

Sample Preparation

The specimens were randomly selected and assigned to 5 experimental groups (n = 20) according to the treatment they re-

ceived: deionized water, humidity chamber, 0.27% mucin, 0.5% casein, or a mixture of 0.27% mucin and 0.5% casein. The specimens were immersed in the prepared saliva for 2 h at 30°C with constant agitation. Afterwards, they were rinsed in deionized water and exposed to the artificial protein solution for another 2 h at 30°C with constant agitation [Plasmans et al., 1994; Yin et al., 2006]. The specimens were rinsed again in deionized water and incubated in a 0.65% citric acid solution (pH = 3.5) for 1 min at 30°C with constant agitation. The specimens were then carefully rinsed with deionized water and dried for 5 s with oil-free air. Each sample was treated once a day for 3 days. The specimens were kept in a humidity chamber at 4°C between treatments.

Surface Microhardness Measurement

Hardness measurements were performed using a Knoop diamond under a load of 50 g and a dwell time of 15 s (MNT-10, Anton Paar, Paar Physica, Graz, Austria). Six indentations were made, with each long axis parallel to the vertical borders of the window, at 50-μm intervals. The length of each indentation was measured with an optical analysis system and transferred to a computer (Leica DMR Microscope, Leica Mikroskopie and Systeme GmbH, Wetzlar, Germany). The apparatus was calibrated before each use. Control indentations of 2 and 5 g were made to detect possible surface loss. The indentation lengths were used for the calculation of the surface microhardness (SMH) values, which were a measure of enamel softening.

Statistical Analysis

The average SMH was calculated for further analysis. The changes in SMH ($SMH_{baseline} - SMH_{after\ erosion}$) between the groups were analyzed using a non-parametric ANOVA followed by pair-wise Wilcoxon Mann-Whitney tests and a Bonferroni-Holm adjustment. All statistical analyses were carried out using SAS 9.1.3 (SAS institute Inc., Cary, N.C., USA). The significance level was set at 5%.

Results

The microhardness loss of the enamel increased after each treatment cycle in all 5 treatment groups ($p < 0.0001$).

Statistically significant effects of the control and experimental groups, as well as the erosion cycle, on the microhardness loss were observed ($p < 0.0001$). The effect of the treatment group on each erosion cycle, calculated using the Kruskal Wallis test, was also significant ($p < 0.0001$).

A pair-wise post hoc test with a Bonferroni-Holm adjustment demonstrated that the microhardness loss was significantly lower after the first-cycle erosion in the protein mixture (mucin-casein) group compared to the mucin, casein, and humidity chamber groups. However, no statistically significant difference was observed when compared with the deionized water group (fig. 1a). After the second and the third erosion cycles, the pellicle in the protein mixture (mucin-casein) group was statistically more protective than the pellicle in the single protein and control groups, which did not differ from each other (fig. 1b, c).

Discussion

The present study aimed to examine whether single or mixed protein treatments can enhance the acquired enamel pellicle resistance to erosion. A prerequisite for the application is the usage of non-toxic and inexpensive protein agent. Therefore, casein from bovine milk and pig gastric mucin were used for this study. Preliminary experiments showed that enamel softening was not affected by mucin or casein concentrations in the range of 0.055–0.27% for mucin and 0.01–1% for casein (online suppl. fig. A, www.karger.com/doi/10.1159/000324803). These data are supported by the findings of Barbour et al. [2008], who demonstrated that the reduction in hydroxyapatite dissolution was not significantly affected by the casein concentration. A concentration of 0.27% mucin and 0.5% casein was chosen for this investigation [Meyer-Lueckel et al., 2004], as this protein content provides a buffering potential similar to human salivary proteins (online suppl. fig. B). The method used in this study included the formation of a saturated pellicle layer, the incubation of the pellicle with protein solutions, and the subsequent exposure to an erosive agent (0.65% citric acid; pH = 3.6). Abrasive effects, such as toothbrushing or tongue movement, were not included in the experimental design because their influence on the pellicle layer is well described [Hannig et al., 2004]. It should therefore be noted that the results of the present study may not be representative of the situation in vivo.

In this study, the erosive challenge caused enamel softening and no material loss was observed. The measurement of the degree of softening of the enamel surface was assessed by microhardness measurements rather than profilometry [Attin, 2006]. The latter measures material loss, and is most suitable for erosion at more advanced stages [Barbour and Rees, 2004]. Our results clearly demonstrate that the protective effect of the pellicle was modified with the casein (0.5%)/mucin (0.27%) mixture compared to the individual proteins or the control groups (fig. 1). This effect was statistically significant after the second erosion cycle. In contrast, no statistically significant microhardness loss was found between the control and single protein groups.

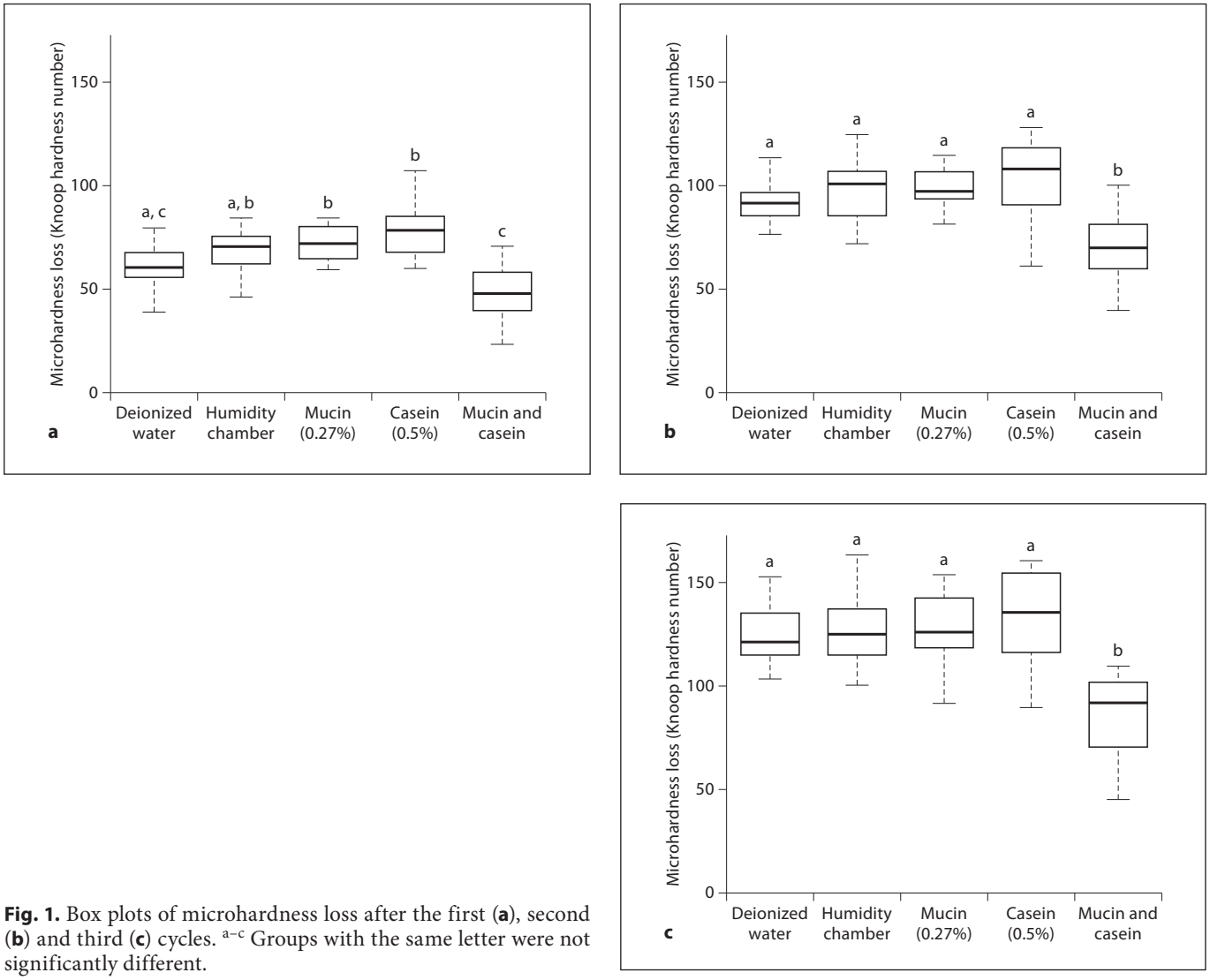

Fig. 1. Box plots of microhardness loss after the first (**a**), second (**b**) and third (**c**) cycles. [a–c] Groups with the same letter were not significantly different.

Thickness, density and continuity of the pellicle layer are three factors that affect the protection of the underlying enamel [Hannig, 1999]. In this study, the thickness of the adsorbed casein molecules was most likely small and could not significantly change the protective properties of the pellicle. The isoelectric point of casein is 4.6; it thus carries a net negative charge at the neutral pH used in this study and may adsorb onto enamel through calcium bridging. Furthermore, it has been reported that casein can adsorb on the tooth surface in exchange for albumin in the enamel pellicle [Schupbach et al., 1996]. According to Barbour et al. [2008], the combination of a human pellicle and 0.02% casein resulted in a significant reduction in hydroxyapatite (HAP) dissolution when immersed into 0.3% citric acid. These results are in contrast to our study, which was carried out using human enamel. Additionally, the casein used in the above-mentioned investigation could affect the HAP by coating its surface or changing the buffering capacity of the erosive solution, which was avoided in the current experiment. Different analytical techniques (pH stat vs. microhardness analysis) could have also contributed to the discrepancies between the results.

The gastric pig mucin used in this study is most probably MUC5AC, rather than MUC5B, the high molecular weight mucin present in the enamel-acquired pellicle. There is a high similarity between cysteine-rich subdomains of MUC5B and MUC5AC. The subdomains of the C-terminal region of the MUC5B also have high sequence similarity to those present in MUC5AC [Zalewska et al.,

2000]. The mucin from gastric pig (2×10^6 Da) is larger than the high molecular weight salivary mucin, with a molecular mass of 10^6 Da and they are both highly glycosylated [Poncz et al., 1988; Thomsson et al., 2002]. However, there may be a limited similarity between the behavior of these proteins.

The lower molecular weight salivary mucin (MUC7) was not considered as a structural component of enamel pellicle, unlike the higher molecular weight (MUC5B) which was detected in 2-hour in vivo pellicle [Al-Hashimi and Levine, 1989]. The role of salivary mucins in protection of the tooth surface from acid challenge was reported. Pellicle formed after 3 days of incubation in mucins gave 100% protection against demineralization by 1% citric acid. Additionally, when submandibular/sublingual saliva was depleted of mucins, lesion formation was reduced only by 30% compared to pellicle-free surfaces [Amerongen et al., 1987].

In this study, no effect of pig mucin on the anti-erosion function of the pellicle was detected, and this can be explained by the weak interactions between mucin and the proteins of the pellicle. In contrast, combined treatment with casein and mucin resulted in a significant reduction in enamel softening after the second treatment cycle. This is most likely the result of protein-protein interactions that promote additional binding sites that allow for more interactions with the pellicle. Previous studies have shown that the affinity of proteins for HAP could be enhanced through interactions with other proteins [Yin et al., 2006]. It has been demonstrated that heterotopic complexation among salivary proteins drives the selective adsorption of proteins to the enamel surface [Tabak, 1990]. For example, high molecular weight mucin (MG1) coordinates with secretory IgA, and this complex contributes to the formation of the globular structure of the pellicle. Therefore, it is possible that a self-assembled micellar structure can be formed by casein and mucin, resulting in increased pellicle thickness and/or density and cross-linkage. There is a need for the systematic investigation of the pellicle structure and its binding pathways, which would contribute significantly to a better understanding of the natural protective mechanisms of the pellicle and stimulate new developmental strategies for clinical application.

Initial bacterial attachment may also be affected by pellicle thickness and composition [Hannig, 1999]. More investigations should also be carried out to elucidate the effect of pellicle modifications on plaque formation.

In conclusion, the present results highlight the complex effects of protein solutions on the acquired enamel pellicle, indicating that protein cross-linking does play an important role in the effectiveness of protein anti-erosion agents. While the addition of mucin or casein alone had no effect on the erosion-inhibiting properties of the pellicle, the combination of mucin and casein significantly increased the efficacy of the pellicle against acid-induced enamel softening.

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Disclosure Statement

There is no conflict of interest.

References

Al-Hashimi I, Levine MJ: Characterization of in vivo salivary-derived enamel pellicle. Arch Oral Biol 1989;34:289–295.

Amerongen AVN, Oderkerk CH, Driessen AA: Role of mucins from human whole saliva in the protection of tooth enamel against demineralization in vitro. Caries Res 1987;21:297–309.

Attin T: Methods for assessment of dental erosion; in Lussi A (ed): Dental Erosion from Diagnosis to Therapy. Basel, Karger, 2006, pp 152–172.

Barbour ME, Rees JS: The laboratory assessment of enamel erosion: a review. J Dent 2004;32:591–602.

Barbour ME, Shellis RP, Parker DM, Allen GC, Addy M: Inhibition of hydroxyapatite dissolution by whole casein: the effects of pH, protein concentration, calcium, and ionic strength. Eur J Oral Sci 2008;116:473–478.

Dawes C: How much saliva is enough for avoidance of xerostomia? Caries Res 2004;38:236–240.

Dickinson ME, Mann AB: Nanoscale characterisation of salivary pellicle. Mater Res Soc Symp Proc 2005;844:113–118.

Fox PF, McSweeney PLH: Milk proteins; in Fox PF, McSweeney PLH (eds): In dairy Chemistry and Biochemistry. London, Chapman & Hall, 1998, pp 146–239.

Hannig M: Ultrastructural investigation of pellicle morphogenesis at two different intraoral sites during a 24-h period. Clin Oral Invest 1999;3:88–95.

Hannig M, Fiebiger M, Güntzer M, Döbert A, Zimehl R, Nekrashevych Y: Protective effect of the in situ formed short-term salivary pellicle. Arch Oral Biol 2004;49:903–910.
Hannig M, Joiner A: Function and properties of the acquired pellicle; in Duckworth RM (ed): The Teeth and Their Environment. Basel, Karger, 2006, pp 29–64.
Hara AT, Ando M, Gonzalez-Cabezas C, Cury JA, Serra MC, Zero DT: Protective effect of the dental pellicle against erosive challenges in situ. J Dent Res 2006;85:612–616.
Jaeggi T, Lussi A: Prevalence, incidence and distribution of erosion; in Lussi A (ed): Dental Erosion from Diagnosis to Therapy. Basel, Karger, 2006;20:44–65.
Kielbassa AM, Oeschger U, Schulte-Monting J, Meyer-Lueckel H: Microradiographic study on the effects of salivary proteins on in vitro demineralization of bovine enamel. J Oral Rehabil 2005;32:90–96.
Larsen MJ, Richards A: Fluoride is unable to reduce dental erosion from soft drinks. Caries Res 2002;36:75–80.
Lendenmann U, Grogan J, Oppenheim FG: Saliva and dental pellicle – a review. Adv Dent Res 2000;14:22–28.
Lussi A: Dental erosion-novel remineralizing agents in prevention or repair. Adv Dent Res 2009;21:13–16.
Lussi A, Jaeggi T: Chemical factors; in Lussi A (ed): Dental Erosion from Diagnosis to Therapy. Basel, Karger, 2006, pp 77–87.
Malone ME, Appelqvist IAM, Norton IT: Oral behaviour of food hydrocolloids and emulsions. 1. Lubrication and deposition considerations. Food Hydrocolloids 2003;17:763–773.
Meyer-Lueckel H, Umland N, Hopfenmuller W, Kielbassa AM: Effect of mucin alone and in combination with various dentifrices on in vitro remineralization. Caries Res 2004;38:478–483.
Plasmans PJ, Creugers NH, Hermsen RJ, Vrijhoef MM: Intraoral humidity during operative procedures. J Dent 1994;22:89–91.
Poncz L, Jentoft N, Ho MC, Dearborn DG: Kinetics of proteolysis of hog gastric mucin by human neutrophil elastase and by pseudomonas aeruginosa elastase. Infect Immun 1988;56:703–704.
Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W: SELDI-TOF-MS of saliva: methodology and pre-treatment effects. J Chromatogr B 2007;847:45–53.
Schupbach P, Neeser JR, Golliard M, Rouvet M, Guggenheim B: Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. J Dent Res 1996;75:1779–1788.
Sreebny LM: Saliva in health and disease: an appraisal and update. Int Dent J 2000;50:140–161.
Svendsen I, Lindh L, Elofsson U, Arnebrant T: Studies on the exchange of early pellicle proteins by mucin and whole saliva. J Colloid Interface Sci 2008;321:52–59.
Tabak LA: Structure and function of human salivary mucins. Crit Rev Oral Biol Med 1990;1:229–234.
ten Cate JM: Current concepts on the theories of the mechanism of action of fluoride. Acta Odontol Scand 1999;57:325–329.
Thomsson KA, Prakobphol A, Leffler H, Reddy MS, Levine MJ, Fisher SJ, Hansson GC: The salivary mucin MG1 (MUC5B) carries a repertoire of unique oligosaccharides that is large and diverse. Glycobiology 2002;12:1–14.
Vacca Smith AM, Bowen WH: The effects of milk and kappa-casein on salivary pellicle formed on hydroxyapatite discs in situ. Caries Res 2000;34:88–93.
Yin A, Margolis HC, Yao Y, Grogan J, Oppenheim FG: Multi-component adsorption model for pellicle formation: the influence of salivary proteins and non-salivary phospho proteins on the binding of histatin 5 onto hydroxyapatite. Arch Oral Biol 2006;51:102–110.
Zalewska A, Zwierz K, Zółkowski K, Gindzieński A: Structure and biosynthesis of human salivary mucins. Acta Biochim Pol 2000;47:1067–1079.
Zero DT, Rahbek I, Fu J, Proskin HM, Featherstone JD: Comparison of the iodide permeability test, the surface microhardness test, and mineral dissolution of bovine enamel following acid challenge. Caries Res 1990;24:181–188.