Assessment of Enamel Erosion and Protective Effect of Salivary Pellicle by Surface Roughness Analysis and Scanning Electron Microscopy

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Purpose: To assess dental erosion caused by 0.1% and 1.0% citric acid *in vitro* and to estimate the protective influence of experimentally formed salivary pellicle.

Materials and Methods: Bovine enamel slabs (n = 80) were polished and embedded in epoxy resin. For the formation of pellicle layer 40 specimens were immersed for 24 h in pooled human saliva. Erosion was caused by immersion in citric acid solution for 1, 5, 10 and 30 min. Erosive alterations on the pellicle-covered and non-covered enamel specimens were scored as a change (Δ) of surface roughness parameters Ra, Rt and RzDIN using contact profilometer and observed in scanning electron microscope.

Results: Profilometric analysis of eroded enamel specimens emphasized the aggressiveness of even low concentrated citric acid with a short period of challenge. The change of roughness parameters after 1-min immersion in 0.1% citric acid were 16.4, 182.6 and 132.2 nm for ΔRa, ΔRt and ΔRzDIN, respectively, and 54.8, 516.6 and 258.2 nm after 1-min immersion in 1.0% citric acid. Changes of the surface roughness were dependent on the exposure time and concentration of acidic solution. Pellicle layer significantly reduced the extent of erosive destruction, which was additionally documented on SEM-micrographs. Residual pellicle-like structures were detected after 5 min of immersion in 0.1% citric acid. However, there were no significant differences in pellicle-covered and non-covered enamel slabs measured profilometrically for 1.0% citric acid with 10 min and 30 min exposure time.

Conclusion: The findings confirm the property of pellicle layer to resist against erosive influence of organic acids, which is, however, limited by duration of acidic treatment and concentration of erosive agent.

Key words: enamel erosion, salivary pellicle, surface roughness, scanning electron microscopy

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Since pathological tooth wear has been recognized as an increasing oral health problem (Imfeld, 1996), continuous attempts have been made to assess erosive alterations on the enamel surface using microhardness tests, microradiographic measurements, calcium release analysis or micromorphological observations (Grando et al, 1996; Lussi et al, 1997; Amaechi et al, 1999). The etiology of dental erosion is usually attributed to excessive consumption of dietary acidic products and beverages or to gastrointestinal disorders and dysfunctions (Scheutzel, 1996). Long-lasting cumulative influence of these factors leads to the irreversible loss of dental hard tissue. Numerous studies underline an erosive potential of acidic foodstuffs, juices and so-called sport drinks (Grobler et al, 1989; Larsen and Nyvad, 1999).

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On the other hand, it is known that human saliva offers buffering and neutralization of acids as well as dilution and clearance of erosive agents (Lendenmann et al, 2000). Salivary proteins, especially glycoproteins and prolin-rich proteins cover the enamel surface and form an organic film called 'acquired salivary pellicle'. This protein layer, formed within a short period of time on the enamel surface, is supposed to protect the underlying structures against destructive influences of organic acids (Hannig, 2002). Recently published results demonstrate the protective nature of salivary pellicle in case of the erosive attack caused by such aggressive organic agents as citric or lactic acid (Hannig, 1998; Hannig and Balz, 2001; Nekrashevych and Stösser, 2003).

A diversity of modern quantitative and qualitative techniques offers the opportunity for an accurate assessment of erosion, but at the same time produces some difficulties when choosing the adequate and most sensitive method or combination of methods (Grenby, 1996). The present study focuses on the quantitative methodology of erosive destruction because, as yet, only a very few authors have described the changes of roughness on the enamel surface (Nieuw Amerongen et al, 1987; Rytömaa et al, 1998). Moreover, there is no profilometric analysis made on the enamel surface in respect of the pellicle layer. Therefore, the purpose of the present study was to perform roughness examinations and to combine this technique with scanning electron microscopy in order to analyze micromorphological alterations on enamel surface after erosive attack of citric acid and to evaluate the protective effect of the experimentally formed salivary pellicle on the erosive process.

MATERIALS AND METHODS

Preparation of Enamel Samples

Freshly extracted bovine permanent incisors were used for the preparation of enamel specimens. The teeth were rinsed with tap water and thoroughly cleaned using a dental brush to remove remnants of periodontal tissues and plaque. Only non-damaged teeth without pigmentation were used in the current study. The dentine-enamel blocks (approx. 4 x 4 x 2 mm) were separated from the labial surfaces using an ultra-thin dental diamond disk with water cooling. Subsequently, enamel slabs were fixed in mounting cups, embedded in self-curing epoxy resin (Epofix Resin, Struers, Denmark) and wet polished with silicon carbide grinding paper (grit size 1200 and 4000) using the 'Metasinex' polishing device (Rathenow Werke, Germany). The superficial enamel layer of 100 – 120 µm determined with a micrometer was removed by the polishing procedures. After polishing the enamel samples were ultrasonically cleansed for 10 min in distilled water and separately preserved in 0.1% thymol solution pending the following investigations.

Pellicle Formation

Saliva was collected between 8.00 and 8.30 into ice-chilled vials from 6 human volunteers who were caries free, had no salivary gland dysfunction, and were not on any drug therapy. Secretion of saliva was stimulated by chewing a block of paraffin. Whole mixed saliva was centrifuged for 10 min at 4°C and 2000 g (Biofuge 22 R, Heraeus Sepatech GmbH, Germany) and filtered (0.2 µm RC25, Sartorius, Germany). Pooled supernatant was used for pellicle formation. Pellicle formation was performed on 40 enamel samples which were separately immersed in clarified saliva (3 ml saliva per each specimen) for 24 hours at 37°C. The second group of 40 specimens without pellicle layer was used as a control.

Erosion of Enamel

Enamel specimens with and without pellicle layer were immersed in 5 ml 0.1% or 1.0% citric acid over the periods of 1, 5, 10 or 30 min. Each of the 16 subgroups comprised 5 enamel samples. After acid treatment enamel specimens were rinsed with distilled water, ultrasonically cleansed, air dried and mounted on the microscopic slides with silicone based impression material (putty) for the following surface roughness analysis.

Profilometric Analysis

Measurements of the surface roughness of non-pellicle-covered control specimens and pellicle-covered specimens were performed before (as a baseline) and after erosive treatment using a computerized contact profilometer Hommel Tester

T 1000 (Hommelwerke GmbH, Germany). Three surface roughness parameters were used for the characterization of the recorded profiles: arithmetic roughness Ra, maximum roughness depth Rt and average roughness RzDIN. Five tracings were made on each specimen using a sampling length of 1.5 mm. The evaluation of registered surface roughness parameters was performed with Turbo Datawin Software for Windows (Hommelwerke GmbH, Germany).

Micromorphological Observations

Randomly selected enamel specimens (n = 48; 3 samples per subgroup) were thoroughly washed with distilled water, cleansed in an ultrasonic bath, air dried, mounted on the metal stubs, gold sputtered and examined in a scanning electron microscope XL 30 ESEM FEG (FEI, The Netherlands) operating at 10 kV. Four differently localized areas were documented on each enamel sample using up to 20,000-fold magnification.

Statistical Analysis

Surface roughness parameters were registered fivefold per enamel specimen before and after acidic treatment and the average of the difference represented the change of surface roughness. Mean values per subgroup were calculated and statistical testing of between group differences with respect to pellicle layer and duration of acid challenge was performed with two-way ANOVA using SPSS (Version 10.0 for Windows). The mean values for each of the tested exposure intervals were additionally analyzed by unpaired Student t-test if the result with respect to the effect of pellicle was statistically significant. This was done for the two acid concentrations separately. If p value was less than 0.05, differences were considered as statistically significant.

RESULTS

Profilometric Measurements

Representative surface profiles of the control specimens and pellicle-covered enamel specimens after erosive treatment are exemplarily summarized in Fig 1. Significant alterations on the enamel surface were registered even after short exposure to 0.1% citric acid when compared with non-acid-treated polished enamel surface; the mean values of change of roughness parameters were 16.4, 182.6 and 132.2 for ΔRa, ΔRt and ΔRzDIN, respectively (Table 1). The 1 min immersion in 1.0% citric acid increased the surface roughness, which was registered as 54.8 nm for ΔRa, 516.6 nm for ΔRt and 258.2 nm for ΔRzDIN. The extent of erosive alterations increased after the prolongation of immer-

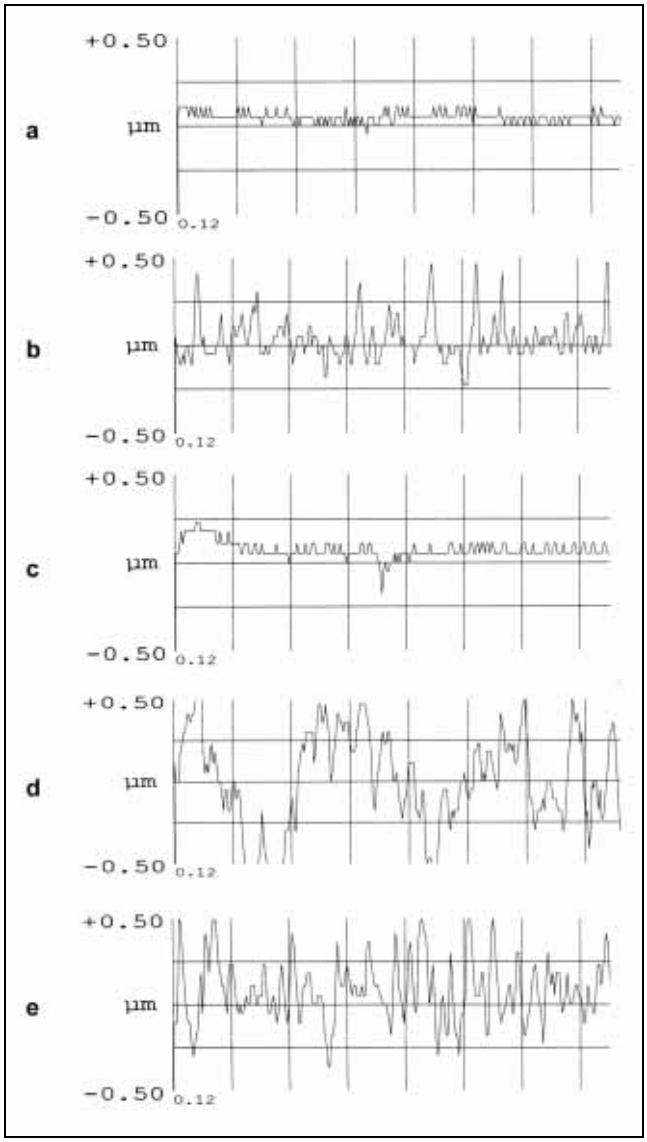

Fig 1 Profiles of pellicle-non-covered (b, d) and pellicle-covered (c, e) enamel surface eroded with 0.1% (b, c) and 1.0% (d, e) citric acid for 1-min immersion time in comparison with uneroded polished surface (a).

Table 1 Changes of surface roughness (Δ nm) of the pellicle-covered and non-covered enamel specimens after erosion in 0.1% and 1.0% citric acid. Mean ± SD.

Acid concentration:		0.1%			1.0%		
Roughness parameter:		ΔR_a	ΔR_t	ΔR_zDIN	ΔR_a	ΔR_t	ΔR_zDIN
Exposure time:							
1 min	without pellicle	16.4 ± 6.7	182.6 ± 64.3	132.2 ± 46.1	54.8 ± 5.8	516.6 ± 138.4	258.2 ± 84.5
	with pellicle	2.4 ± 0.6	42.2 ± 15.7	22.6 ± 8.2	14.4 ± 6.2	172.4 ± 28.3	82.6 ± 22.1
5 min	without pellicle	32.6 ± 11.3	202.46 ± 41.5	176.4 ± 32.7	122.4 ± 26.7	842.8 ± 204.3	394.6 ± 62.4
	with pellicle	18.2 ± 2.3	64.0 ± 21.4	37.6 ± 19.1	82.6 ± 12.4	534.6 ± 92.7	112.8 ± 38.2
10 min	without pellicle	102.8 ± 26.9	412.6 ± 66.3	370.2 ± 71.2	338.8 ± 54.3*	1412.6 ± 316.2*	648.2 ± 124.5*
	with pellicle	46.4 ± 17.7	361.8 ± 91.2	289.6 ± 68.2	322.6 ± 12.4*	1444.8 ± 262.7*	616.8 ± 118.7*
30 min	without pellicle	288.8 ± 64.5	912.0 ± 223.4	407.2 ± 56.7	622.8 ± 162.7*	1862.6 ± 442.1*	706.2 ± 182.3*
	with pellicle	166.4 ± 44.5	788.6 ± 186.3	312.4 ± 64.1	646.4 ± 202.3*	1944.2 ± 318.4*	742.4 ± 203.4*

* – no statistical significance

sion time for both acid concentrations (Table 1). In particular, the 1.0% citric acid led to the considerable change in surface roughness after 10 and 30 min of immersion; mean Δ values of roughness parameters Ra, Rt and RzDIN underlined destructive process on the enamel surface.

Experimentally formed pellicle layer protected the enamel surface against erosion caused by low-concentrated citric acid and at the initial stages of erosion challenged by more concentrated solution. In fact, there was a significant protection of the enamel surface for all immersion time groups treated with 0.1% acid (Table 1). Pellicle-induced protection was registered in all specimens shortly immersed in 1.0% citric acid. However, pellicle layer did not reduce the change of surface roughness after 10 min or 30 min of acidic treatment. There were no significant differences in changes of surface roughness due to acid treatment between pellicle-covered and non-covered samples in these groups (Table 1).

Surface Morphology

Superficial demineralization of non-pellicle-coated enamel surfaces was detected after 1 min immersion in 0.1% citric acid when compared to uneroded polished surfaces (Fig 2a, b). Higher concentrated acid solution caused definitely stronger enamel erosion (Fig 2d). The 5 min exposure caused moderate erosive destruction of the non-pellicle-covered specimens that progressed after 10 min and 30 min of immersion to severe erosion characterized by dissolution of the enamel prism sheaths and cementing interrod substance (Fig 2e, f).

In terms of morphology, there were no erosive alterations on the pellicle-covered enamel samples after 1 min exposure to 0.1% citric acid. Usually visible traces, remaining after polishing procedures, were covered by pellicle layer. Pellicle reduced erosive alterations in all groups eroded with 0.1% citric acid. The pellicle-induced protection of the enamel surface eroded by 1.0% citric acid was recorded in the specimens treated for 1 min or 5 min (Fig 2c). However, pellicle-covered enamel samples investigated after 30 min of immersion in 1.0% acid were characterized by surface pattern, usually typical for the manifest form of dental erosion. Strongly eroded prism crystallites and affected interprismatic spaces were detected on the most SEM-micrographs.

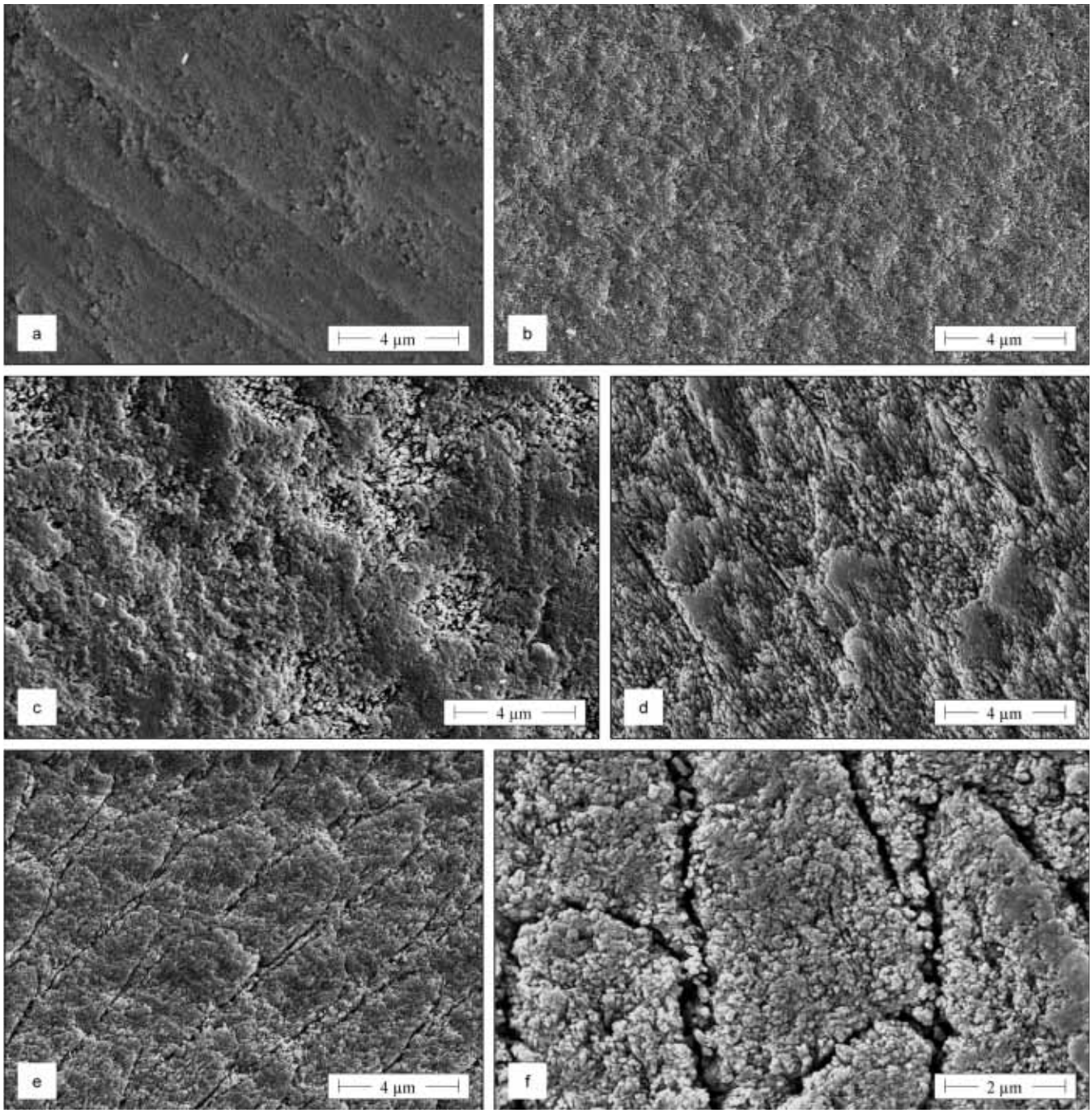

Fig 2 SEM-micrographs (magn. 5,000 fold with exception of 2f – magn. 12,000 fold) of erosive alterations on the enamel surface with (c) and without (b, d, e, f) pellicle caused by 0.1% (b) and 1.0% (c, d, e, f) citric acid after 1 min (b), 5 min (c, d), and 30 min (e, f) min of immersion in comparison with non-eroded polished enamel surface (a).

DISCUSSION

The application of surface roughness analysis, which was one of the primary objectives of this study, contributed to the already described assessment methods of dental erosion (Grenby, 1996). Modern profilometric technique used in our investigations provides an accurate measurement with a sensitive registration of surface roughness. This study has also definitely shown that the extent of

erosive alterations on the enamel surface could be reduced by salivary pellicle.

Surface topography of sound enamel varies individually due to its micromorphological structure (Arends et al, 1980). This feature can usually have an influence on the conducted measurements. Therefore, the enamel specimens were polished with an ultra-fine wet grinding paper in order to standardize the enamel surfaces for baseline tests. Furthermore, the diamond stylus of the profilometer was always traversed from the cervical margin towards the incisal margin of the sample. Surface roughness was characterized by arithmetic roughness Ra, which is the arithmetic average height of roughness irregularities registered from the mean line within the sampling length. This most commonly used parameter was supported by maximum roughness depth Rt and average roughness RzDIN. The first parameter reflects the deepest points of the registered profile; the second one represents the average distance between the five highest peaks and five deepest valleys within the sampling length. According to Mummery (1993), the average roughness RzDIN is more sensitive to occasional peaks and contributes to the exactness of usually reported parameter Ra. Because of differences in applied profilometric techniques and in the nature of the tested samples, the findings of our study could not be compared with previously published results.

Moreover, there are no published profilometric investigations in dental research applied according to the international guidelines to surface roughness analysis and concerning the influence of pellicle layer on the erosive processes. Al-Omari et al (2001) investigated the surface roughness and wettability of dentine after the preparation procedures with dental burs, while Davis and Winter (1980) or Ganss et al (2000) exclusively measured the erosion depth. Rytömaa et al (1988), who observed the changes on enamel surface caused by commonly used acidic drinks and milk products, have scored the maximum depth of erosive lesions by Dektac profilometer. Finally, the attempts of West et al (1998) and of Hughes et al (1999) to record dental erosion profilometrically were not based on the systematic profile analysis.

Surface roughness values registered after erosive attack have clearly demonstrated the high destructive potential of citric acid. The increase of surface roughness was dependent on acid concentration and immersion time (Table 1). In contrast to the minor variations in the baseline roughness values, some values registered after erosive treatment were characterized by relatively high standard deviations. It could be supposed that enamel zones with different level of mineralization were responsible for this phenomenon due to the irregular substance loss from the affected enamel surface. Subsequently, stronger mineralized zones were less affected after acidic attack, which was supported by micromorphological observation (Fig 2). The SEM-documentary of shortly immersed specimens showed initially eroded enamel surface with regularly localized non-eroded areas.

Erosion progressed readily in non-pellicle-covered specimens. Most of micrographs showed dissolution in prism sheet areas already after 10 min immersion in 1.0% citric acid. This finding is in accordance with previously obtained micromorphological observations (Meurman and Frank, 1991).

Furthermore, the results of this study support the postulate of protective function of acquired salivary pellicle. Profilometric findings contribute to the number of recently published data on the protective nature of pellicle that were obtained by application of such investigative techniques as microhardness measurements, microradiography or analytical methods (Amaechi et al, 1999; Hannig and Balz, 2001). However, it must be recognized that *in vitro* formed pellicle layer, which was used in the present study, varies from the *in vivo* pellicle formation and from the conditions existing in the oral cavity. The formation conditions as well as the duration of pellicle maturation could have an influence on the efficacy of pellicle-induced protection (Zahradnik et al, 1976). On the other hand, recently published findings support the suggestion that pellicle layer formed *in vivo* within 1 h (Amaechi et al, 1999) or already within a few minutes (Hannig, 2002) can protect the enamel surface. In our study 24 h *in vitro* pellicle effectively suppressed acidic challenge of both 0.1% and 1.0% citric acid concentrations. However, erosive alterations caused by 1.0% after 10 or 30 min of immersion were not significantly reduced by pellicle layer (Fig 2). It is also appropriate to consider the influence of centrifugation and filtration on the protective properties of saliva. Nieuw Amerongen et al (1987) showed that the ultracentrifugation procedures removed mucins from human whole saliva, which subsequently reduced the protective potential of *in vitro* pellicle by up to 70%. Usage of the micropore filter in our study could be also a reason that leads to the isolation of salivary

mucins and partial reduction of pellicle-induced protection. As a result, maximum protection against 1.0% citric acid at the final stages of immersion was not achieved. Nevertheless, the most presented findings have definitely reflected a considerable protective potential of salivary pellicle.

In conclusion, profilometric technique presented in this study appears to be a sensitive way to quantify destructive alterations caused by erosion and could be systematically applied in the further studies on pathological loss of dental hard tissues. The results of this study strongly suggest that the extent of erosive destruction could be definitely retarded by pellicle.

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