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Protective effect of the in situ pellicle on dentin erosion–an ex vivo pilot study

Christian Hannig [a,b,*], *Klaus Becker* [b,c], *Nico Häusler* [b], *Wiebke Hoth-Hannig* [d], *Thomas Attin* [b,c], *Matthias Hannig* [d]

[a] *Department of Operative Dentistry and Periodontology, University of Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany*
[b] *Department of Operative Dentistry, Preventive Dentistry and Periodontology, University of Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany*
[c] *Clinic for Preventive Dentistry, Periodontology and Cariology, University of Zürich, Plattenstr. 11, CH-8032 Zürich, Switzerland*
[d] *Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital, Saarland University, Building 73, D-66421 Homburg/Saar, Germany*

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ABSTRACT

Aim: The acquired pellicle is well known as an anti-erosive proteinaceous layer on enamel, but its protective properties on dentin have not been investigated in detail until now. The aim of the present ex vivo study was to evaluate the erosive effects on pellicle coated dentin.
Methods: Bovine dentin slabs were exposed to the oral cavity of one subject for 120 min for in situ pellicle formation. Subsequently, the slabs were incubated with HCl (pH 2.3) in vitro for 5 min and erosive calcium-release was measured photometrically. In addition, the acid treated specimens were evaluated by transmission electron microscopy (TEM). Pellicle free samples served as controls.
Results: Calcium erosion from the pellicle coated dentin slabs amounted to 23.5 ± 2.9 μg Ca/min (pellicle free samples: 32.2 ± 4.2 μg Ca/min). The difference was statistically significant ($p \leq 0.05$). In pellicle coated as well as in uncoated dentin samples, TEM-evaluation showed a demineralised dentinal surface layer which thickness ranged between 3 and 6 μm. The pellicle itself was partially dissolved but not removed by hydrochloric acid treatment.
Conclusion: The protective properties of the acquired pellicle against an erosive challenge of the dentinal surface are limited. The dentinal pellicle functions like an ion permeable network rather than a barrier.

1. Introduction

Loss of enamel and dentin due to chemical processes without involvement of microorganisms has been defined as dental erosion in the literature.[1,2] Acidic substances represent the main etiological factor.[2] Erosions may be classified as intrinsic or extrinsic according to the origin of the acids.[3] The main intrinsic source is gastric juice which is of considerable pathological impact in patients suffering from anorexia nervosa, bulimia, or chronic gastro-esophageal reflux.[4,5] In modern western societies main extrinsic cause for dental erosions is diet.[3] There is an increasing consumption of acidic beverages, such as sport drinks, juices or lemonades.[6] Also fruits and vegetables, which are regarded as healthy food, may cause significant erosive effects.[2,3]

The extent of erosive effects depends mainly on the pH of the acidic solution, but also on the type of acid.[7] However, the amount of titratable acid in an acidic solution is only of

* *Corresponding author*. Tel.: +49 761 270 4888; fax: +49 761 270 4762.
E-mail address: christian.hannig@uniklinik-freiburg.de (C. Hannig).
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relevance, if the acid is consumed or if the amount of acid is limited, respectively.[7]

Several physiological mechanisms are considered to expose anti-erosive properties, such as salivary buffer systems or the acquired pellicle.[2,8,9,10,11] The acquired pellicle is a thin proteinaceous layer of high uniformity covering all solid substrata exposed to the oral fluids within very few minutes.[12–14] Beside proteins, amongst them several enzymes, it contains mucins and glycoproteins.[12,13] The pellicle is assumed to have significant protective properties as shown for enamel surfaces in a couple of studies based on a broad spectrum of methods such as electron microscopy, atomic absorption spectroscopy based determination of calcium or evaluation of micro hardness.[8,9,11,15,16] Salivary enamel pellicles significantly reduce erosion induced decrease of surface micro hardness and the increase of surface roughness.[8,9,15]

Even short term enamel pellicles formed in situ within 3 min offer protection against erosive demineralisation to the same extent as 120-min pellicles irrespective of localization in the oral cavity as shown via calcium release and micro hardness changes.[17] In addition, calcium releases from pellicle coated enamel samples after in situ formation times of 2–24 h did not differ considerably as measured by atomic absorption spectroscopy.[18] However, TEM investigations yielded that the acquired enamel pellicle is dissolved during acid exposure despite the fact that a residual pellicle layer could be detected on the enamel surface.[8,9,18] Interestingly, there were no significant differences in calcium release between pellicle covered and uncoated enamel in an in vitro approach.[15] In another in vitro-study,[20] the protective effect of the pellicle depended on the pellicle formation time in contrast to the results mentioned above based on in situ-pellicles.[17,18]

In spite of its clinical significance, pellicle formation on dentin surfaces has attracted little attention in the past in contrast to enamel.[11,19–21] This is also true for the analysis and quantification of erosive effects on dentinal structures.[20] However, this topic is of great clinical relevance due to the susceptibility of free cervical margins or uncovered dentin in general to erosive attacks because dentin is more susceptible to erosive demineralisation than enamel.[20,22] Nevertheless, only very sparse data point to a certain protective effect of the dentinal pellicle against erosive challenge.[19,23]

Electron-microscopy allows visualization of erosive effects in different views, however quantification of the mineral loss is not possible.[8,9,12] Highly sensitive photometric approaches allow the quantification of nano-erosive effects via determination of calcium or phosphate release as shown in previous studies on erosive impact of different acids on enamel specimens.[7,24,25]

The aim of the present pilot study was to quantify erosive effects on pellicle coated dentin by determination of calcium release in a photometric assay. In addition, pellicle's ultrastructure and behaviour during acid treatment as well as the dentin-demineralisation were evaluated electron-microscopically.

The specific hypothesis tested in this study was that the in situ formed salivary pellicle protects the dentin against erosive challenge.

2. Materials and methods

2.1. Specimens preparation

Dentin slabs with a diameter of 5 mm were prepared from freshly extracted bovine incisor teeth (2-year old cattle). The enamel was ground away to expose the sound dentin. Specimens were etched laterally and at the bottom site for 15 s with 37% phosphoric acid gel (Etching gel, DMG, Hamburg, Germany) and treated for 30 s with Optibond Primer (Kerr, Karlsruhe, Germany). Optibond Adhesive was applied afterwards and light cured for 30 s. Application of adhesive was performed three times.[7] Subsequently, the unsealed surfaces were ground flat and polished (grit 4000). The smear layer was removed by 1-min ultrasonication of the dentinal slabs in 17% EDTA solution (pH 7.4).[26,27]

2.2. Subject

One healthy male volunteer, member of the laboratory staff (age: 31), participated in the study. Visual oral examination was carried out by an experienced dentist. The subject showed no signs of gingivitis or caries. Informed written consent had been given by the subject about participation in the study. The study design was reviewed and approved by the Ethic Committee of the University of Göttingen in Germany (Proposal 16/6/05).

2.3. In situ pellicle formation and in vitro erosion

For in situ pellicle formation, an individual upper jaw splint was vacuum-formed from 1.5 mm thick methacrylate foil. Cavities were prepared in the buccal aspects of the splints (region 16, 15, 14). Before exposure in the oral cavity, slabs were stored in aqua dest. for 24 h.

The slabs were fixed on the splints with polyvinyl siloxane impression material (President light body, Coltene, Switzerland) so that only the surfaces were exposed to oral fluids. The splints were carried intraorally for 120 min to allow pellicle formation on the specimens' surfaces.[7,28]

After intraoral exposure, the slabs were quickly removed from the splints and thoroughly rinsed with running water for 5 s. In vitro erosion was performed in 1000 μl hydrochloric acid (pH 2.3, 4.5 mmol/l) to provide an excess of acid in order to maintain constant pH during incubation for 5 min as described previously.[7]

2.4. Transmission electron microscopy (TEM)

Immediately after the erosion experiments the dentin slabs were fixed in 2.5% glutaraldehyde for 2 h. Post fixation took place in 1% osmium tetroxide for 2 h. The specimens were dehydrated in increasing concentrations of alcohol and embedded in Araldite M (Serva, Darmstadt, Germany).

Ultra thin sections were cut from embedded specimens without decalcification of the dentin. The ultra thin sections were collected on Pioloform-F coated copper grids and stained with uranyl acetate and lead citrate. Transmission electron microscopic investigation took place at 3000–50,000-fold magnification in an EM 109 microscope (Zeiss, Oberkochen, Germany).

2.5. *Photometric determination of calcium release*

Mineral dissolution caused during HCl-treatment was determined by assessing calcium release into the solution photometrically in double assays using the Arsenazo III method (Fluitest®, Ca-A-II, Analyticon, Lichtenfels, Germany) as described previously.[7,24]

Arsenazo III reacts with calcium in an acid solution to form a blue purple complex. Intensity developed is proportional to the calcium concentration. Absorption can be determined at $\lambda = 650$ nm according to standard curves.[7,24] The reagent for determination of calcium was composed of 100 mmol/l Imidazol buffer (pH 6.5) and 0.12 mmol/l Arsenazo III.

2.6. *Preparation and gaining of solutions for the photometric assays*

After 1–5 min, 100 μl were taken from the acidic solutions and replaced by 100 μl of fresh acid to maintain the volume and to keep pH constant at 2.3. This was controlled with a pH electrode (RadiometerA/S, Copenhagen, Denmark). The addition of 100 μl in the respective time led to a dilution of the samples with respect to the actual calcium concentration. This dilution was taken into consideration in later performed calculation of calcium concentration in the solutions.

From the volume taken from the solution, 10 μl were admixed to 100 μl of the calcium assay.[7]

2.7. *Statistics*

Data were evaluated with Mann–Whitney *U*-test (Statistica, StatSoft, Hamburg, Germany). Level of significance was set at $p < 0.05$.

3. Results

3.1. *Transmission electron microscopic images*

Representative electron microscopic micrographs are shown in Figs. 1 and 2. In all images, dentinal structures and pellicle could be distinguished clearly (Figs. 1 and 2). The dentinal pellicle manifested itself as a loosely arranged, globularly structured layer of 300–750 nm thickness (Fig. 1c and d). Immediately onto the dentinal surface, the adsorbed biomolecules formed an electron dense basal layer of 30–60 nm.

Hydrochloric acid treatment of pellicle free dentin specimens caused demineralisation of the dentinal surface leaving a 3–6 μm thick layer of densely packed demineralised collagenic fibrils (Fig. 1a and b). Also exposure of pellicle coated samples to hydrochloric acid yielded layers of densely packed demineralised collagenic fibrils measuring 3–6 μm in thickness (Fig. 2a–d). No major differences could be observed between the ultra structural appearance of the demineralised dentin on pellicle coated and on uncoated samples (Figs. 1a

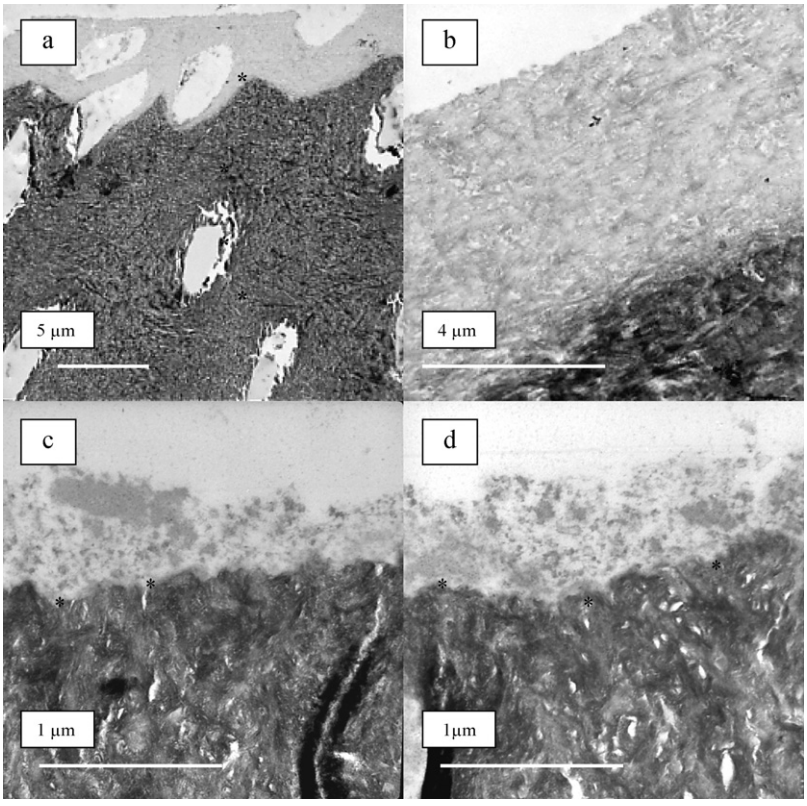

Fig. 1 – (a) Dentin specimen without pellicle after 5 min treatment with hydrochloric acid, dentinal tubules can be seen in intact as well as in demineralised dentin (original magnification: 3000-fold). (b) Dentin specimen without pellicle after 5 min treatment with hydrochloric acid. Network of collagenic fibrils can be seen in demineralised dentin. (Original magnification: 12,000-fold.) (c and d) Dentinal 120-min pellicles without acid treatment. The pellicle layer is characterised by a globular structure. Note the electron dense basal layer (*) (original magnification: 30,000-fold).

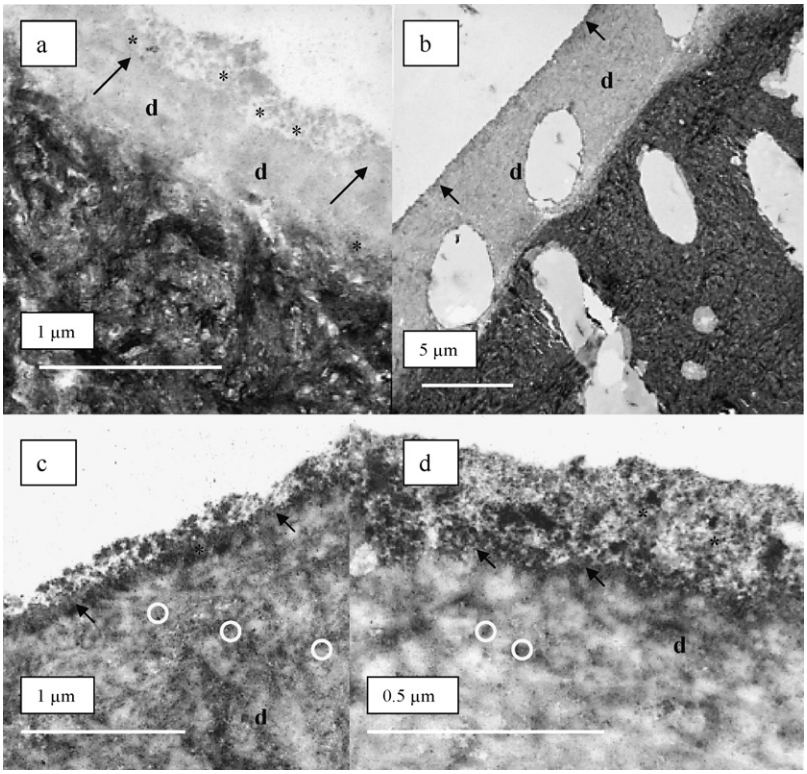

Fig. 2 – Dentin samples with 120 min pellicles after treatment with hydrochloric acid for 90 s (a) and 5 min (b–d). Pellicle residues (*) on top of the demineralised dentin (d). Arrows indicate the borderline between demineralised dentin and pellicle. Note the electron dense granular structure of the pellicle residues in c and d. Electron dense agglomerates in nanometre size can be seen between the collagenic fibrils of the demineralised dentin (o) (a) original magnification: 30,000-fold; (b) original magnification: 3000-fold; (c) original magnification: 30,000-fold; (d) original magnification: 50,000-fold.

and b and 2a–d). However, electron dense agglomerates of 2–10 nm diameter could be detected between the collagenic fibrils of the demineralised dentinal surface layer in pellicle coated samples treated with hydrochloric acid for 5 min (Fig. 2c and d). Due to exposure to hydrochloric acid, the pellicle layer itself was partially dissolved and removed from the dentinal surface. However, continuous pellicle residues, measuring 100–300 nm in thickness, were still detectable on all specimens surface even after 5-min acid exposure (Fig. 2a–d). Pellicle residues on specimens exposed to hydrochloric acid for 5 min were characterised by a granular ultrastructure and a higher electron density as compared with the unaffected 120 min control pellicles (Figs. 1c and d and 2a–d).

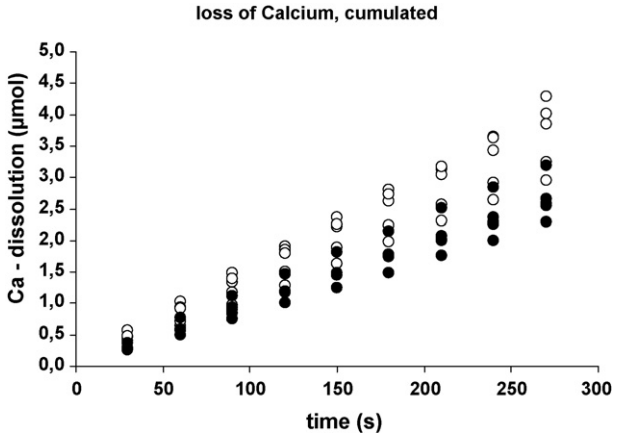

Fig. 3 – Loss of calcium (μmol) during erosion of the samples in HCl, pH 2.3. (○) Samples without pellicle ($n = 5$) (●) samples with an in situ formed pellicle ($n = 5$).

3.2. *Calcium release*

Calcium release from dentin samples could be determined well. The treatment of dentin samples with hydrochloric acid yielded linear kinetics of calcium release irrespective of the pellicle-layer (Fig. 3). Calcium erosion from the pellicle coated dentin slabs was 23.5 ± 2.9 μg Ca/min and 32.2 ± 4.2 μg Ca/min for the uncoated samples. The difference was statistically significant ($p \leq 0.05$). The pellicle caused a reduction of calcium erosion of about 27%.

4. Discussion

Electron microscopic analyses are well established for investigation of the acquired pellicle as well as of dental erosions.[7–9] However, the methods require extensive preparation of the samples and quantification of the erosive effects is very limited.[7–9] In contrast, the photometric determination of calcium or phosphate release allows investigation of minimal

erosive effects during short time exposition to erosive substrates.[7,24,25] With this method, even minimal erosive effects occurring within 1 min can be analyzed quantitatively.[7,24,25] In comparison with other methods such as profilometry or determination of micro-hardness, the present photometric measurement of calcium release is advantageous since it allows evaluation of erosions' kinetics without causing any artifacts.[29–31]

Bovine hard tissue was used as a substitute for human teeth as done in other investigations on erosions or pellicle, respectively.[8,9,28] After flattening and polishing of the dentinal specimens by wet-grinding with abrasive paper, a smear layer covers the specimens' surfaces. In order to remove this smear layer prior to in situ pellicle formation without significant demineralisation, the dentin specimens were treated with EDTA. This procedure has been shown to remove the smear layer effectively.[26,27] Pellicle formation was performed for 120 min. In previous studies on the acquired pellicle formed on enamel, 120-min pellicle differed neither from 24-h biofilms nor from 3-min short time pellicles regarding the protective potential.[8,9,18] The present investigation was restricted to one acid only as previous studies on calcium and phosphate release yielded that the type of the acid is of little impact on dental erosions.[7] Due to the fact that many acidic beverages and gastric juice have pH-values between 2 and 3, pH 2.3 was chosen in the present study.[2] Dilution and buffering effects of saliva that are relevant factors determining the final effect of an acid under in vivo conditions were not considered in this pilot study.

Moreover, the experiments were based on a single subject. Accordingly, the reported findings should be interpreted with caution concerning generalisation, but the data give valuable information for future research. The most important finding is that 2-h acquired pellicles formed on the dentinal surface reduce erosive calcium loss only to a small extent under the ex vivo/in vitro conditions of the present study. These findings are in accordance with the results of a profilometric in vitro-study indicating a reduction of the erosion depth due to an experimental dentinal pellicle to an extent of about 20% as compared with samples stored in water.[19]

In contrast, a recently published in situ study yields that the 2-h salivary pellicle provides no protection of the underlying dentin against erosive challenge by orange juice lasting over 10–30 min.[11] The difference between these findings and the present results might be explained by the fact that Hara et al. considered long-term exposure to orange juice over 30 min, whereas in the present investigation exposure to HCl was limited to 5 min.[11] It has been stated by Hara et al.[11] that the lack of protection found for the dentinal pellicle might be related to the higher porosity and solubility of dentin compared with enamel. Thus, the dentin could be demineralised relatively faster than enamel, not allowing the pellicle to act as a protective barrier against the erosive challenge.[11]

It is evident from the present TEM micrographs that the pellicle layer is not dissolved completely from the dentinal surface due to the 5-min exposure to acid, whereas the underlying dentin is demineralised. This observation clearly demonstrates that the dentinal pellicle layer is highly permeable for hydrochloric acid as well as the dissolved calcium and phosphate. Early in vitro studies have shown that pellicle like protein layers on enamel possess a selective permeability function, thus regulating the de- and remineralisation processes at the dental surfaces.[32–35] Due to its perm-selective and permeable nature the pellicle layer possess the ability to modify acid diffusion and the transport of calcium and phosphate ions into and out of the enamel surface.[8,9,12,36,37] It has been suggested by Busscher et al.[36] that salivary pellicles are open structures allowing ion exchange at the enamel surface. This suggestion is in accordance with high-resolution SEM and AFM images of the pellicle layer,[38–40] indicating a porous mesh-like surface pattern. The in situ-formed pellicle layer reduces and retards enamel demineralisation during acid exposure, but does not completely inhibit acid-related changes to the enamel surface.[8,9,15,18,39,41]

For the first time, the present data and TEM-images yield clear evidence that pellicles expose similar properties on the dentinal surfaces. In this context, it is of interest that the thickness of the demineralised dentinal surface layer yielded no distinct differences in pellicle coated and uncoated dentinal samples as observed in TEM-micrographs despite the fact that significant differences in calcium release were measured photometrically. Maybe, the pellicle serves as a reservoir and retainer for calcium and phosphate ions released during the erosive process to a certain extent. The electron dense agglomerates observed within the pellicle and the demineralised dentin surface after acid treatment in the present TEM-images may represent calcium and phosphate precipitates.

Differences in substrates' physico-chemical surface properties are assumed to influence the formation, composition and ultrastructure of the salivary pellicle.[42–45] Notwithstanding, amino acid composition of pellicles on different substrata expose only slight differences in the amino acid profile.[43] Furthermore, amylase and lysozyme activity within pellicles on dentin and enamel do not differ significantly.[28] These findings indicate that the pellicle is a proteinaceous layer of high uniformity on different substrata in the oral cavity.[12] In the present study, the 2-h dentinal pellicle formed in situ was characterised by a predominately globular ultrastructure resembling the ultra morphology described previously for in situ formed enamel pellicles.[12,46]

5. Conclusion

In conclusion, the present data indicate that the dentinal in situ pellicle layer has a limited potential to protect the underlying dentinal surface against erosion by hydrochloric acid. The in situ formed 2 h dentinal pellicle acts mainly as an ion permeable network rather than a diffusion barrier.

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