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Effect of Polyphenols on the Ultrastructure of the Dentin Pellicle and Subsequent Erosion

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Keywords
Polyphenols · Salivary pellicle · Dentin · Erosion · Transmission electron microscopy · Fluoride

Abstract

Introduction: Erosive tooth wear is a highly prevalent dental condition that is modified by the ever-present salivary pellicle. The aim of the present in situ study was to investigate the effect of polyphenols on the ultrastructure of the pellicle formed on dentin in situ and a subsequent erosive challenge. ***Methods:*** The pellicle was formed on bovine dentin specimens for 3 min or 2 h in 3 subjects. After subjects rinsed with sterile water (negative control), 1% tannic acid, 1% hop extract, or tin/fluoride solution containing 800 ppm tin and 500 ppm fluoride (positive control), specimens were removed from the oral cavity. The erosive challenge was performed on half of the specimens with 1% citric acid, and all specimens were analyzed by transmission electron microscopy. Incorporation of tannic acid in the pellicle was investigated by fluorescence spectroscopy. ***Results:*** Compared to the negative control, ultrastructural analyses reveal a thicker and electron-denser pellicle after application of polyphenols, in which, according to spectroscopy, tannic acid is also incorporated. Application of citric acid resulted in demineralization of dentin, but to a lesser degree when the pellicle was pretreated with a tin/fluoride solution. The pellicle was more acid-resistant than the negative control when modified with polyphenols or tin/fluoride solution. ***Conclusion:*** Polyphenols can have a substantial impact on the ultrastructure and acid resistance of the dentin pellicle, while the tin/fluoride solution showed explicit protection against erosive demineralization.

Introduction

The spread of acidic food and beverages had a major impact on the oral cavity, not least on erosive tooth wear [1]. Erosive tooth wear can affect all dental hard tissues but is more complex in dentin due to the heterogenous morphology [2]. Different preventive measures were developed to protect dentin against dental erosion, with stannous fluoride being considered the gold standard. Tin has a dual mechanism: on the one hand, it reduces demineralization by being incorporated in dentin and forming a protective layer [3], and on the other hand, it reduces the degradation of dentin collagen by inhibiting endogenous collagenases [4]. Despite the promising effects, access to fluoridated products or oral health services is limited in some populations [5]. Therefore, natural alternatives that are readily available are needed.

In dental research, polyphenols are increasingly being investigated for their preventive effects [6]. Polyphenols are plant secondary metabolites that are ubiquitous found in nature. This heterogeneous group consists of several

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Table 1. Test substances

Test substance	Additional information	pH	Manufacturer
Sterile water	Negative control	5.5	Ampuwa, B. Braun Melsungen AG, Melsungen, Germany
Tannic acid	1%	2.9	Sigma-Aldrich, Taufkirchen, Germany
Hop extract	1%	2.4	Flavex, Rehlingen-Siersburg, Germany
Tin/fluoride solution	Positive control, contains 800 ppm tin chloride and 500 ppm fluorides	4.5	Elmex Zahnschmelz Professional, CP GABA GmbH, Hamburg, Germany

thousand substances, which contain more than one phenolic ring [7]. Thanks to their functional groups, polyphenols can interact with proteins via hydrophobic interactions and hydrogen bonds [8], and thus are of interest for protection of dentin against dental erosion.

Polyphenols interact with dentinal proteins, such as collagen and collagenases. During erosive demineralization of dentin, the collagen network is exposed. This network reduces further demineralization but is easily removed through enzymatic degradation by endogenous collagenases [9]. By cross-linking collagen and inhibition of collagenases, polyphenols stabilize the collagen network and finally protect dentin from further demineralization [10].

Polyphenols also interact with salivary proteins, which are substantially involved in the formation of the pellicle, a proteinaceous layer that immediately covers the dental surface. The pellicle and in particular the basal layer acts as a diffusion barrier, thereby protecting enamel against dental erosion [11]. In contrast, the protective properties of the dentin pellicle are controversially discussed [12], which could be related to the missing basal layer [13]. A promising approach to enhance the protective properties of the pellicle is the modification of its ultrastructure by polyphenols. According to studies on the enamel pellicle, polyphenols lead to the formation and deposition of salivary protein aggregates and, simultaneously, to aggregation of proteins within the pellicle. As a result, the thickness and density of the enamel pellicle are increased that provide better protection against dental erosion [6, 14–17].

Prevention of erosive tooth wear by polyphenols was also shown for dentin. In the studies by Magalhães et al. (2009) and De Moraes et al. (2015), the application of the polyphenolic green tea to pellicle-covered dentin significantly reduced erosive tooth wear [18, 19]. In a similar study with green tea and other natural extracts rich in polyphenols, erosive tooth wear of dentin was investigated in the absence and presence of the dentin pellicle. The protective effect depends on the specific polyphenol and also on the presence of the pellicle. Even if the modified dentin was more resistant to erosion, the greatest protective effect was observed in the presence of the pellicle, indicating that modification of the pellicle with polyphenols plays a crucial role in the prevention of erosive tooth wear [20].

Polyphenols modify the pellicle by interacting with proteins from saliva and the pellicle, but so far, ultrastructural studies have only been performed on the enamel pellicle [15–17]. Therefore, the aim of the present study was to investigate the effect of polyphenols on the ultrastructure of the dentin pellicle and a subsequent erosive challenge. The substances investigated were tannic acid, a potent polyphenol with many functional groups [21], and a hop extract, which contain different polyphenols such as flavanols, flavan-3-ols, and phenolic carboxylic acid [22]. In addition, the incorporation of tannic acid into the pellicle was examined by fluorescence spectroscopy.

Materials and Methods

Subjects

The present study involved three volunteers who had no carious lesions, no periodontitis, no systemic diseases, did not smoke, drank alcohol, or took medication that affect saliva. Informed written consent was obtained from all volunteers. The Medical Ethics Committee of the Medical Association of Saarland approved the study design (238/03, 2016).

Specimens and Splints

The roots of lower incisors from 2-year-old calves (Slaughterhouse, Zweibrücken, Germany) were cut into several pieces. After the cementum and circumpulpal dentin were removed, the pieces were wet ground into a rectangular form (3 mm × 3 mm) and scored in the outer third of the dentin. The smear layer was removed by ultrasonication with 3% NaOCl for 30 s, followed by disinfection with 70% isopropyl alcohol for 15 min and rehydration in sterile water for 24 h. Then, they were fractured along the score line in order to generate a surface with open dentinal

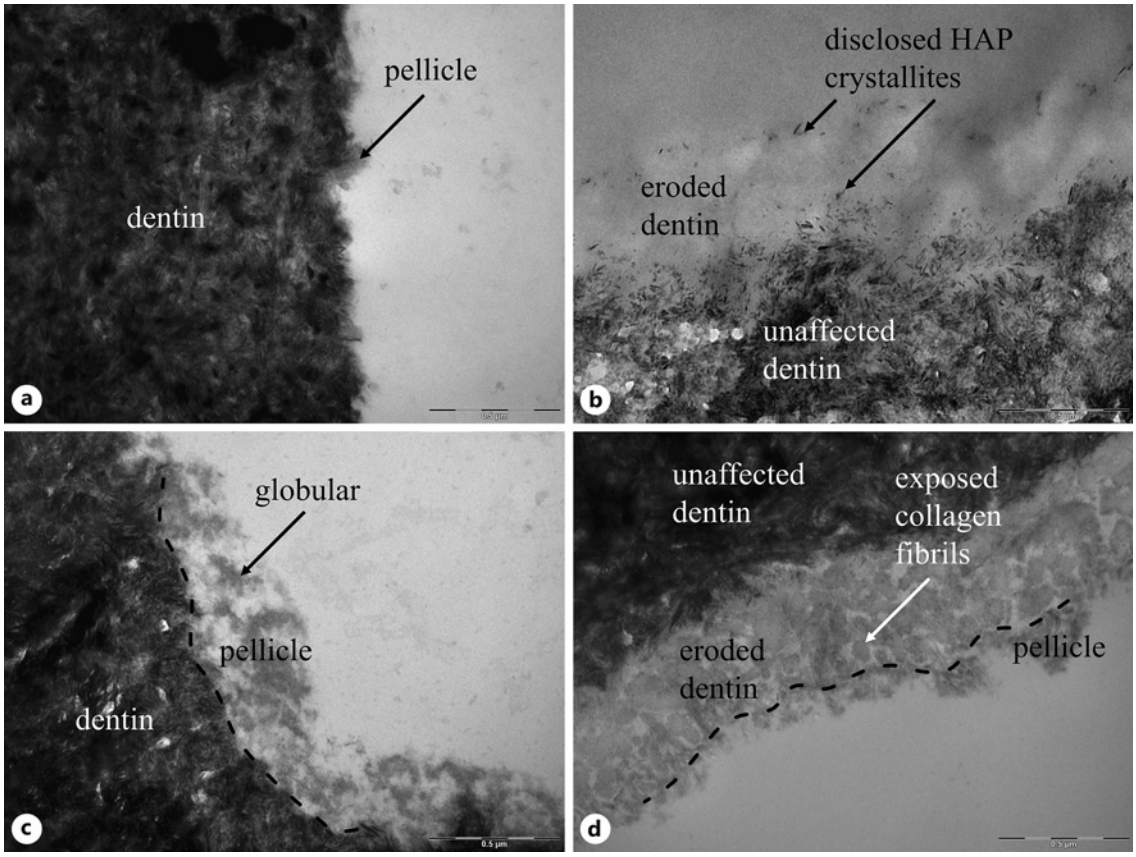

Fig. 1. Transmission electron micrographs of 3-min (**a**, **b**) and 2-h pellicles (**c**, **d**) formed on buccally mounted dentin specimens followed by treatment with water (negative control). After the intraoral exposure, specimens were eroded with citric acid (**b**, **d**) or stayed untreated (**a**, **c**). The unaffected dentin has a higher electron density than the eroded dentin with exposed collagen fibrils. The dentin is covered by a pellicle with granular and globular structures. The interface between the pellicle and dentin is marked by a dashed line. Disclosed hydroxyapatite (HAP) crystallites appear both in the pellicle and the intermediate zone between the sound and eroded dentin. Original magnification: 30,000-fold.

tubules. The resulting specimens (n = 48 in total) were mounted with silicone impression material (President light body, Coltène/Whaledent GmbH + Co. KG, Langenau, Germany) in the buccal posterior region of individual upper splints made of methacrylate foils (DURAN®, Scheu Dental GmbH, Iserlohn, Germany). Four specimens were used for each subject and substance, with two specimens each in the first and second quadrants.

Pellicle Formation and Erosion

Each trial started in the morning after a 2-h fast and cleaning of the teeth without dentifrice. During the trial, oral hygiene and eating or drinking were not allowed. The splints were inserted in the oral cavity for 3 min or 2 h to allow pellicle formation, followed by rinsing with 10 mL of the respective test substance for 30 s (Table 1). Then, specimens were dismounted, and saliva remnants were removed by gentle rinsing with distilled water. Half of the specimens were eroded with 5 µL 1% citric acid (pH 2.2) (SERVA Electrophoresis GmbH, Heidelberg, Germany) for 1 min. The washout phase in between the trials was at least 5 days.

Transmission Electron Microscopy

Specimens were fixed with 1% glutaraldehyde, 1% paraformaldehyde, and 0.1 M cacodylate for at least 2 h, washed in 0.1 M cacodylate, and postfixed with 2% osmium tetroxide for 2 h. After dehydration in an ascending ethanol series and acetone, specimens were stored overnight in Araldite, dodecenylsuccinic anhydride, and tris buffer and then embedded in Araldite. Ultrathin sections were cut using an ultramicrotome with a diamond knife, mounted on pioloform-coated cooper grids, and contrasted with uranyl acetate and lead citrate. The morphology of the pellicle and dentin was investigated with a transmission electron microscope (Tecnai 12, FEI, Eindhoven, Netherlands) in up to 98,000-fold magnification.

Fluorescence Spectroscopy

The presence of tannic acid in the pellicle was investigated with fluorescence spectroscopy using MoO3-x quantum dots (QDs) and a multifunctional microplate reader (Tecan infinite® 200, Tecan Austria GmbH, Austria). QDs were synthesized by adding 0.5 g molybdenum in 7.5 mL hydrogen peroxide. The dispersion

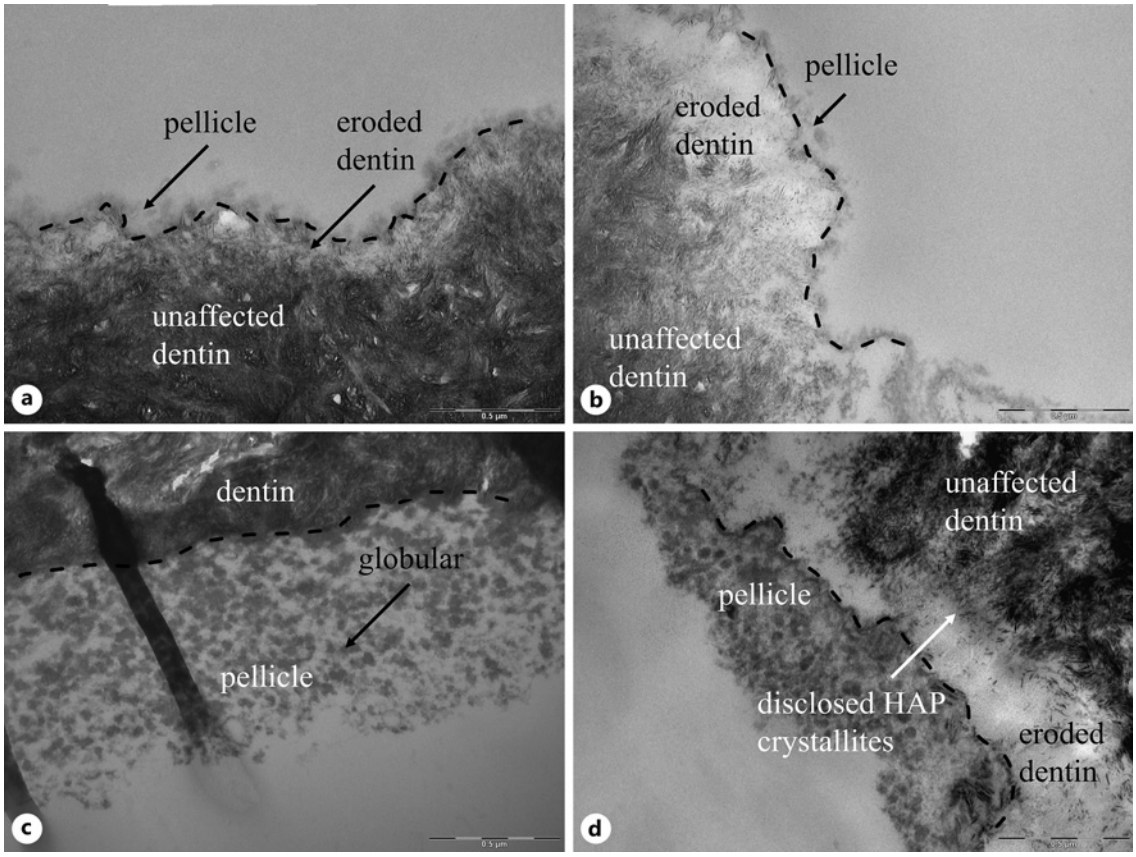

Fig. 2. Transmission electron micrographs of 3-min (**a**, **b**) and 2-h pellicles (**c**, **d**) formed on buccally mounted dentin specimens followed by treatment with tannic acid. After the intraoral exposure, specimens were eroded with citric acid (**b**, **d**) or stayed untreated (**a**, **c**). The unaffected dentin has a higher electron density than the dentin eroded by citric acid or even by tannic acid itself (**a**). The dentin is covered by a condensed pellicle with granular and predominantly densely packed globular structures. The interface between the pellicle and dentin is marked by a dashed line. Disclosed hydroxyapatite (HAP) crystallites appear both in the pellicle and the intermediate zone between the sound and eroded dentin. In contrast to water (negative control), the modified pellicle is thicker and withstands the erosion by citric acid. Original magnification: 30,000-fold.

was diluted with 30 mL double-distilled water, and unreacted hydrogen peroxide was removed with 6.5 g manganese oxide, which was also removed by centrifugation at 10,000 rpm for 20 min. After adding 1 g chitosan, the mixture was incubated at 80°C for 24 h. After cooling to room temperature, the mixture was centrifuged at 12,000 rpm for 20 min, and the yellow supernatant was dialyzed against water (100–500 Da). Finally, the yellow solution was lyophilized, and the resulting powder was dissolved in 1 mL of water. Characterization of QDs was performed by recording the absorption and fluorescence spectra [23]. Dentin specimens ($5 \times 5 \times 1.5$ mm^3) were polished from all sides up to 4,000 grit and pretreated as stated above. Specimens ($n = 4$) were placed in the vestibule of the lower jaw, and after 3 min of pellicle formation, subjects ($n = 3$) rinsed for 30 s with 10 mL water (negative control) or 1% tannic acid. The pellicle was eluted with Tris/RIPA buffer (pH 7.5) to obtain a solution of 1.5 mL per subject and substance [24]. The 2-h pellicle was not investigated because a pellicle with a formation time of more than 30 min cannot be completely removed with the present elution protocol. After mixing 100 µL of the eluted pellicle with 100 µL of QDs (0.5 mg/mL), tannic acid was detected by recording the fluorescence spectra with an excitation wavelength at 350 nm in triplicate.

Results

Transmission Electron Microscopy

Exposure of dentin specimens to the oral cavity resulted in pellicle formation. When specimens were exposed for 3 min followed by rinsing with water (negative control), the thin pellicle was discontinuous so that the dentin was not completely covered by a pellicle layer. In contrast, the granular and globularly structured 2-h pellicle was continuous, with bacteria occasionally adhering to the pellicle through their fimbriae. When

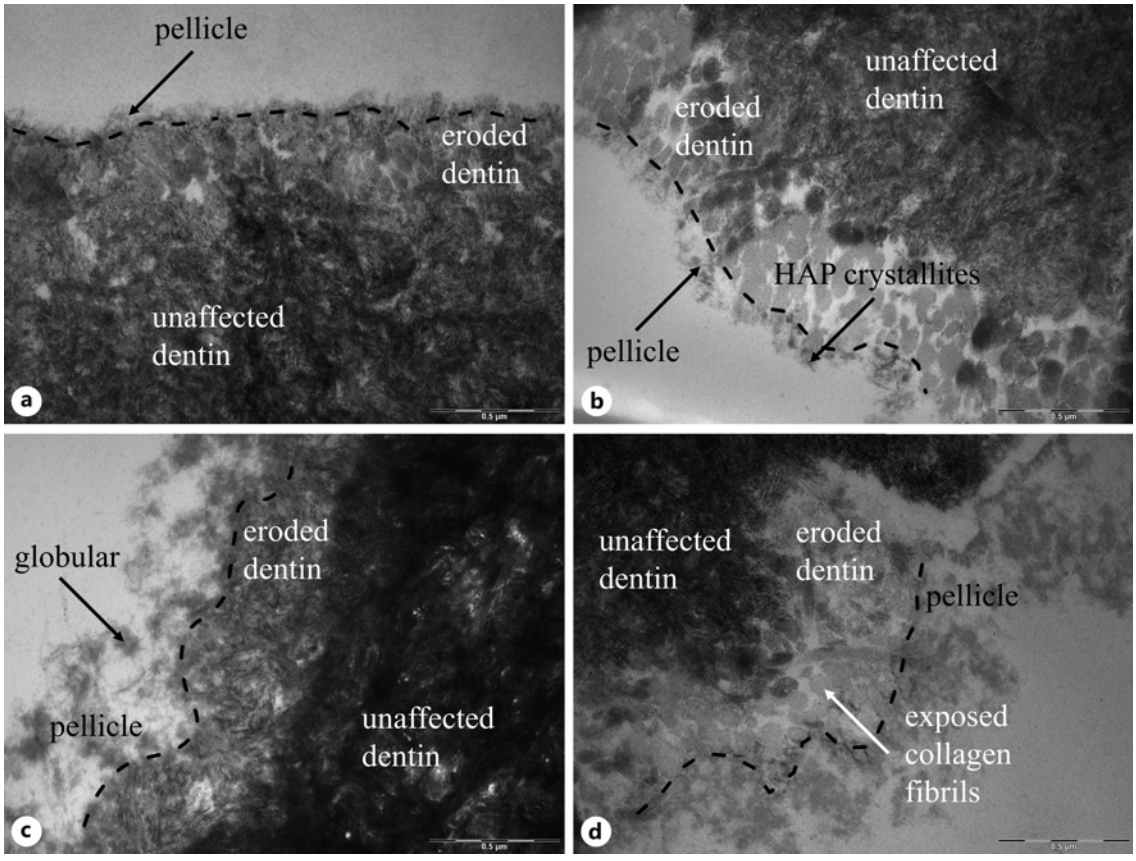

Fig. 3. Transmission electron micrographs of 3-min (**a**, **b**) and 2-h pellicles (**c**, **d**) formed on buccally mounted dentin specimens followed by treatment with hop extract. After the intraoral exposure, specimens were eroded with citric acid (**b**, **d**) or stayed untreated (**a**, **c**). The unaffected dentin has a higher electron density than the dentin eroded by citric acid or even by hop extract itself. The dentin is covered by a pellicle with granular and globular structures. The interface between the pellicle and dentin is marked by a dashed line. Disclosed hydroxyapatite (HAP) crystallites appear both in the pellicle and the intermediate zone between the sound and eroded dentin. In contrast to water (negative control), the modified pellicle withstands the erosion by citric acid. Original magnification: 30,000-fold.

specimens were eroded with citric acid, the thickness of the 3-min and 2-h pellicles was reduced, but the pellicles were not completely removed. In the basal region of the pellicle, needle-shaped electron-dense particles appeared, representing hydroxyapatite crystals. The dentin was demineralized, which differed from the unaffected dentin in the reduced electron density and exposure of collagen fibrils. The demineralization front was characterized by disclosure of hydroxyapatite crystals (Fig. 1). When subjects rinsed with tannic acid, the thickness and electron density of the 3-min and 2-h pellicles increased. The 2-h pellicle was predominantly globularly structured with densely packed agglomerates. Rinsing with tannic acid also caused demineralization of the dentin. Erosion with citric acid further increased the demineralization but did not reduce the pellicle thickness (Fig. 2). After rinsing the oral cavity with hop extract, the alterations were less pronounced, but similar to tannic acid, the pellicle had a globular structure, and the dentin was demineralized, which increased after application of citric acid. The pellicle was not affected by the erosion (Fig. 3). The demineralization of dentin with a 3-min pellicle was reduced when the tin/fluoride solution (positive control) was applied and even completely inhibited when a 2-h pellicle was present. The morphology of the pellicle was altered after application of citric acid from a dense granular to a loose globular and granular structure (Fig. 4).

Fluorescence Spectroscopy

The characterization of QDs with a microplate reader showed an absorption peak at 250 nm, fluorescence emission peak at 436 nm with excitation at 350 nm, and fluorescence excitation peak at 352 nm with emission at

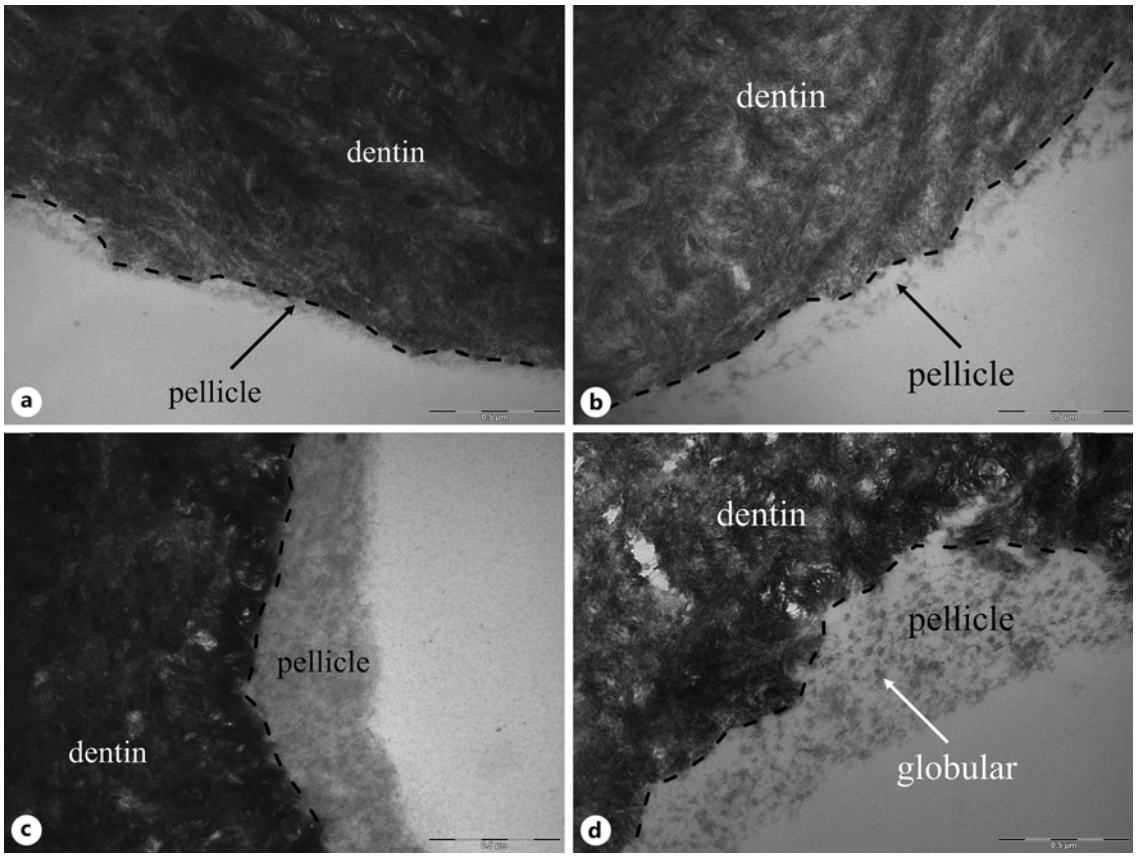

Fig. 4. Transmission electron micrographs of 3-min (**a**, **b**) and 2-h pellicles (**c**, **d**) formed on buccally mounted dentin specimens followed by treatment with tin/fluoride solution (positive control). After the intraoral exposure, specimens were eroded with citric acid (**b**, **d**) or stayed untreated (**a**, **c**). The dentin is covered by a pellicle with a dense granular structure (**c**) or granular and globular structures (**a**, **b**, **d**). The interface between the pellicle and dentin is marked by a dashed line. In contrast to water (negative control), the modified pellicle withstands the erosion by citric acid, and the dentin is not demineralized (**b**, **d**). Original magnification: 30,000-fold.

435 nm. The fluorescence intensity of the eluted pellicle that was treated with tannic acid in situ was lower than the negative control (Fig. 5), indicating the presence of tannic acid in the treated pellicle.

Discussion

All surfaces in the oral cavity that are exposed to saliva are readily covered by a pellicle [25]. Regarding enamel, the pellicle consists of an inner electron-dense basal layer and a globular and granularly structured outer layer, which is formed by deposition of further salivary proteins and protein aggregates [26]. In the present study, a basal layer was occasionally detected on dentin. The visualization was complicated by the high electron density of dentin itself. Unlike enamel, which is dissolved for ultrastructural analysis of the pellicle [26], dentin specimens are embedded once and sectioned as a whole [27]. After 2 h of pellicle formation, a heterogenous layer was observed that has a similar ultrastructure to the enamel pellicle [26]. The number of globular agglomerates and, thus, the thickness of the pellicle increased after rinsing with the polyphenol tannic acid or, to a lesser extent, the polyphenolic hop extract. Tannic acid has a high tendency to interact with proteins since the efficacy of binding increases with the number of functional groups, and tannic acid, being a high-molecular-weight polyphenol with a pentagalloyl-D-glucose core, has at least five functional groups [8]. The present fluorescence spectroscopic analysis with selective and sensitive QDs indicates that tannic acid is also incorporated in the pellicle [23]. In contrast to tannic acid, the polyphenol content of the hop extract is low, and the containing polyphenols, such as

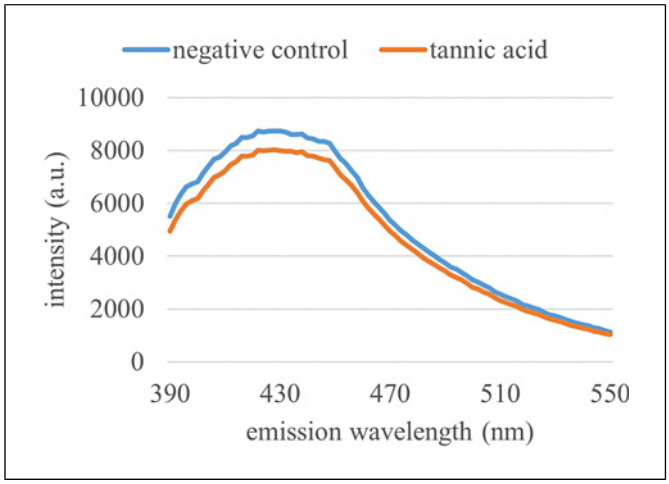

Fig. 5. Mean fluorescence emission spectra with an excitation wavelength at 350 nm of eluted 3-min dentin pellicles after treatment with water (negative control) or tannic acid.

flavanol, flavan-3-ols, and phenolic carboxylic acid, have fewer functional groups than the hydrolyzable tannin tannic acid, offering an explanation for the lower but still present effect on the pellicle [21, 22]. It is not known to what extent other components of hop extract, in particular humulones and lupulones, contribute to protein aggregation. According to the literature, treatment with polyphenols not only leads to formation of thicker pellicles but also to increased density [16, 17]. Two mechanisms can be involved in this observation. On the one hand, rinsing with tannic acid leads to aggregation of proteins in the pellicle. On the other hand, salivary proteins can form aggregates [14], which might adsorb to the preexistent pellicle. The demineralization of dentin could be determined by the lower electron density of the demineralized dentin compared to the unaffected dentin. Due to the low pH value of tannic acid and hop extract, treatment with polyphenols resulted in demineralization of dentin. The erosive potential of the acidic solution of polyphenols can be reduced by adjusting the pH value. Another study showed that at least the anti-biofilm effect of polyphenols is not impaired by increasing the pH value [14], so that the anti-erosive potential of polyphenols cannot be assumed to be impaired either. In similar studies, erosion was not observed when enamel specimens with a pellicle were treated with polyphenols [16, 17, 28]. Since dentin has a different composition and morphology, it is more susceptible to initial erosion than enamel [29]. But unlike enamel, as erosion progresses, the exposed collagen network of the demineralized dentin can act as a diffusion barrier and protect the underlying dentin [9]. As shown in the present study, the collagen fibrils are exposed after the inorganic material is chemically dissolved, but since specimens were fixed directly after erosion, an enzymatic degradation of collagen fibrils by collagenases was not observed. At the junction between the demineralized and unaffected dentin, fine electron-dense particles were detected, representing disclosed hydroxyapatite crystals in the partially dissolved demineralization front. These crystals were also found in the pellicle. During an erosive attack, calcium and phosphate ions dissolve and diffuse to the surface under a concentration gradient, where they can deposit again [30]. The crystals found in the pellicle can also be precipitated ions. These findings underline that the pellicle acts as a depot for calcium and phosphate ions [11]. This protective property of the pellicle could be maintained even under an erosive challenge when the pellicle is treated with polyphenols, since the modified pellicle is distinctly more acid-resistant than the native pellicle as shown in the present study.

Erosive demineralization of dentin was observed both in the negative control and the polyphenol groups. The evaluation of the erosion-protective properties of the pellicle is limited by the present qualitative study design with a single application of polyphenols and an erosive agent. In quantitative studies on pellicle-covered dentin using an erosive cycling model and periodic application of polyphenols, significant protective effects were shown for some polyphenolic substances [18–20]. Furthermore, the collagen network can be stabilized by polyphenols. Thanks to their functional groups, polyphenols can also interact with proteins of dentin and, e.g., cross-link collagen and inhibit collagenases, thereby protecting dentin from degradation and demineralization [29, 31].

In contrast to the negative control and the polyphenol groups, an erosive demineralization was only occasionally observed when the positive control containing fluoride and tin was applied to dentin with a 3-min pellicle and was even completely absent when a 2-h pellicle-covered dentin. Fluorides protect teeth from erosion by forming a protective calcium fluoride layer, inhibiting demineralization, promoting remineralization, and by incorporation in the dental hard tissue by forming fluorapatite [32]. The formation of a calcium fluoride layer is strongly dependent on the pH value of the solvent and the concentration of fluorides used [33, 34]. Although the pH value of the present tin/fluoride solution (pH 4.5) was low enough, no calcium fluoride layer was observed. In contrast to the study by Scholz et al. (2019), when high concentrated fluorides (12,500 ppm) were used and a layer was formed even on enamel with a 2-h in vitro

pellicle, the concentration of fluorides in the present study (500 ppm) with additional dilution by saliva during the intraoral application seems to be not high enough to form a calcium fluoride layer. Although fluorides were detected in the study by Scholz et al. [33] (2019), precipitation of fluorides was significantly reduced when a pellicle was formed before. Another point to consider is that dentin, as used in the present study, differs from enamel in morphology and composition that could have an impact on pellicle formation [12]. In a study in the rat, only traces of fluoride were detected after application of 50,000 ppm sodium fluoride to pellicle-covered dentin [35]. The preventive effect observed in the present study is thus associated with tin. Tin forms a protective layer and is also incorporated in the dentin and the pellicle [3, 36], which may explain the observed alterations in the pellicle ultrastructure. The pellicle of the positive control had a dense granular structure, which changed into a loose globular and granular structure after the application of citric acid.

The present study is limited by the low number of subjects. Due to the descriptive investigation, a power analysis was not performed, and instead the number of subjects was based on similar studies [16, 17, 28].

In conclusion, the present microscopic analyses showed that polyphenols can have a substantial impact on the ultrastructure of the dentin pellicle, which is caused by the formation and deposition of aggregates and, according to the present spectroscopic analysis, by the incorporation of polyphenols themselves, as shown for tannic acid. Application of citric acid resulted in demineralization of dentin, but to a lesser degree when the pellicle was pretreated with a tin/fluoride solution, which provided sufficient protection.

Acknowledgment

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Statement of Ethics

This study protocol was reviewed and approved by the Medical Ethics Committee of the Medical Association of Saarland (238/03, 2016). Written informed consent was obtained from participants to participate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Anton Schestakow and Wadim Rasputnis contributed to data acquisition and interpretation and drafted the manuscript. Matthias Hannig contributed to conception, design, interpretation and critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

Data Availability Statement

Data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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