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Erstveröffentlichung in / First published in:

Caries Research. 2017, 51 (1), S. 34 – 45 [Zugriff am: 19.05.2020]. Karger. ISSN 1421-976X.

DOI: https://doi.org/10.1159/000451036

Original Paper

Caries Research

Caries Res 2017;51:34–45
DOI: 10.1159/000451036

Received: February 5, 2016
Accepted after revision: September 25, 2016
Published online: December 14, 2016

Effect of Tannic Acid on the Protective Properties of the in situ Formed Pellicle

Susann Hertel[a] Sandra Pötschke[a] Sabine Basche[a] Judith Delius[b]
Wiebke Hoth-Hannig[c] Matthias Hannig[c] Christian Hannig[a]

[a]Clinic of Operative and Pediatric Dentistry, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, [b]Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Freising, and [c]Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital, Saarland University, Homburg/Saar, Germany

Keywords
Bioadhesion · Erosion · Pellicle · Polyphenols · Tannic acid

Abstract
Objectives: In the present in situ/ex vivo study the impact of tannic acid on the erosion-protective properties of the enamel pellicle was tested. Additionally, the antiadherent and antibacterial effects of tannic acid were evaluated. ***Methods:*** The pellicle was formed in situ on bovine enamel samples fixed on individual splints worn by 6 subjects. Following 1 min of pellicle formation the volunteers rinsed for 10 min with tannic acid. After further oral exposure for 19 min, 109 min, and 8 h overnight, respectively, slabs were incubated in HCl ex vivo (pH 2.0, 2.3, 3.0) over 120 s. Subsequently, kinetics of calcium and phosphate release were measured photometrically. Samples after a 1-min fluoride mouth rinse as well as enamel samples with and without a 30-min in situ pellicle served as controls. Antiadherent effects were evaluated after a 1-min rinse with tannic acid and oral exposure of the slabs overnight. DAPI (4′,6-diamidino-2-phenylindole) combined with concanavalin A staining and live/dead staining was used for fluorescence microscopic visualization and quantification of adherent bacteria and glucans. Modification of the pellicle's ultrastructure by tannic acid was evaluated by transmission electron microscopy (TEM). ***Results:*** Tannic acid significantly improved the erosion-protective properties of the pellicle in a pH-dependent manner. Bacterial adherence and glucan formation on enamel were significantly reduced after rinses with tannic acid as investigated by fluorescence microscopy. TEM imaging indicated that rinsing with tannic acid yielded a sustainable modification of the pellicle; it was distinctly more electron dense. ***Conclusion:*** Tannic acid offers an effective and sustainable approach for the prevention of caries and erosion.

Both dental caries and erosion are common oral diseases leading to the continuous loss of dental hard substance. Caries initiation is triggered by acids generated by bacteria following metabolism of dietary carbohydrates, whereas dental erosion is due to nonbacterial acid attacks from intrinsic (reflux esophagitis or bulimia nervosa) or extrinsic (dietary habits, acidic medicines) sources [Jaeggi and Lussi, 2006; Schlueter and Tveit, 2014; West and Joiner, 2014]. A recently published epidemiological study indicated an increasing prevalence of erosions in all age groups with a trend towards younger patients and

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E-Mail karger@karger.com
www.karger.com/cre

Susann Hertel
Clinic of Operative Dentistry, Medical Faculty Carl Gustav Carus
Technische Universität Dresden, Fetscherstrasse 74
DE–01307 Dresden (Germany)
E-Mail susann.hertel@uniklinikum-dresden.de

erosion progressing with age [Jaeggi and Lussi, 2006]. It can be assumed that a common vegetarian diet or frequent consumption of acidic soft drinks are the main reasons for this trend, especially in the younger generation [Barbour and Lussi, 2014; Herman et al., 2011]. To date, the use of dental products containing fluoride is the most common and established approach to prevent both caries and erosion [Bartlett, 2009; Tenuta and Cury, 2010; Huysmans et al., 2014]. Nonetheless, additive or alternative biological and biomimetic approaches are desirable for prevention.

The physiological pellicle formed by the selective adsorption of salivary proteins and glycoproteins to the tooth surface serves as a natural protection layer during acidic attacks. Pellicle components mainly responsible for the inhibition of enamel demineralization are proline-rich proteins (PRPs), mucins, and statherins as they have a high affinity to hydroxyapatite and are able to maintain high saturations of calcium and phosphate at the tooth surface during erosive attacks [Hannig and Hannig, 2014; Vukosavljevic et al., 2014]. However, complete inhibition of erosive effects on dental hard tissues is not possible [Hannig and Joiner, 2006; Hannig and Hannig, 2014; Vukosavljevic et al., 2014]. A promising approach to enhance the pellicle's protective properties is the modification of its composition and ultrastructure. It was assumed that natural agents such as lipids or polyphenols were suitable for this purpose [Hannig and Hannig, 2014]. Unexpectedly, in situ experiments yielded lack of preventive impact of rinses with edible oils or even adverse effects on the pellicle's ultrastructure [Hannig et al., 2012; Kensche et al., 2013b]. However, as recently shown in a combined in situ/in vitro study, secondary plant extracts rich in polyphenols showed promising potential to improve the pellicle's protective properties. After rinsing with a combination of watery extracts from wild *Origanum* and wild *Ribes nigrum folium,* transmission electron microscopic (TEM) analysis of the pellicle indicated an increase in the pellicle's thickness and a more electron-dense pellicle layer compared with controls [Weber et al., 2015]. Moreover, the calcium and phosphate loss of enamel during an erosive attack was reduced by the application of the combined plant extracts. Thus, a higher tenacity of the modified pellicle was postulated [Weber et al., 2015]. These observations are in accordance with previous in vitro experiments and substantiate the assumption that polyphenols facilitate the precipitation and aggregation of salivary proteins and subsequently the adsorption and incorporation of polyphenol-protein aggregates in the pellicle layer [Joiner et al., 2003, 2006].

Tannins are water-soluble polyphenols commonly found in plant-derived foods like fruits, vegetables, or teas [Soares et al., 2011]. They can be classified according to their chemical structure into 2 categories: hydrolyzable and nonhydrolyzable (condensed) tannins. Tannic acid is a specific gallotannin that belongs to the hydrolyzable class characterized by weak acidity. Extracted from plants such as tara pods *(Caesalpinia spinosa)*, gallnuts from *Rhus semialata* or *Quercus infectoria*, or Sicilian Sumac leaves *(Rhus coriaria)*, tannic acid is rated as a "GRAS" (generally recognized as safe) viand additive [Akiyama et al., 2001b] and finds application in food and beverages as a flavoring substance with an astringent taste. In addition, it is used in medicine, in particular as an ingredient of dermatological ointments for local treatment of burn wounds, skin infections, and chronic diseases such as eczema [Mangus et al., 1977; Mukhtar et al., 1988; Akiyama et al., 2001b].

Thus far, besides astringency, little is known about the intraoral effects of tannic acid. Kandra et al. [2004] demonstrated inhibitory effects of tannic acid on human salivary α-amylase in vitro; therefore, anticariogenic effects can be assumed.

The aim of the present in situ/ex vivo study was to evaluate the effect of pure tannic acid on the protective properties of the in situ formed pellicle. To evaluate the impact on erosive mineral loss an established ex vivo model for the determination of calcium and phosphate release was adopted. Furthermore, antibacterial properties of tannic acid were investigated on the in situ formed pellicle using fluorescence microscopy. Moreover, the potential influence of tannic acid on the pellicle's ultrastructure was visualized by TEM.

Materials and Methods

Subjects

Six healthy volunteers (aged 24–42 years) agreed to participate in this study. Visual oral examination by an experienced dentist could exclude oral diseases as unrestored carious lesions or periodontitis. All subjects showed physiological salivary flow rates and good oral hygiene with plaque index scores close to zero. Informed written consent was given by the volunteers about participation in the study. The study design was reviewed by the Ethics Committee of the Medical Faculty, Technische Universität Dresden, Dresden, Germany (Vote EK 147052013).

Specimen Preparation

For intraoral pellicle formation, enamel samples (diameter of 5 mm) were obtained from bovine incisor teeth of 2-year-old cattle. The specimens were etched at all sites except for the outer enamel surface for 30 s with 37% phosphoric acid gel (Etching gel;

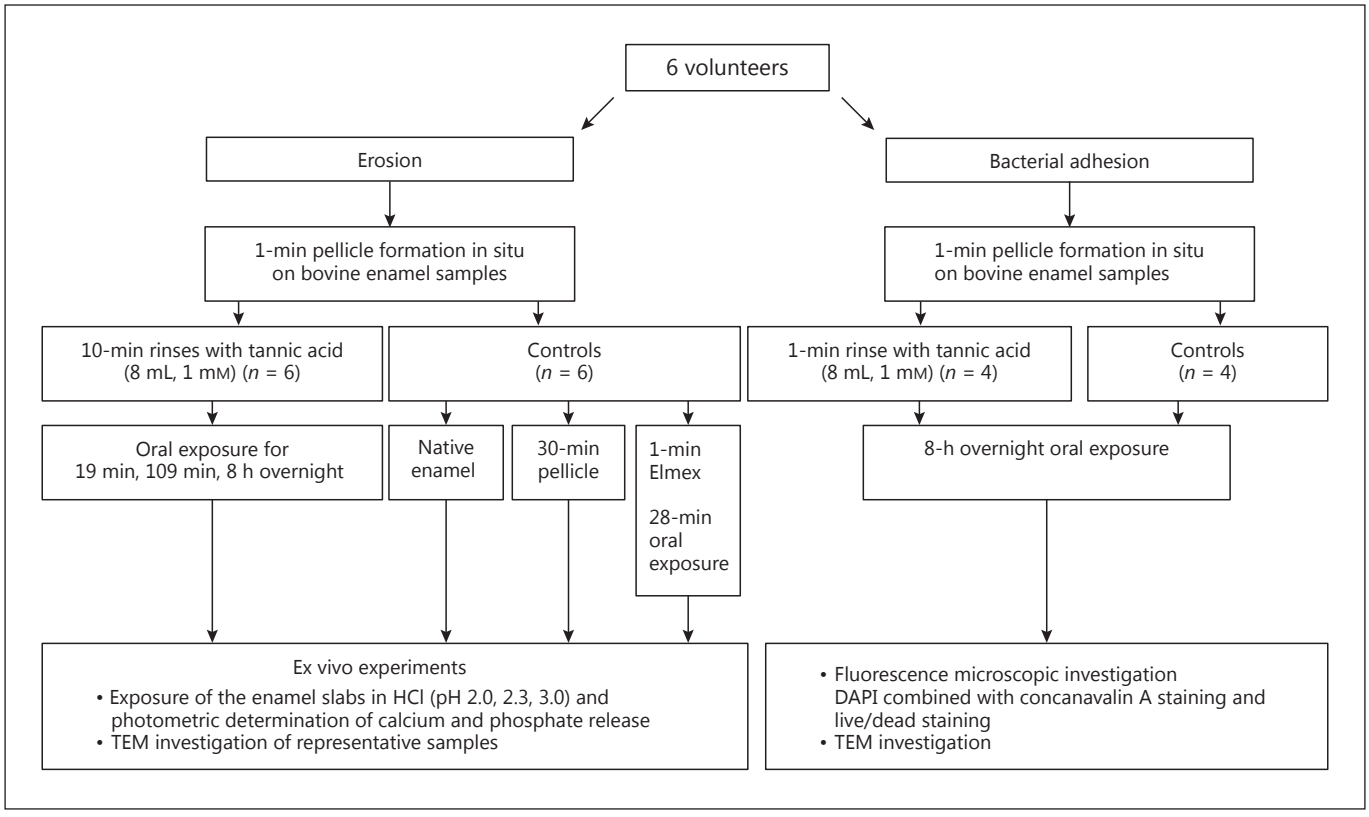


Fig. 1. Flowchart of the in situ/ex vivo experiments.

DMG, Hamburg, Germany). Afterwards, Optibond Primer (Kerr, Karlsruhe, Germany) was applied for 30 s before Optibond Adhesive was added and the specimens were light-cured in a halogen light furnace for 30 s. After that, the unsealed enamel surfaces were wet-ground and polished in a standardized grinding procedure with up to 4,000-grit abrasive paper, until approximately 200 µm of the enamel was removed [Hannig et al., 2007a, 2012]. In a next step, the smear layer on the samples was removed by ultrasonication in sodium hypochlorite (3%) for 3 min [Hannig et al., 2012]. After the samples had been washed twice in distilled water for 5 min using an ultrasonic bath, they were disinfected in ethanol (70%) for another 10 min under ultrasonication. Finally, the samples were washed again and stored in distilled water for 24 h at 2°C before exposure in the oral cavity [Jung et al., 2010].

Pellicle Formation in situ

For in situ pellicle formation individual upper jaw splints were made of methacrylate foils for each volunteer. At the buccal sites of the splints bovine enamel samples were fixed in the regions of the upper premolars and molars using polyvinyl siloxane impression material (President Light Body, Coltène, Switzerland) so that only the enamel surfaces were exposed to the oral cavity. During the test period the participants were instructed to refrain from eating and drinking. First of all, the splints were carried intraorally for 1 min to allow initial pellicle formation on the enamel surfaces.

For evaluation of the antierosive effects of tannic acid volunteers were requested to rinse thoroughly for 10 min with 8 mL tannic acid (1 mM) (tannic acid, pharmaceutically pure, Caelo; Caesar & Loretz GmbH, Hilden, Germany). Afterwards, the splints with a total of 6 enamel samples remained in the oral cavity for a further 19 min, 109 min, or 8 h overnight; this approach resulted in a total intraoral exposure time of 30 min, 120 min, and 8 h. In the following ex vivo experiments each 2 samples were incubated in $HCl_{(aq)}$ at pH 2.0, 2.3, and 3.0, respectively [Hannig et al., 2005, 2012; Weber et al., 2015]. For control, volunteers had to rinse with Elmex Kariesschutz (GABA GmbH, Lörrach, Germany) for 1 min and then carry the splints for a further 28 min intraorally. Enamel slabs with a physiological 30-min pellicle and no additional rinsing as well as native enamel slabs that were not exposed in the oral cavity served as controls (Fig. 1).

For investigation of the influence of tannic acid on the initial oral bioadhesion, the participants were asked to rinse thoroughly for 1 min with 8 mL tannic acid (1 mM) after usual toothbrushing for the overnight trial. Overnight exposure of the splints was conducted for 8 h. Specimens carried intraorally overnight without any rinse served as controls. For each trial a total of 4 enamel samples were fixed on the splints. The following morning the splints were wrapped in a moist paper towel and subsequently brought to the laboratory for direct microbial evaluation. The 4 enamel samples were applied for visualization of adherent bacteria using DAPI

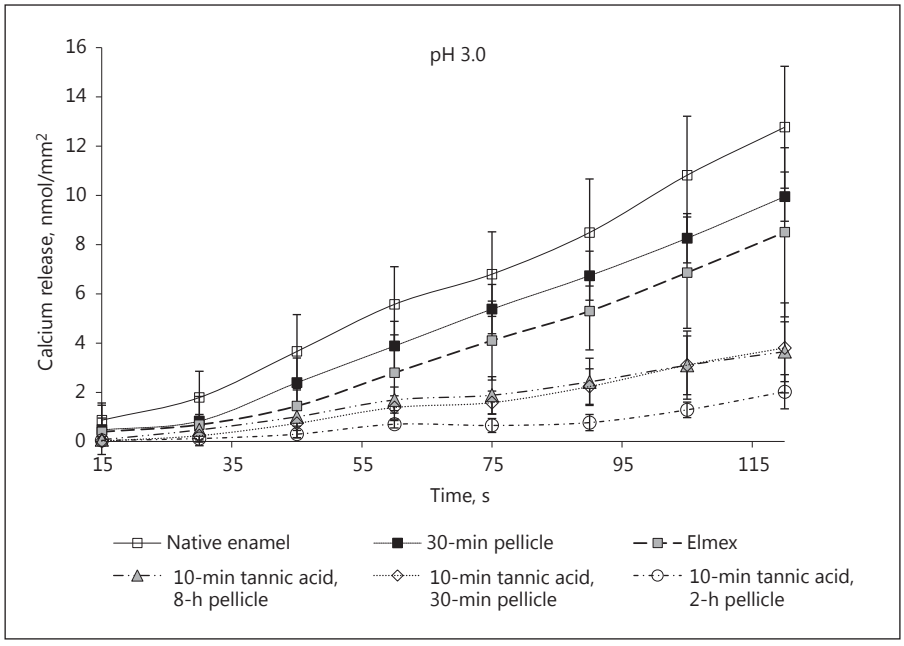


Fig. 2. Kinetics of calcium loss at pH 3.0 during incubation of enamel slabs for 120 s with and without application of tannic acid in situ. Samples with a physiological 30-min pellicle, samples after 1-min mouth rinse with Elmex Kariesschutz and 28-min intraoral exposure, as well as native enamel served as controls. $n = 6$ subjects, $n = 12$ enamel samples per subgroup (2 samples from each subject).

(4′,6-diamidino-2-phenylindole) combined with concanavalin A staining and live/dead staining (BacLight™) – 2 samples each (Fig. 1).

Ex vivo Erosion and Photometric Determination of Calcium and Phosphate Release

For the performance of the ex vivo erosion test the samples were embedded in silicone impression material at the bottom of a 2-mL Eppendorf cup [Hannig et al., 2012]. As described previously, ex vivo erosion was performed by incubating each sample in 1,000 µL hydrochloric acid (pH 2.0, 2.3, 3.0) to provide an excess of acid in order to maintain constant pH during the incubation period of 120 s [Hannig et al., 2012]. Constant acid application was ensured by pumping with a 100-µL pipette (1 lift/s). In this procedure, 100 µL of the acid was removed every 15 s for photometric analysis and replaced by 100 µL of fresh acid. Specimens after rinsing with Elmex Kariesschutz, samples with 30-min pellicle without any rinses, and native enamel samples without pellicle served as controls. Mineral loss was determined photometrically via calcium and phosphate release into the solution. Therefore, a double assay based on the Arsenazo III method (Fluitest®, Ca-A-II; Analyticon, Lichtenfels, Germany), and the malachite green assay was performed [Attin et al., 2005a, b; Hannig et al., 2013a]. In an acidic medium Arsenazo III forms a blue-purple complex with Ca^{2+} and can be used to quantify free calcium ions photometrically by measuring its absorption at $\lambda = 650$ nm.

The reagent for the determination of calcium was composed of 100 mM imidazole buffer (pH 6.5) and 0.12 mM Arsenazo III. A 10-µL aliquot of the sample was added to 100 µL Arsenazo reagent, always performed as a duplicate test, with the average absorption being calculated [Hannig et al., 2013a]. Malachite green interacts with phosphate to form a colored complex that can be determined photometrically at $\lambda = 650$ nm. For the test reagent 0.045 mg of malachite green dissolved in 100 mL aqua bidistilled water was mixed with 12.69 g of ammonium molybdate dissolved in 300 mL $HCl_{(aq)}$ (4 M). The reagent was stirred for 30 min afterwards and filtered (pore size 0.22 µm) [Hannig et al., 2012]. A10-µL aliquot of the sample was pipetted to 200 µL malachite reagent. The absorption was measured after 15 min; 2 measurements were performed for each specimen, and the average absorption was calculated. Calcium and phosphate release were calculated based on the mean photometric absorption values for the specimens and their surface areas (based on diameter of 5 mm).

Transmission Electron Microscopy

In order to visualize the influence of tannic acid on the pellicle's ultrastructure TEM investigation was performed. The in situ pellicle samples were obtained as described above. Firstly, the enamel slabs were fixed in glutaraldehyde for 2 h (2.5% glutaraldehyde, 1.5% formaldehyde in phosphate buffer, pH 7.4). Afterwards, the slabs were washed 5 times in phosphate buffer. For visualization of organic structures postfixation was performed by incubation in 1% osmium tetroxide for 2 h. Then the specimens were dehydrated in increasing concentrations of alcohol and embedded in Araldite M (Serva, Darmstadt, Germany). After the removal of dentine from the samples using a diamond bur and decalcification in 1 M $HCl_{(aq)}$, the samples were reembedded with Araldite. Finally, ultrathin sections of the pellicle were cut in series with an ultramicrotome (Ultracut E; Reichert, Bensheim, Germany) using a diamond knife. After mounting on Pioloform-coated copper grids the sections were contrasted with uranylacetate and lead citrate. The investigation was performed at 3,000- to 50,000-fold magnification in a TEM (TECNAI 12 Biotwin; FEI, Eindhoven, The Netherlands) [Hannig et al., 2012].

Fluorescence Microscopic Assays

The epifluorescence microscopic analyses were performed at 1,000-fold magnification (Axioskop II; Zeiss, Oberkochen, Ger-

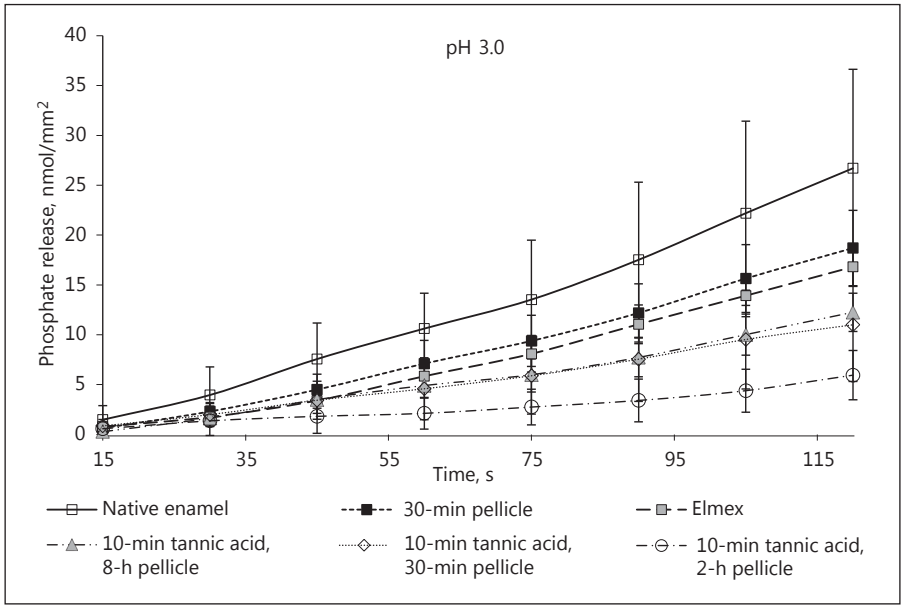


Fig. 3. Kinetics of phosphate loss at pH 3.0 during incubation of enamel slabs for 120 s with and without application of tannic acid in situ. Samples with a physiological 30-min pellicle, samples after 1-min mouth rinse with Elmex Kariesschutz and 28-min intraoral exposure, as well as native enamel served as controls. $n = 6$ subjects, $n = 12$ enamel samples per subgroup (2 samples from each subject).

many) as in previous studies. The number of cells observed in 10 randomized microscopic ocular grid fields per sample was counted. The area of an ocular grid field (0.0156 mm^2) allowed the calculation of the number of cells per centimeter squared [Hannig et al., 2007b; Al-Ahmad et al., 2009; Jung et al., 2010].

DAPI Combined with Concanavalin A Staining

The fluorescent dye DAPI (Merck, Darmstadt, Germany) is commonly used for the evaluation of the total number of adherent bacteria as it binds to adenine-thymidine regions of double-stranded DNA, thereby forming fluorescent complexes [Hannig et al., 2007b; Jung et al., 2010]. The maximum fluorescence is observed at $\lambda = 461$ nm. Glucans are a major part of the extracellular matrix and were stained using Alexa Fluor 594-conjugated concanavalin A, a lectin that specifically binds to the α-mannopyranosyl and α-glucopyranosyl residues of glucans [Kensche et al., 2013a]. First, the enamel samples were washed in 0.9% sodium chloride. Then 1.5 µL DAPI stock solution (1 mg/mL methanol) and 10 µL of concanavalin A stock solution (5 mg/mL concanavalin A-Alexa Fluor in 0.1 M sodium hydrogen phosphate, stored at –20°C, centrifuged, supernatant) were added to 488.5 µL buffer solution of 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ in a phosphate-buffered solution [Kensche et al., 2013a]. After 15 min of incubation in a dark chamber at room temperature the samples were rinsed several times with sodium chloride and air dried before fluorescence microscopic analysis.

BacLight Viability Assay

Experiments in situ

In order to differentiate between vital and avital adherent bacteria the BacLight bacterial viability kit (Invitrogen) was adopted as in previous studies [Hannig et al., 2013a, b]. The test kit consists of two nucleic acid stains – green fluorescent SYTO® 9 stain and red fluorescent propidium iodide stain [Hannig et al., 2010]. Similar amounts of component A (SYTO 9 dye 1.67 mM/propidium iodide 1.67 mM and 300 µL dimethyl sulfoxide, DMSO) and component B (SYTO 9 dye 1.67 mM/propidium iodide 18.3 mM and 300 µL DMSO) were mixed, and 2 µL were pipetted to 1 mL of saline solution. The slabs were incubated in this solution for 10 min and rinsed with saline solution afterwards. Finally, the samples were investigated by fluorescence microscopy using a fluorescein diacetate filter and an ethidium bromide filter.

Experiments in vitro

A suspension of *Streptococcus mutans* in saline solution was cultivated overnight. An amount of the bacteria was inactivated by heating (at 95°C for 1 h). Next, dilutions of the tannic acid were prepared with saline solution (0.9% sterile NaCl). The vital bacteria were mixed 1:1 with the diluted tannic acid and subsequently incubated for 10 min. These suspensions were mixed with heat-inactivated bacteria (0:100; 10:90; 25:75; 50:50). A volume of 0.5 µL of BacLight staining solution (components A and B 1:1) was added to 250 µL of the mixtures. The staining was performed over a period of 10 min in a dark chamber. Then, a volume of 100 µL from each sample was transferred into a microtiter plate, and the fluorescence was measured. The excitation wavelength was 470 nm; emission was recorded at 530 nm for the vital bacteria and 620 nm for the avital bacteria. The measurements were carried out in duplicate. For evaluation of the recorded data, the ratio of vital and dead/avital cells was calculated as emission vital/emission avital bacteria. Experiments with saline solution served as a reference/negative control [Hannig et al., 2013a, b].

Statistics

Statistical analysis of the fluorescence microscopic data was performed by the Mann-Whitney U test due to the lack of normal distribution ($p < 0.05$). Statistical analysis of the calcium and phosphate release data was calculated using the t test with Bonferroni-Holm correction ($p < 0.01$) considering the means of the data from 2 enamel samples per subject of each subgroup. The software used was SPSS 22.0 (IBM, Ehningen, Germany).

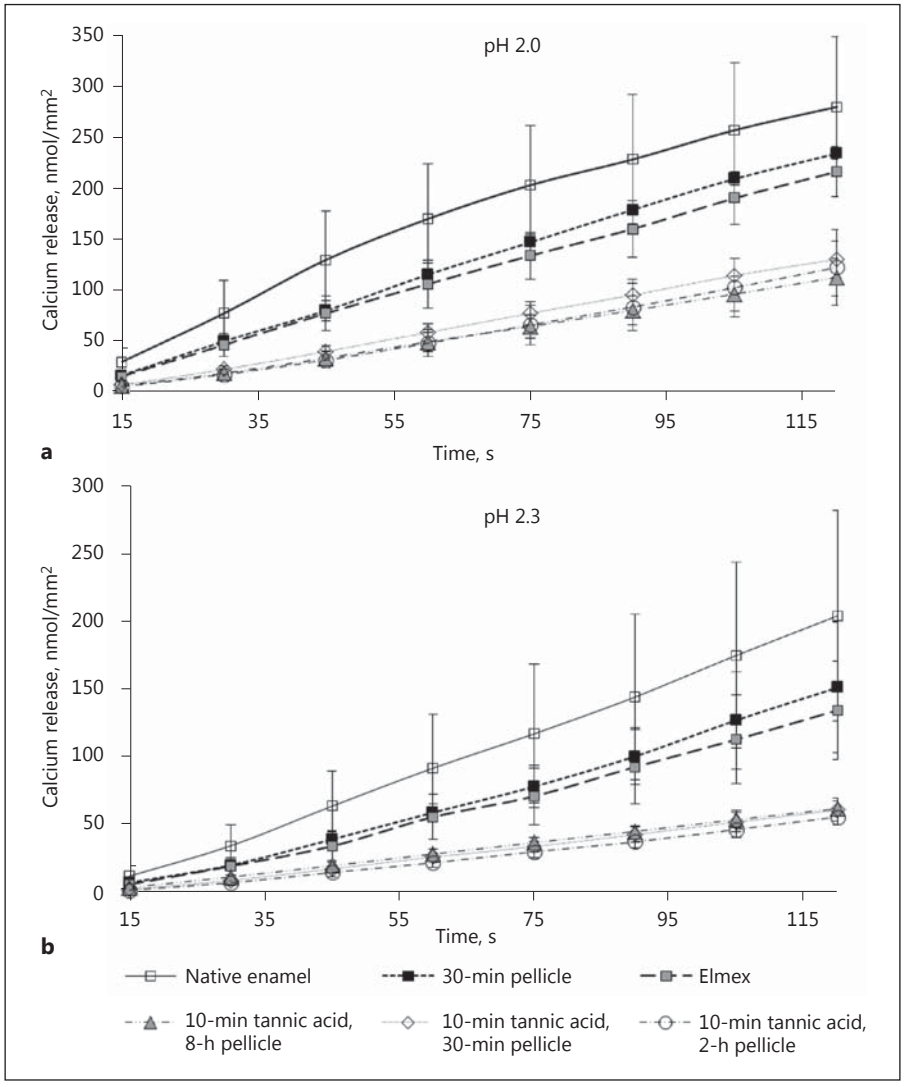


Fig. 4. Kinetics of calcium loss at pH 2.0 (**a**) and 2.3 (**b**) during incubation of enamel slabs for 120 s with and without application of tannic acid in situ. Samples with a physiological 30-min pellicle, samples after 1-min mouth rinse with Elmex Kariesschutz and 28-min intraoral exposure, as well as native enamel served as controls. Note the different scales. n = 6 subjects, n = 12 enamel samples per subgroup (2 samples from each subject).

Results

Calcium and Phosphate Release

In general, all specimens showed a linear kinetics of calcium and phosphate release dependent on the pH values (2.0, 2.3, 3.0) as depicted exemplarily for pH 3.0 in Figures 2 and 3. Kinetics for calcium and phosphate release at pH 2.0 and 2.3 are presented in Figures 4 and 5 (see also the online suppl. Appendix; for all online suppl. material, see www.karger.com/doi/10.1159/000451036). However, the physiological 30-min pellicle without any rinse reduced mineral loss significantly compared with native enamel. As expected, rinses with fluoride mouth rinse (Elmex Kariesschutz) enhanced the protective properties of the pellicle. A significant reduction of calcium and phosphate loss was recorded after rinses with tannic acid, already after 30 min of pellicle formation time (Fig. 2, 3). The protective effect of tannic acid was even better compared with the gold standard (Elmex Kariesschutz). Statistical evaluation revealed a significant impact of tannic acid rinses on the cumulative mineral loss over 120 s (t test, $p < 0.01$) (Tables 1, 2; online suppl. Appendix).

Transmission Electron Microscopy

In order to evaluate the impact of tannic acid on the pellicle's ultrastructure, TEM imaging was carried out. Representative micrographs are shown in Figure 6. As expected, the physiological 30-min as well as 2-h pellicles are characterized by an electron-dense basal layer covered by fine granular structures (Fig. 6a, b). After 10-min rinses with tannic acid the pellicle was of higher electron density

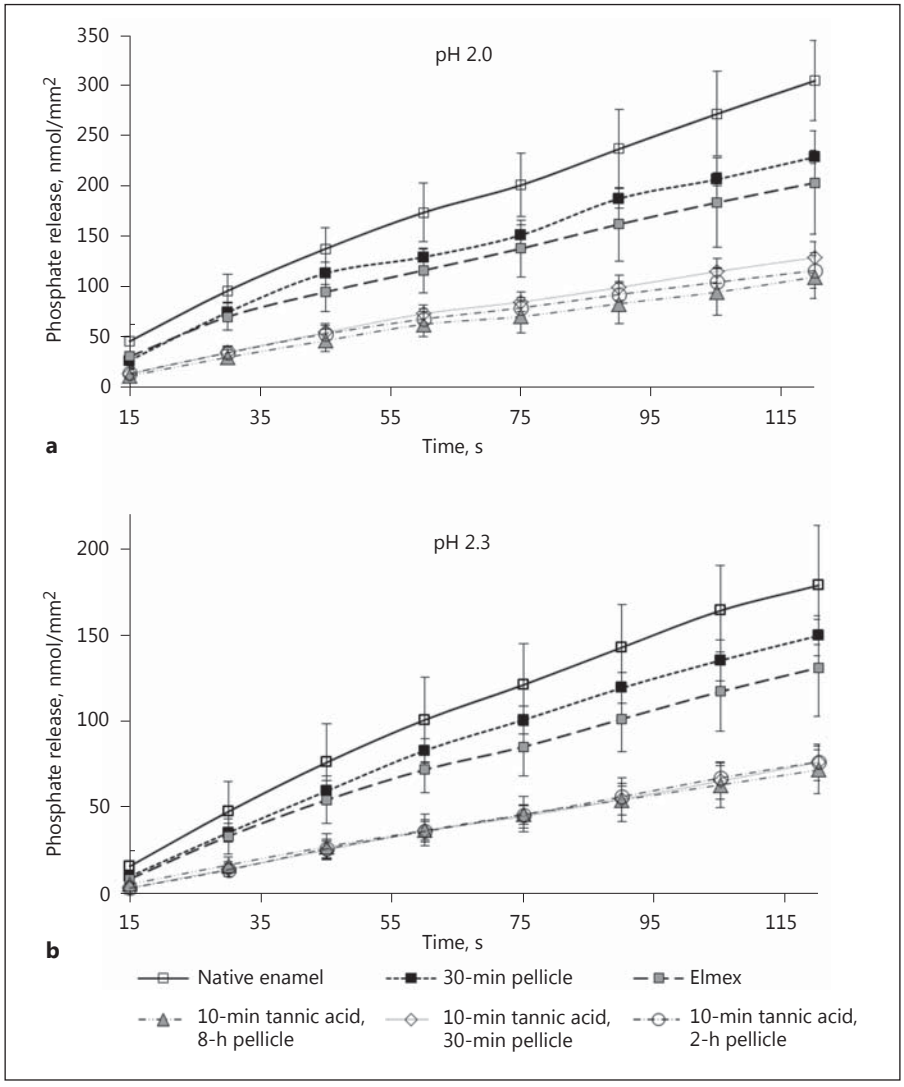


Fig. 5. Kinetics of phosphate loss at pH 2.0 (**a**) and 2.3 (**b**) during incubation of enamel slabs for 120 s with and without application of tannic acid in situ. Samples with a physiological 30-min pellicle, samples after 1-min mouth rinse with Elmex Kariesschutz and 28-min intraoral exposure, as well as native enamel served as controls. Note the different scales. $n = 6$ subjects, $n = 12$ enamel samples per subgroup (2 samples from each subject).

already after 30 min, and even more distinctly after 2 h of pellicle formation time (Fig. 6d, e). After 8 h of biofilm formation there was still an impact of the 10-min tannic acid rinse compared with the 8-h control (Fig. 6c, f).

Fluorescence Microscopic Assays

DAPI Combined with Concanavalin A Staining

The total amount of bacterial adherence and glucan formation was visualized together by simultaneous DAPI and concanavalin A staining. Rinsing with tannic acid significantly reduced the amount of adherent bacteria on enamel surfaces after 8 h of biofilm formation (Mann-Whitney U test, $p = 0.01$) (Fig. 7, 8). Additionally, glucan formation generally occurred in immediate proximity to the adherent bacteria and was reduced considerably after rinsing with tannic acid (Fig. 8).

BacLight Assay

In situ Experiment

The BacLight viability test confirmed the general reduction of adherent bacteria after rinsing with tannic acid on enamel samples. Furthermore, significant effects of tannic acid for dead bacteria were observed ($p = 0.037$) (Fig. 7). The amount of adherent viable bacteria was not diminished significantly ($p = 0.55$) (Fig. 8).

In vitro Experiment

The in vitro experiment with *S. mutans* indicated antibacterial properties for tannic acid in a dose-dependent manner. The proportion of dead bacteria increased as indicated by the ratio of viable to dead bacteria. Thereby, even stronger effects for pure, 1:2, and 1:5 diluted tannic acids were observed compared with chlorhexidine (Fig. 9).

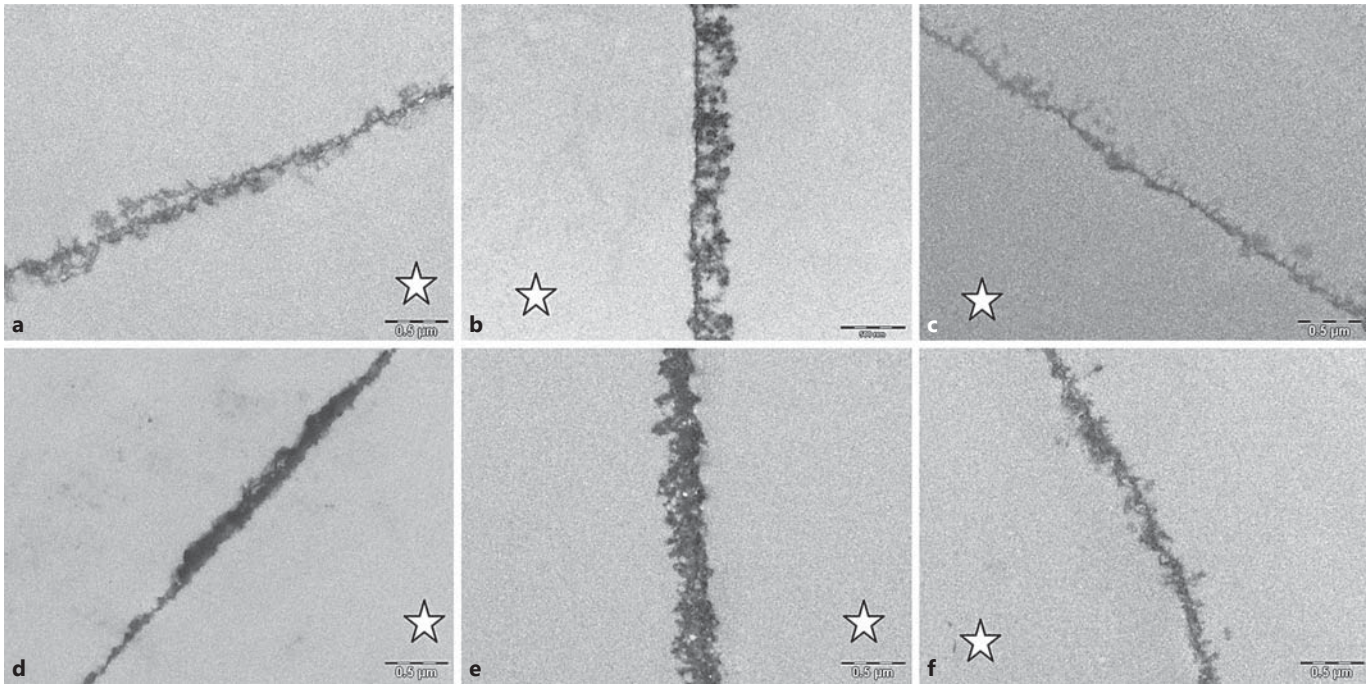


Fig. 6. TEM micrographs of representative physiological 30-min (**a**), 2-h (**b**) and 8-h pellicle samples (**c**) before and after 10-min rinses with tannic acid (**d–f**). Both the 30-min and the 2-h pellicle consist of an electron-dense basal layer covered by fine granular structures (**a**, **b**). Application of tannic acid for 10 min yielded an increase in the pellicle's electron density already after 30 min (**d**) and considerably after 2 h of pellicle formation time (**e**). The impact of tannic acid rinses is still visible after 8 h of pellicle formation (**f**). Original magnification. ×30,000. Note that enamel was removed during the preparation of the samples for TEM. The former enamel site is marked with stars.

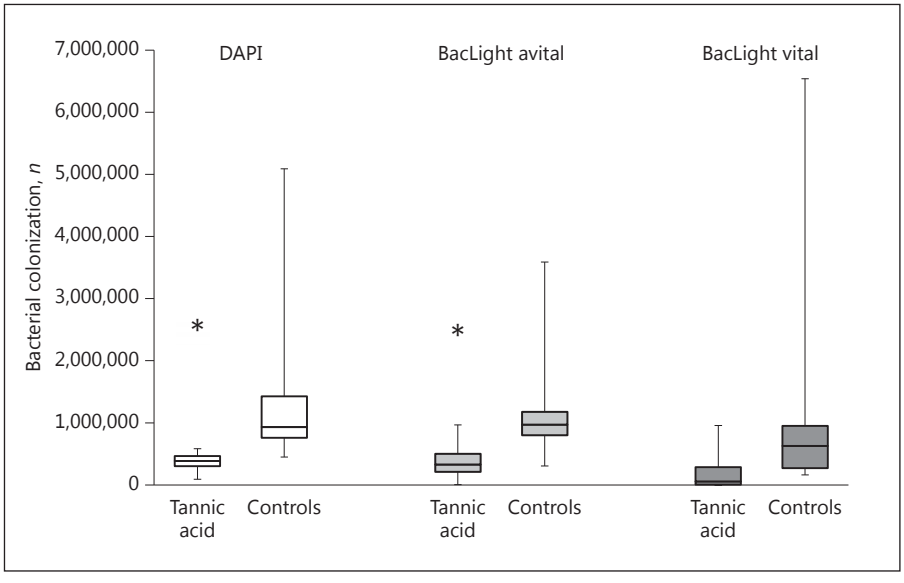
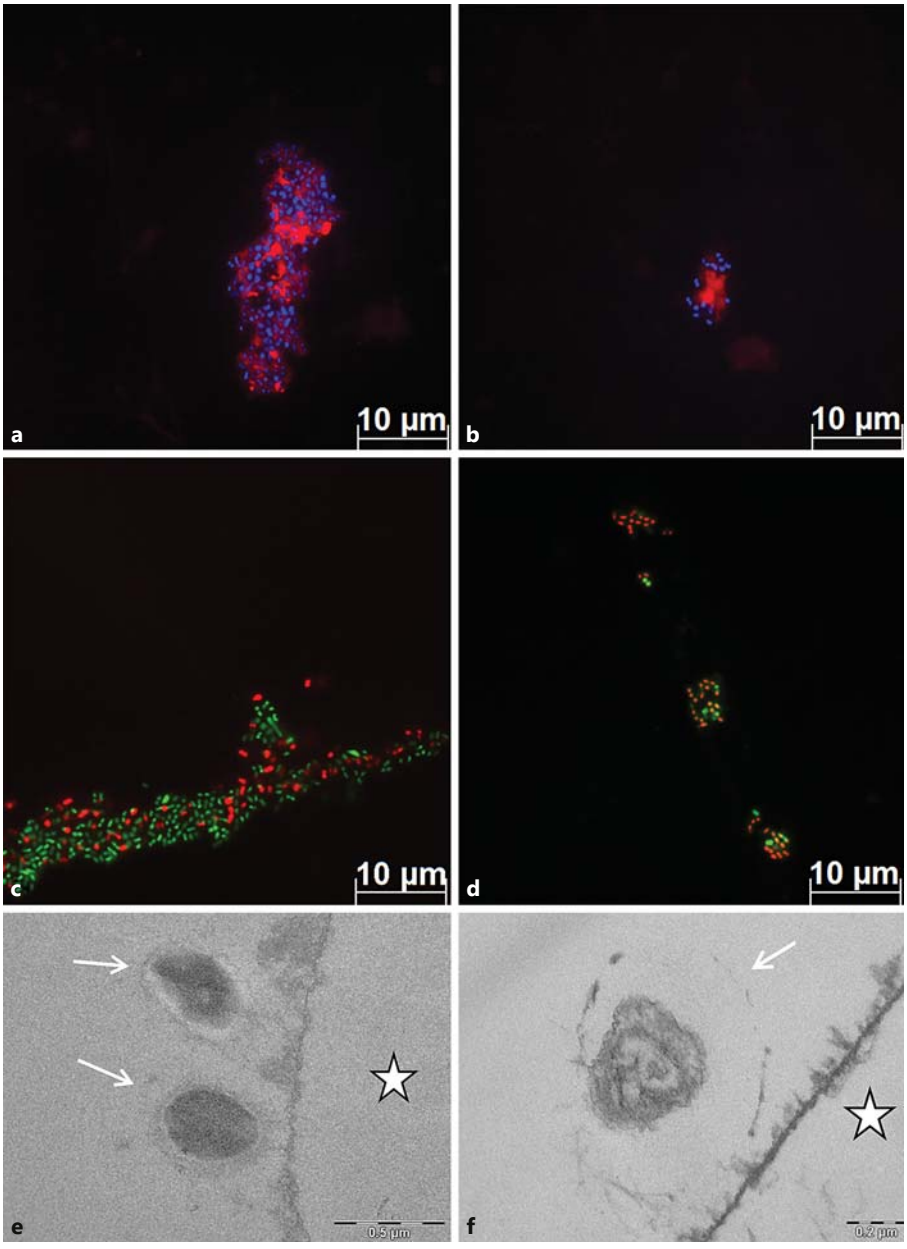


Fig. 7. Impact of tannic acid on bacterial colonization of enamel samples in situ: DAPI staining, BacLight for live/dead staining. After 1-min pellicle formation volunteers (n = 6) rinsed for 1 min with tannic acid and carried the specimens for 8 h overnight in the oral cavity. Enamel samples without any rinse served as controls. Statistically significant differences are marked with an asterisk (Mann-Whitney U test, $p < 0.05$).



Color version available online

Fig. 8. Effect of tannic acid on the initial bacterial colonization of enamel in situ. Representative fluorescence microscopic as well as TEM images of 8-h pellicle (**a**, **c**, **e**) after 1-min rinse with tannic acid and overnight exposure of the enamel samples (**b**, **d**, **f**) are depicted. DAPI and concanavalin A staining enabled the combined visualization of adherent bacteria (blue; color in online version only) and glucans (red). **a**, **b** Glucan formation typically occurred in direct proximity to the adherent bacteria. **c**, **d** The BacLight viability test was applied for differentiation of viable (green) and dead (red) bacteria. Rinses with tannic acid for 1 min significantly reduced bacterial adherence (**b**, **d**) and hence glucan formation (**b**). TEM investigation showed adherent bacteria in the 8-h pellicle (**e**, arrows). Tannic acid application led to lysis of the bacterial cell wall (**f**, arrow). Original magnification. ×49,000. The former enamel site is marked with stars.

Discussion

The present study investigated the impact of tannic acid on the protective properties of the in situ formed pellicle. Thereby, the influences of tannic acid on erosive mineral loss as well as initial bioadhesion were considered. Regarding previous studies on the erosion-preventive potential of natural agents in dentistry, thus far plant extracts or tea decoctions have been adopted [Hannig et al., 2008b, 2012; Weber et al., 2015], but usually not pure substances. To the best of our knowledge tannic acid in pharmaceutical pure quality has been applied for the first time in situ. Since fluorides are known as the gold standard in caries and erosion prevention, they served as positive controls.

The present results indicate clearly that tannic acid has the potential to improve the pellicle's protective properties against both erosive enamel demineralization and

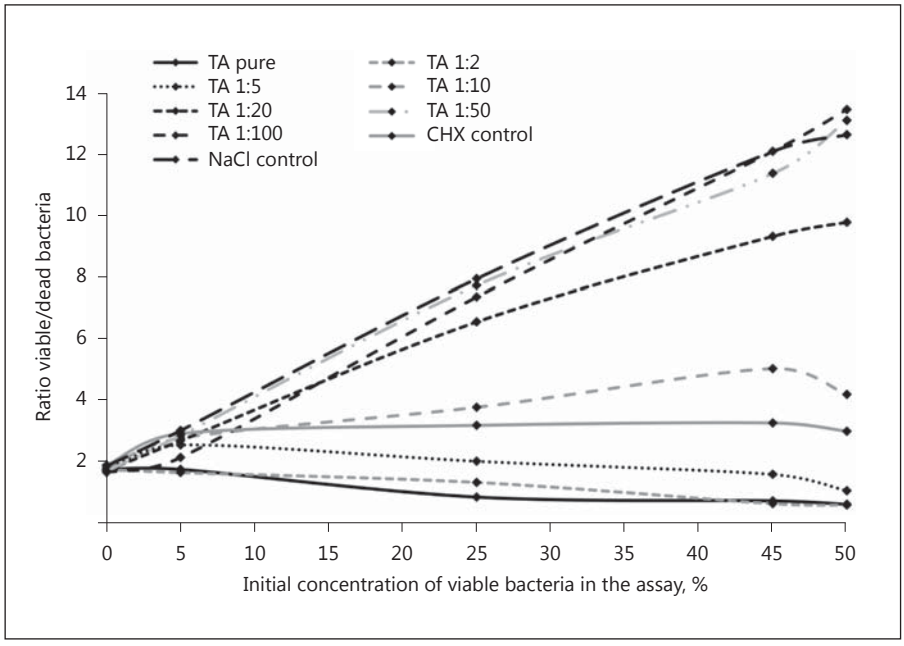


Fig. 9. Effect of tannic acid (TA), chlorhexidine (CHX), and saline solution (NaCl) on the viability of *S. mutans* in vitro. A suspension of *S. mutans* was incubated with tannic acid and chlorhexidine solutions in different concentrations. After incubation, the samples were admixed to proportions of the basic suspension. The amount of vital (emission 530 nm) and dead bacteria (emission 620 nm) was determined by the BacLight bacterial viability assay. The measurements were carried out in duplicate and the ratio was calculated [Hannig et al., 2013a, 2013b].

bacterial attachment. It is worth noting that rinses with tannic acid showed long-lasting effects as the protective impact is not only verifiable in the short-time pellicle but also in the 2-h and the 8-h pellicle. As observed by TEM, the 8-h pellicle was completely thin irrespective of the application of tannic acid. This can be attributed to the fact that the pellicle formation was carried out overnight. It is well known that salivary flow is reduced considerably during sleep. This can lead to diminished pellicle formation.

In general, the naturally formed pellicle serves as a protection layer against acid-related alterations as observed in previous in vitro and in situ investigations [Hannig and Balz, 1999; Hannig et al., 2007a; Hannig and Hannig, 2014; Wiegand et al., 2008] and, last but not least, in the present study. PRPs have even been identified as acid-resistant pellicle components that are increased 2-fold in the in vivo formed pellicle after acid exposure [Delecrode et al., 2015]. It is further known that PRPs are enhanced in the saliva of caries-free subjects compared with caries-susceptible ones, which underlines the protective role of the proteins against acidic dissolution [Vitorino et al., 2006; Delecrode et al., 2015]. Tannins generally have diverse effects on the biological system as they are protein-precipitating agents and biological antioxidants [Kandra et al., 2004]. Lu and Bennick [1998] described the effective precipitation of salivary PRPs by tannins. In view of potential harmful tannin effects in both animal and man it has been proposed that salivary PRPs form complexes with dietary tannins and, hence, prevent their absorption from the intestinal canal [Mehansho et al., 1983, 1987; Lu and Bennick, 1998]. It can therefore be assumed that the PRP-tannic acid aggregates might be incorporated in the pellicle layer and sustain elevated concentrations of calcium by stabilizing the calcium-based salivary layers [Hannig and Hannig, 2014].

The present TEM findings clearly indicate that tannic acid modifies the pellicle's ultrastructure. The increased electron density of the pellicle layer might be due to the facilitated adsorption of the PRP-tannic acid complexes in the pellicle layer, resulting in stronger protective effects against erosion [Joiner et al., 2006; Vukosavljevic et al., 2014].

The adopted in situ/in vitro model allows the investigation of the immediate impact of mouth rinses on the functional pellicle properties during short-time erosive effects. Mimicking the clinical situation while consuming acidic beverages, the dental hard tissue is only exposed to the acidic noxae for a few seconds. The photometric assays for the determination of dissociated calcium and phosphate is characterized by high sensitivity and precision and has already been used previously [Hannig et al., 2008a, 2012; Weber et al., 2015]. Nevertheless, it has to be admitted that the clinical situation is not mirrored completely.

The influence of tannic acid on biofilm formation was evaluated using fluorescence microscopic approaches

such as DAPI combined with concanavalin A and live-dead staining as successfully proven previously [Hannig et al., 2008b, 2010]. Despite the small number of subjects, the present data indicate a clear reduction of adhering bacteria to the pellicle layer due to the 1-min rinse with tannic acid. TEM imaging indicated that tannic acid might destroy the bacterial cell wall integrity, thereby causing lysis of adherent bacteria. Further, the in vitro experiment yielded antibacterial properties of tannic acid against *S. mutans*.

Former studies concerning the influence of tea extracts on oral health described antibacterial effects, especially of polyphenolic compounds in green and oolong tea that were associated with so-called "tea tannins". In fact, however, these effects are attributed to the class of nonhydrolyzable tannins referred to as catechins, but not to tannic acid [Hamilton-Miller, 1995, 2001; Yam et al., 1997]. Akiyama et al. [2001a] examined the antibacterial activity of several tannins on intestinal bacteria. They pointed out that the antibacterial effect of tannic acid is due to its binding efficiency to iron that makes iron unavailable to microorganisms. For microorganisms growing under aerobic conditions iron is essential for a variety of functions [Chung et al., 1998]. In contrast, catechins from tea are known to have anticariogenic properties by damage of the bacterial membrane, inhibition of bacteria-derived glucosyltransferases, and, last but not least, inhibitory effects on salivary α-amylase [Kakiuchi et al., 1986; Ikigai et al., 1993; Zhang and Kashket, 1998]. The specific antibacterial mechanism of tannic acid against oral bacteria such as *S. mutans* is not known yet and needs to be evaluated in further studies.

Widely used as a flavoring additive in soft drinks and juices and, moreover, added in sweets, gums, and baking mixes as an antioxidant and adjuvant, tannic acid is of growing relevance in nutrition, especially for the younger generation. The present findings indicate positive impacts of tannic acid on oral health, but further research is required to substantiate these observations. It is of special interest whether tannic acid might influence pellicle enzymes relevant for caries initiation such as amylase and glucosyltransferase. Furthermore, the effect of tannic acid on dentin erosion needs to be explored. All in all, tannic acid offers a relevant approach in the prevention of dental erosion and bacterial colonization based on modification of the pellicle but requires further research.

Acknowledgment

The study has been partly supported by the German Research Foundation (DFG, SFB 1027).

Author Contributions

Susann Hertel: literature research and writing the paper. Sandra Pötschke: laboratory work, determination of calcium and phosphate release, and statistics. Sabine Basche: laboratory work and fluorescence microscopic analysis. Judith Delius: literature research and laboratory work. Wiebke Hoth-Hannig: performance of TEM work. Matthias Hannig: electron microscopic imaging and interpretation and discussion of the TEM results. Christian Hannig: interpretation of the data and coordination and planning of the research project.

Disclosure Statement

The authors declare no conflicts of interest.

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