

In vitro salivary pellicles from adults and children have different protective effects against erosion

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Abstract

Objectives We aimed at analyzing the protective effects of salivary pellicles, formed with saliva from adults or children, on enamel from permanent or deciduous teeth.

Materials and methods Ninety human enamel specimens (45 permanent premolars and 45 deciduous canines) were ground, and the outer 200 µm of enamel was removed. We divided the teeth into three further subgroups: no salivary pellicle (control), adult salivary pellicle (AP), and child salivary pellicle (CP). We collected stimulated saliva from adults and children and placed 160 µl of either saliva on enamel specimens from AP and CP, respectively. Control specimens received no saliva. Specimens were stored at 37 °C for 2 h and then submitted to an erosive challenge (10 mL; 1 % citric acid; pH 3.6; 25 °C, 1 min). Pellicle formation and erosion was repeated for a total of 4 cycles. After every cycle, relative surface reflection intensity (rSRI) and surface microhardness (rSMH) were calculated.

Results On permanent enamel, AP presented significantly better protective effects, with less rSMH loss ($p < 0.001$) and less rSRI loss ($p < 0.001$). On deciduous enamel, CP presented significantly better protective effects than AP and control ($p < 0.05$), for both measured parameters.

Conclusion We conclude that pellicles from adults and children promote different erosion protective effects, where adult pellicle provides better protection for permanent enamel, and child pellicle promotes better protection on deciduous enamel.

Clinical relevance The present results provide a better understanding toward the protective effect of salivary pellicle against dental erosion and brings light to one more factor involved in the erosion of deciduous teeth.

Keywords Dental erosion · Permanent teeth · Primary teeth · Saliva · Acquired enamel pellicle

Introduction

Initial enamel erosion occurs both at the enamel/acid interface, as well as within a partly demineralized thin softened layer of enamel, in a process called near-surface demineralization [1]. Surface microhardness and the chemical analysis of calcium are the most common methods to measure initial enamel erosion [2, 3], but another recently developed method uses an optical reflectometer [4, 5]. This optical reflectometer can even be used to measure the erosion-inhibiting effects of salivary pellicles [6].

Salivary pellicles begin to form as soon as the enamel comes into contact with saliva. It incorporates mainly salivary proteins (mostly mucins), but it also contains peptides, and, to a lesser extent, enzymes, glycoproteins, carbohydrates, and lipids [7, 8]. The pellicle is composed of two main layers: a densely packed basal layer and a more loosely packed but more complex and heterogeneous globular layer [9]. Initially, peptides and proteins will adsorb onto the enamel surface, thus forming the initial pellicle (basal layer) almost instantly. Subsequent protein-protein interactions allow further adsorption of single proteins or protein agglomerates, leading to maturation and modulation of the salivary pellicle and the formation of its globular layer. The presence of the salivary pellicle is one of the patient-related factors in the protection against enamel erosion [10].

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Different factors can influence, or modulate, the formation of the salivary pellicle, namely the protein content of the individual's saliva and the type of enamel substrate present in the individual's mouth (permanent or deciduous teeth) [11, 12]. On the one hand, protein content in saliva differs with age [13, 14]. Cabras and coworkers observed significantly lower protein concentrations in younger children (aged 6 to 9 years) than in older children and adults [11], and Ben-Aryeh and coworkers found a significant increase in total protein concentration, amylase activity, and salivary IgA with increasing age [12]. These distinctions can lead to different protein contents in the salivary pellicles from children and adults, which, in turn, lead to different protective effects. On the other hand, salivary pellicles formed on deciduous teeth are considerably thinner and have significantly different protein contents than those formed on permanent teeth [8, 15]. These differences will also lead to variations between pellicles formed in adults and children, which, in turn, could lead to different protective effects. So, our study aimed at analyzing the differences in the protective effect of the salivary pellicles formed with saliva from adults or children on permanent and deciduous enamel surfaces. The null hypothesis is that there is no difference in the protective effect of pellicles formed with either adult or child saliva on deciduous or permanent teeth.

Materials and methods

Ethics

The present experiment was carried out in accordance with the approved guidelines and regulations of the local ethical committee (Kantonale Ethikkommission: KEK). Different patients donated teeth and saliva to be used in this study. Stimulated saliva was collected from healthy adults and children, while the teeth were extracted by dental practitioners in Switzerland. Before the donation, the patients (and parents, in case of children) were informed about the use of their teeth or saliva for research purposes. Oral consent was obtained by all patients (and parents) for the use of the teeth or saliva in research. Because we are using saliva and teeth from pooled bio-banks, the local ethics committee categorized these specimens as "irreversibly anonymized," so no previous approval from the committee was necessary.

Enamel specimen selection and preparation

Ninety caries-free human enamel specimens were used in this study: 45 permanent (premolars) and 45 deciduous (canines) human teeth. The patients had been informed about the use of their teeth for research purposes. The crown of each tooth was sectioned in the vertical mesiodistal plane, and the buccal surfaces were used in the experiment. All enamel specimens

had their surfaces covered with a layer of nail polish, and they were later individually embedded in acrylic resin (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). In order to obtain a flat and highly polished enamel surface, the specimens were serially ground (LabPol 21, Struers, Ballerup, Denmark) with water-cooled silicon carbide paper disks (from grit #500 to #4000) and polished with diamond paste under constant cooling. This procedure removed a standardized layer of 200 μm of the outer enamel. The specimens were then divided into three groups (each group containing 15 specimens of permanent and 15 specimens of deciduous teeth): 1. Control group (no pellicle), 2. Adult pellicle, and 3. Child pellicle and stored in a mineral solution (1.5 mmol/l CaCl_2 , 1.0 mmol/l KH_2PO_4 , 50 mmol/l NaCl, pH=7.0) until the time of the experiment [16].

Saliva collection

Stimulated saliva was collected from healthy adults and children, aged between 20–30 and 7–13 years, respectively. Both groups of saliva donors, as well as the children's parents, gave their oral consent to use their saliva in the experiment.

Saliva was collected 2 h after the volunteers' last meal or oral hygiene. The volunteers were asked to chew on a piece of paraffin for 10 min, and the stimulated saliva was collected into chilled vials. The vials were kept in ice throughout the whole saliva collection period so that the properties of the saliva were not altered. Afterwards, the saliva from each group of donors (adults or children) was separately pooled to obtain one pool of adults' and another pool of children's saliva. The two saliva pools were centrifuged for 20 min at 4 $^{\circ}\text{C}$ (4000 $\times g$), and the supernatant was stored in 2.5 ml aliquots at -80°C until the time of experiments.

As described above, our study used pooled enamel and saliva specimens, which is considered as "bio-banking," so we are not able to trace information from individual specimens to their donors.

Experimental procedure

Initially, we measured enamel surface microhardness (SMH) and surface reflection intensity (SRI) on all enamel specimens (permanent and deciduous). Then, salivary pellicles were formed on the enamel specimens using saliva either from adults or from children, according to the pellicle group. For pellicle formation, one aliquot of adult saliva and one aliquot of child saliva were taken from the freezer and thawed at 37 $^{\circ}\text{C}$. Respectively to the group, each enamel specimen received an aliquot of 160 μl of either adult saliva or child saliva, and the enamel specimens were stored in a humid chamber at 37 $^{\circ}\text{C}$ for 2 h. In the control group, no salivary pellicle was formed, but the enamel specimens were also kept in a humid chamber at 37 $^{\circ}\text{C}$ for 2 h.

After pellicle formation, the excess saliva was removed from the enamel surface by rinsing with deionized water (20 s) and then drying them with air (5 s). All specimens were then individually submitted to an erosive challenge, with 10 mL of 1 % citric acid (pH 3.6), at 25 °C, for 1 min, under still condition (not shaking). Afterwards, the specimens were rinsed with deionized water (20 s) and dried with air (5 s). The specimens were then immersed for 30 s in sodium hypochlorite (3 % concentration, 25 °C, shaking) [6], before SMH and SRI analyses (SMH_i and SRI_i). Afterwards, the specimens were again submitted to the experimental cycle, consisting of pellicle formation, erosion, and SMH and SRI measurements. A total of four experimental cycles were carried out.

Enamel surface microhardness

Surface microhardness (SMH) was measured with a Knoop microhardness tester (UHL VMHT Microhardness Tester, UHL technische Mikroskopie GmbH & Co. KG, Aßlar, Germany), using a load of 10 g and dwell time of 10 s. For each SMH measurement, six indentations were made on the enamel surface, at 25 μm intervals, and the mean value from these six indentations was considered as the SMH value for the respective enamel surface. For statistical analyses, relative SMH (rSMH) was calculated using the formula $rSMH = (SMH_i / SMH_0) \times 100$, where SMH_0 is the initial SMH measured at baseline (before any erosive challenge) and SMH_i is the value after the i th erosive challenge ($i = 1, 2, 3, \text{ or } 4$).

Enamel surface reflection intensity measurement

Enamel surface reflection intensity (SRI) measurements were carried out using an optical pen-size reflectometer (OPSR) [17]. The OPSR was connected to a computer running a specific software that registers the point of highest reflection

intensity. This reflection intensity is expressed as a SRI value. As erosion progresses, the enamel surfaces become rougher and SRI values decrease. For statistical analyses, we considered the relative change in SRI (rSRI) after the erosive challenges. In practical terms, higher rSRI values mean less change in surface reflection and hence less enamel erosion. The rSRI values were calculated using the formula $rSRI = (SRI_i / SRI_0 \times 100)$, where SRI_0 is the initial SRI measured at baseline (before any erosive challenge) and SRI_i is the value after the i th erosive challenge ($i = 1, 2, 3, \text{ or } 4$).

Statistical analyses

First of all, a global non-parametric ANOVA for longitudinal data [18] was applied to assess whether the groups had a global impact on any of the outcomes (rSMH, rSRI), as well as interactions with factor time (sequential erosive challenges). Experiment-wise probability for significance (α) was set at 0.05, and multiple testing was corrected by the method of Holm.

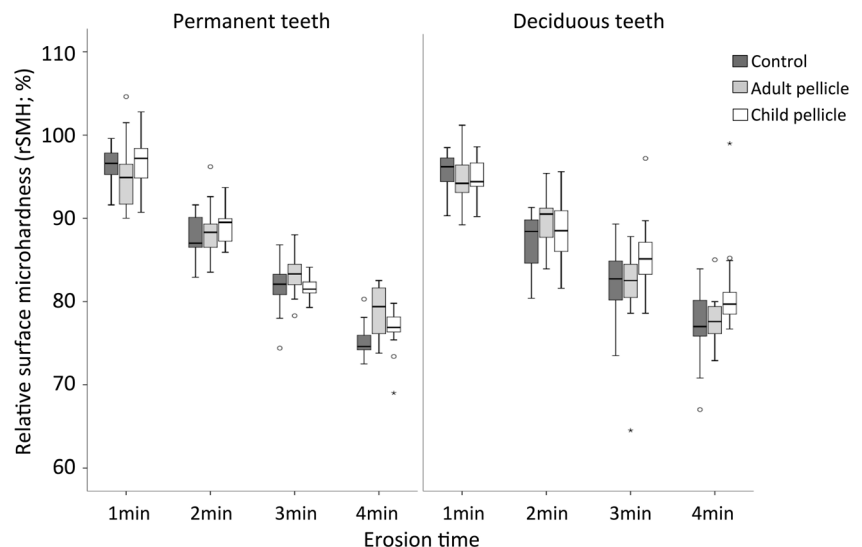
If the global test showed significant values, Wilcoxon rank-sum post hoc tests were performed to assess differences of group medians for each outcome variable after each time point (erosive challenge).

The statistical analyses were carried out first on permanent teeth and then on deciduous teeth. We also later compared the differences between the two types of enamel.

Results

In regards to the permanent enamel, all groups presented significant decrease in rSMH ($p < 0.001$) as the experiment progressed (Fig. 1). Adult pellicle presented the best protection for permanent enamel against erosion, with significantly

Fig. 1 Enamel relative surface microhardness (rSMH) for the different pellicle groups (no pellicle control, adult pellicle, or child pellicle), after different erosion times, according to the type of enamel (permanent or deciduous teeth)



less rSMH loss ($p < 0.001$) than the other groups. At the end of the experiment, the adult pellicle group presented significantly smaller rSMH loss in comparison to the child pellicle group ($p = 0.040$) or the control group ($p = 0.010$); and no significant difference was observed between the latter two groups ($p = 0.250$). Relative surface reflection intensity (Fig. 2) also significantly decreased in all groups ($p < 0.001$). In terms of rSRI, adult pellicle also showed a significant protection for permanent enamel against erosion during the first and second cycles, with lower rSRI decrease than the child pellicle group ($p < 0.001$) or the control group ($p < 0.010$). However, at the end of the experiment, no differences were observed between adult and child pellicles ($p = 0.770$), despite both groups having better protection than the control group ($p < 0.001$).

Regarding the deciduous teeth, all groups presented a significant decrease in rSMH ($p < 0.05$) and rSRI ($p < 0.001$). At the end of the experiment, the child pellicle group presented significantly better erosion protection for deciduous enamel than adult pellicle group ($p = 0.021$), but there was no difference between the latter and the control group ($p = 0.917$). The protective effect of the pellicles was more clearly observed in the rSRI results, where already in the second cycle, the pellicles presented significantly better protection for deciduous enamel against erosion compared to the control group ($p = 0.026$ and $p = 0.001$, for adult pellicle and child pellicle, respectively). The child pellicle, however, presented the best protection until the end of the experiment ($p < 0.050$), when compared to the control and adult pellicle groups.

Comparing permanent and deciduous enamel, we observed that child pellicle promoted significantly better protection on deciduous teeth than on permanent teeth ($p > 0.010$, in both rSMH and rSRI). Likewise, the rSRI results show that adult pellicle promoted significantly better protection on permanent

teeth ($p < 0.001$). In addition, considering the control group (no pellicle), deciduous teeth presented greater change in rSMH ($p < 0.010$) and rSRI ($p < 0.010$) than permanent teeth.

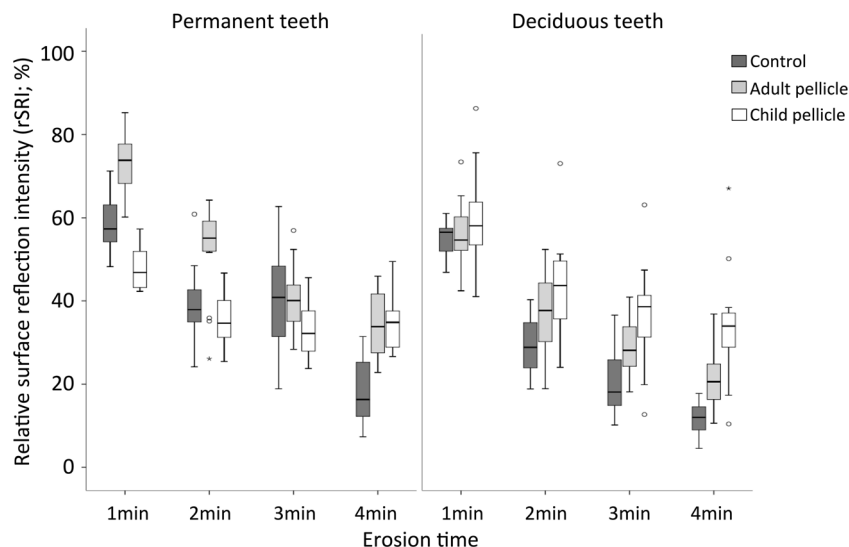
Since there was a significant difference in the protective effect of pellicles formed either with saliva from adults or from children on both deciduous or permanent teeth, the null hypothesis was rejected.

Discussion

The protective effect of the salivary pellicles against dental erosion has long been studied [9, 19, 20], but specific erosion-protective effects of salivary pellicles from children and adults are not yet fully understood. The differences in salivary protein contents between adult saliva and child saliva [11, 12] can lead to different salivary pellicles, which, in turn, could influence the protective effect of these pellicles against dental erosion. With the present results, we were able to show that both adult pellicle and child pellicle promote different protective effects on the different types of enamel, so we could establish that the protective effect of saliva is dependent not only on the type of salivary pellicle itself but also on the type of enamel substrate.

The enamel in deciduous and permanent teeth have different crystal arrangement, mineral composition, and organic contents (for a review see Carvalho, Lussi, Jaeggi, and Gambon [21]). So, deciduous enamel will have different salivary protein adsorption and, consequently, different pellicles. This was demonstrated by Sønju Clasen, Hannig, Skjørland, and Sønju [15], who collected in situ pellicle from children with mixed dentition and showed that the pellicle collected from deciduous enamel was thinner and presented a slower protein adsorption process than the pellicle collected from

Fig. 2 Enamel relative surface reflection intensity (rSRI) for the different pellicle groups (no pellicle control, adult pellicle, or child pellicle), after different erosion times, according to the type of enamel (permanent or deciduous teeth)



permanent enamel. Zimmerman, Custodio, Hatibovic-Kofman, Lee, Xiao, and Siqueira [8] also showed that pellicles formed on deciduous and permanent enamel only had 42 % of the proteins in common. Because salivary pellicles will form differently on different enamel substrates, we used both types of saliva with both types of enamel. For methodological reasons, we used deciduous and permanent teeth together with both types of saliva. This allowed us to draw a full comparison regarding both types of saliva on the different enamel substrates. Moreover, children from 7 to 13 years have mixed dentitions, so it is important to analyze the erosion-protective effect of child pellicle on both permanent and deciduous teeth.

In the present study, we used surface microhardness (SMH) and surface reflection intensity (SRI) to assess the erosion process. Both methods are simple and reliable for accurate measurements of initial enamel erosion [2, 4–6, 17]. SRI is particularly sensitive to measure initial enamel erosion, and previous studies have shown that it highly correlates with calcium release and surface hardness [4], but this method is also highly influenced by the salivary pellicle. Lussi et al. [5] argued that salivary proteins from the pellicle can fill up the spaces between the etched crystals of the eroded enamel, thus resulting in an overall “smoother” surface that masks the analyses of the reflectometer. For this reason, we used NaOCl after each cycle to clean the enamel samples before each SRI and SMH measurement. This procedure reduces this artifact of the pellicle [6], allowing more reliable reflectivity results.

Interestingly, our results showed that adult saliva presented superior protective effects on permanent enamel and that child saliva presented superior protective effects on deciduous enamel. Considering the rSMH results, these differences are mostly detected after 4 min erosion, and the differences are much more clearly observed with the SRI measurements. Rakhmatullina et al. [4] had already discussed that the SRI method is more sensitive than SMH for initial erosion experiments. Here, we observed a considerably lower rSRI decrease on permanent enamel covered with adult pellicle, suggesting better protection than the other groups, whereas on deciduous enamel, child pellicle showed a slower rate in rSRI decrease, presenting a significantly better erosion-protective effect. Moreover, considering the control groups (where no pellicle was used), we observed a greater change in rSMH and rSRI in deciduous enamel than in permanent enamel, suggesting that, under the present experimental circumstances, deciduous teeth had a slightly greater susceptibility to initial erosion than permanent teeth. Since the pellicle was able to significantly protect both kinds of teeth from erosive dissolution, the presence of the enamel pellicle is a major protecting factor against dental erosion.

The differences in the protective effects of adult pellicle and child pellicle are either due to calcium content or to protein content in the saliva/pellicle. On the one hand, the calcium

content in saliva and pellicle can reduce erosive demineralization of the enamel [22]. Calcium concentration in adult saliva is significantly higher than in saliva from children [23]. This higher calcium concentration is also mirrored on the respective pellicles (unpublished results). However, despite the higher calcium content, our results show that adult pellicle was not as effective in preventing erosion on deciduous teeth when compared to child pellicle. This shows that different calcium concentrations in saliva, or in salivary pellicles, play a very limited, minor role in enamel erosion. However, the different protein content in saliva from adults and children may better explain the differences in the protective effect observed in our study. Although pellicle formed on deciduous teeth is thinner and has a slower protein adsorption process than pellicle formed on permanent teeth [15], Hannig and Hannig [9] observed that there are contradicting results on reports regarding the pellicle thickness and its acid-protective properties. The authors also stated that during an acid attack, the pellicle is dissolved from its outer layers toward its more densely arranged basal layer, and the latter has a higher stability against acid dissolution [9]. So, despite any difference in pellicle thickness or calcium content, child pellicle formed on deciduous teeth could have different protein content and, in turn, have different erosion protection properties, as observed in this study. Our results are, therefore, in accordance to what we have previously suggested [21], that inorganic factors in saliva, like calcium concentration, play only a minor role in erosive wear in children; whereas, the organic factors in saliva (protein adsorption onto enamel) have a more noticeable protective effect on deciduous enamel. Furthermore, future proteomic studies are still necessary to systematically analyze pellicles from both kinds of saliva, on both kinds of teeth. Previous studies have analyzed pellicles from children’s saliva on deciduous teeth [8] and pellicles from adults’ saliva on permanent teeth [24, 25]. A systematic study with child and adult saliva, on deciduous and permanent teeth, would allow the identification of the protein groups most abundant in the different scenarios and which are most likely influencing erosion protection on permanent and deciduous teeth.

It is also important to bear in mind that our experiment was made using ground and polished enamel surfaces. This grinding procedure provides a more homogeneous measurement area for comparisons between the groups [26], but on the other hand, it removes the outer native enamel layer. Enamel mineral content [27, 28], susceptibility to dissolution [29], and surface charge will differ according to enamel depth. Also, Hannig and Hannig [9] stated that the pellicle is formed due to van der Waals forces, hydrophobic interactions, as well as electrostatic interactions between calcium and phosphate ions on the surface of the tooth, and the charged groups of the protein macromolecules. So, different surface charges [30], and different mineral constitution of the enamel, could affect

the protein adsorption. Furthermore, deciduous and permanent teeth have different mineral contents [31], and in our study, we observed a significant difference ($p=0.048$) in the initial surface hardness between deciduous (348.06 ± 17.97 KHN) and permanent (372.62 ± 11.69) enamel. This suggests that the different mineral constitution between permanent and deciduous teeth remained even after the grinding procedures and removal of 200 μm of enamel. These different mineral compositions consequently led to different protein adsorption and different pellicles, but further studies are still necessary to verify differences in pellicle formation between native and ground enamel.

To conclude, the present experiment shows that both adult and child pellicles promote different protective effects against erosion, depending on the type of teeth. Adult pellicle provided better protection for permanent enamel, and child pellicle promoted better protection for deciduous enamel.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article did not require the approval of an ethical committee, as detailed in the “Materials and methods” section.

Informed consent For this type of study, formal written consent is not required.

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