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Differences in the natural enamel surface and acquired enamel pellicle following exposure to citric or hydrochloric acid

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Differences observed between weak and strong acids on the acquire enamel pellicle

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Abstract

Objectives

The aim of this study was to investigate variations in the interaction between enamel, the acquired enamel pellicle (AEP) and citric or hydrochloric acid.

Materials and Methods

A 24 hour AEP was formed on natural enamel specimens (*n* = 40) from pooled whole mouth human saliva. Samples were randomly allocated to citric (0.3%, pH 3.2) or hydrochloric (HCl) acid (0.01M, pH 2.38) exposure for 30 or 300 seconds. The total protein (TPC), phosphorous and calcium concentrations of the pellicle were determined before and after acid exposure, and again after re-immersion in saliva. Surface roughness and TSM imaging were used to assess enamel changes.

Results

After 300 seconds of citric acid exposure, the mean ± SD TPC reduced from 5.1 ± 1.1 to 3.5 ± 1.1 mg/mL ($p < 0.05$). In contrast, after 300 seconds of HCl exposure, the mean TPC did not reduce significantly from baseline (6.6 ± 1.1 to 5.7 ± 0.7 mg/mL) but was significantly reduced in the reformed pellicle to 4.9 ± 1.2 mg/mL ($p < 0.001$). This reduction occurred after significant release of calcium and phosphorous from the enamel surface ($p<0.001$). Thirty seconds of exposure to either acid had no detected effect on the AEP. The surface roughness of the enamel decreased after acid exposure but no differences between groups was observed.

Conclusions

These findings indicate that citric acid interacted with proteins in the AEP upon contact, offering enamel protection. In contrast HCl appeared to bypass the pellicle and reduced protein was observed only after changes in the enamel chemical composition.

Introduction

The role of saliva and the salivary pellicle in the protection against enamel erosion has yet to be fully explained. On a clinical level, the effect of individual salivary parameters, such as salivary flow rates or saliva buffering capacity, have been unconvincing. Some studies have reported increased erosive tooth wear in those with low salivary flow rates and low saliva buffering capacity [Dugmore, and Rock, 2003; Dynesen et al., 2008; Moazzez et al., 2004], others have not [Moazzez et al., 2000; Lussi, 2012]. The relationship becomes more complex when one considers the influence of the salivary pellicle. Amaechi et al. 2009 observed that a pellicle formed *in situ* on polished enamel samples for 1 hour provided protection against 5 minutes of orange juice exposure 6 times daily for 4 days, a total exposure period of 2 hours, when specimens were placed in artificial saliva between exposures [Amaechi et al., 1999]. However, Hara et al 2006 investigated the protective effect of a 2 hour *in situ* formed pellicle on enamel or dentine before subjecting specimens to a continuous 10, 20 or 30 minute acid challenges [Hara et al., 2006]. They observed a limited protective effect, only on enamel, which was not observed after greater than 10 minutes of an erosive challenge.

The variation in protection may be in part explained by individual variation in the composition of the salivary pellicle [Moazzez et al., 2014; Carpenter et al., 2014]. Carpenter et al. performed an *in-situ* case-control study on 30 patients with dietary erosive tooth wear compared to 30 matched healthy controls. The pellicle was collected from the surface of polished human enamel surfaces held on a splint worn in the mouth and also collected from the patients natural enamel surfaces [Carpenter et al., 2014]. There were no differences in the pellicle between groups from the natural enamel surfaces. However, on the polished enamel there was reduced protein content in the pellicle in those with dietary erosive tooth wear [Carpenter et al., 2014]. The majority of laboratory and *in-situ* experiments to date have been on polished enamel. Evidence suggests that natural enamel surfaces behave differently to polished surfaces [Ganss et al., 2000; Mullan et al., 2018b]. In a recent *in vivo* paper examining natural eroded vs uneroded enamel surfaces on the same subjects with dietary erosive tooth wear [Mutahar et al., 2017b], reduced total protein concentration and reduced calcium regulating statherin in the pellicle of eroded surfaces were observed when compared to the uneroded surfaces.

Another explanatory factor in the variation observed in clinical studies may be the source of the acid exposure. Most laboratory studies have used citric acid to represent dietary acids and hydrochloric acid (HCl) for gastric causes of erosive tooth wear. Laboratory studies have shown that saliva can play a

greater protective role against citric acid than HCl [Wiegand et al., 2008]. Total protein concentrations have been observed to remain high after acid exposure suggesting that the HCl penetrates through the pellicle [Taira et al., 2018]. Interestingly, statherin has been observed to remain stable after HCl exposure which is different to the *in vivo* studies testing dietary acids [Taira et al., 2018]. This has important implications when researching preventive mechanisms as it is unknown if salivary pellicle composition influences erosive tooth wear or eroded tooth surfaces influence the salivary pellicle composition.

The aim of this experiment was to investigate the variations in total protein concentration of the acquired enamel pellicle and subsequent changes in the natural enamel surface after exposure to an intrinsic acid source (HCl) or an extrinsic acid source (Citric Acid). The null hypothesis proposes that the pellicle composition after acid exposure will not be different between the two acids.

Methods

Enamel specimen preparation

Extracted caries-free human molars (*n* = 40) were collected with informed written consent (REC Ref 12/LO/1836) and sterilized in 1% sodium hypochlorite (Milton's solution, Proctor & Gamble, USA). Buccal enamel surfaces were sectioned using a water-cooled diamond wafering blade (XL 12205, Benetec Ltd., London, UK) and mounted in bis-acrylic composite (Protemp™, 3M, USA), leaving the natural curved buccal surface exposed. Samples were brushed using a manual toothbrush and placed in an ultrasonic bath (GP-70; Nusonics, Lakewood, US) for 15 minutes before finally being wiped with ethanol to remove the smear layer and debris.

Saliva and pellicle collection procedures

Whole mouth human saliva, stimulated by chewing on flavourless paraffin wax for 10 minutes, was collected from three healthy volunteers (REC Ref: 14/ EM/0183). Volunteers were told to abstain from food and drinks for at least two hours prior to collection and samples were immediately frozen at -80°C after collection. Saliva was defrosted on ice overnight prior to pellicle preparation. Thawed whole mouth saliva (WMS) was vortexed, pooled and centrifuged (4°C, 2000 RPM, 10 minutes). The *ex vivo* acquired enamel pellicle (AEP) was created by fully immersing enamel specimens in WMS (8ml per specimen) for 24 hours at room temperature. To collect the AEP, the enamel samples were rinsed for one minute in deionised water and the free saliva was removed from the enamel surface using a single sized Whatman filter paper (21 x 3 mm) by a single investigator at the same location for each surface: 3 mm at the most bulbous point of the buccal surface (Sigma Aldrich, UK). Paper sialostrips (Oraflow Inc. US) were soaked in 3 µL SDS (0.5% w/v) and mechanically rubbed against the enamel surfaces for 15 seconds to collect the AEP as described in previously published protocols [Svendsen et al., 2008; Carpenter et al., 2014]. Following pellicle collection, 15 µL of SDS (0.5% w/v) was added to each Sialostrip and placed into punctuated 0.5 mL microtube placed inside a 1.5 mL microtube. Centrifugation (8 minutes at 8000 rpm) in a table-top centrifuge (Thermo Fisher Scientific, Leicestershire, UK) was carried out and the elution collected in the 1.5 mL microtube was stored at -20 °C.

Assessing the repeatability of baseline pellicle formation procedure

Due to natural high variation in both enamel properties and pellicle properties between samples, the repeatability of pellicle composition on the same surface was established. Prior to acid exposure, the AEP was formed on 10 samples as described above. The pellicle was then collected from each of these samples for analysis. The same samples were then re-immersed in the same saliva for 2 hours to reform the pellicle before repeating the collection procedure.

Experimental procedure

Enamel specimens were immersed in natural saliva for 24 hours prior to collection of the enamel pellicle (baseline pellicle). Samples were rinsed in deionised water for one minute which was followed by re-immersion in saliva for a period of 2 hours to replenish the pellicle which may have been removed after collection. This replenished pellicle was formed by immersing the samples in the same solution of natural saliva for 2 hours at room temperature. The enamel samples were then randomly allocated into two groups; citric acid and HCl. Within each group, the enamel specimens were further divided into two groups for immersion in pH 3.2 0.3% citric acid or pH 2.38 0.01M HCl for 30 or 300 seconds (n=10). Immediately following the acid exposure, the samples were rinsed in 100ml deionised water for 1 minute and the pellicle was collected according to protocol (acid exposed pellicle). The enamel samples were then re-immersed in saliva for a further 24 hours followed by collection of the enamel pellicle (Reformed pellicle).

Measurements

Total protein concentration (TPC) was calculated using spectrophotometry (Nanodrop, Thermo Fischer) reading at 280 nm. Five out of the ten teeth in each study group were randomly selected for ICP-MS analysis. For each tooth, one µL of eluted enamel pellicle was diluted in 1% trace metal grade nitric acid (FischerScientific) in UHQH_2O for a final volume of 2 mL. Phosphorous and calcium concentration was determined by inductively coupled plasma–mass spectrometry (ICP-MS) using a NexION 350D mass spectrometer (PerkinElmer) with Cetac AS520 autosampler (ThermoFisher) & running Syngistix software version 1.0 (PerkinElmer). The main settings were: main & auxiliary argon flows set at 18 & 1.2 L/min; nebuliser argon flow set at 0.92 – 0.98 L/min, optimised daily; RF power: 1600W.The following were set as the acquisition parameters: He collision gas at 4.2 ml/min; 100 ms dwell time for

each element; elements measured 50 times per replicate. Element concentrations were determined by standard additions that were prepared by serial dilutions starting at 0.1 mg/L.

Baseline surface roughness (Sa) was assessed before addition of the pellicle using a red light non-contact laser confocal profilometer (Xyris 4000TL, Taciaan, Southamptom UK). A 4 μm lateral scanning interval was used to scan five randomly selected 0.04 mm^2 areas from the centre of the enamel specimen as previously described in published protocols [Mullan et al., 2018b]. A 25μm Gaussian filter was applied, and the mean Sa roughness of each enamel specimen was calculated (MountainsMap, Digital Surf, Besançon, Frances). Following the experimental cycle, the pellicle would have been removed with the SDS and samples left to dry overnight before repeat profilometry was performed.

Tandem Scanning Confocal Microscopy (TSM) (Noran Instruments, Middleton USA) was used to qualitatively analyse the enamel surface. Two randomly selected enamel specimens were selected from each group to be imaged with an M-plan 40x SLWD (Brightfield Objective x 40/0m35 NA objective). A digitally mounted camera (Andor iXon 885, Andor Technology Ltd, Belfast, UK) and a 550 nm green filter was used with iAndor software for image capture.

Statistical analysis

The TPC, calcium and phosphate concentrations of the AEP, and enamel surface roughness data were tested for normality using the Shapiro-Wilks normality test and subsequently assessed using a 3-way ANOVA followed by Bonferroni corrected multiple comparisons. Enamel surface roughness changes were assessed using a two-way ANOVA followed by Bonferroni corrected multiple comparisons. Statistical analyses were carried out on Prism 6 software (GraphPad). The following were used to denote statistically significant differences in the figures: **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$.

Results

Repeatability of total protein concentration on baseline pellicle formation on the same natural surfaces.

The mean ± SD AEP TPC of 10 enamel samples after a 24-hour pellicle formation was 5.5 mg/ml ± 1.16). Following removal of the pellicle and reformation after a 2-hour period the mean ± SD TPC was 4.99 mg/ml ± 1.08. This difference was not statistically significant ($p = 0.104$).

The effects of acid exposure on the total protein concentration of the AEP

The mean TPC in the AEP at the different time points are presented in Figure 1. After 300 seconds of citric acid exposure, the mean ± SD TPC reduced from 5.1 ± 1.1 mg/mL to 3.5 ± 1.1 mg/mL ($p < 0.05$). This was followed by a slight increase to 4.0 ± 0.5 mg/mL in the reformed pellicle. There was no statistical difference between the TPC in the baseline pellicle and the reformed pellicle after 300 seconds of citric acid exposure. In contrast, after 300 seconds of HCl exposure, the mean ± SEM TPC in the pellicle reduced non-significantly from the baseline pellicle from 6.6 ± 1.1 mg/mL to 5.7 ±0.7 mg/mL but was followed by a significant reduction to 4.9 ±1.2 mg/mL in the reformed pellicle after acid exposure ($p < 0.001$) (Figure 1). No changes in the mean TPC of the AEP were observed after 30 seconds of citric acid or HCl exposure either after acid exposure or in the reformed pellicle. There were significant differences in the baseline mean TPC between the four groups. Baseline TPCs significantly varied from 4.2 ± 1.1 mg/mL in the 30 second HCl group to 6.6 ± 1.1 mm/mL to the 300 second HCl group.

The effects of acid exposure on calcium and phosphorous concentrations in the AEP

The mean calcium concentration in the AEP at the different time points are presented in Figure 2. The AEP Calcium concentration increased significantly from the baseline AEP to 6.3 mM after a 300 second exposure to HCL, significantly higher than the calcium concentration of the AEP after a 300 second citric acid exposure. A similar observation was made regarding the phosphorous concentration in the AEP (Figure 3). The mean phosphorous concentration after a 300 second exposure to HCl was 3.2 mM, also significantly higher than the 1.8 mM observed after a 300 second citric acid exposure. The calcium and phosphorous concentrations after exposure to 30 seconds of HCl and 300 seconds of citric

acid were greater than baseline levels but lacked statistical significance. Furthermore, the calcium and phosphorous concentration in the reformed pellicle samples in the 30 and 300 seconds of HCl rinsing groups were raised from baseline levels, but these also lack statistical significance. Unlike baseline TPC, the baseline calcium and phosphorus concentrations were comparable across the groups.

The effect of acid exposure on the enamel surface: Surface roughness and TSM imaging

The mean Sa roughness pre- and post-acid exposure is shown in Figure 4. There was a significant decrease in Sa roughness of each enamel surface after all acid exposures overall ($p = 0.02$). However, there were no significant differences between acid types or the times of exposure.

The TSM images are reported on in Figure 5. Visually, minimal dissolution of the enamel prisms was observed when the enamel samples were exposed to citric acid for 30 seconds in the presence of saliva (Figure 5a-b). As the exposure time increased, some prisms underwent demineralisation, but intact prisms and natural surfaces were seen after 300 seconds citric acid exposure (Figure 5c-d). In contrast, exposure to HCl resulted in visual changes after 30 seconds (Figure 5e-f). After 300 seconds of HCl exposure evidence of inter-prismatic enamel destruction was observed (Figure 5g-h).

Discussion

The data presented in this study demonstrates that the different acids impacted the pellicle at disparate stages therefore the null hypothesis is rejected. After 300 seconds of HCl exposure, the TPC did not decrease after acid exposure (Figure 1). However, following acid mediated calcium/phosphate release from the surface, the TPC in the reformed pellicle decreased. This indicates that for HCl acid, damage to the enamel surface precedes changes in the salivary pellicle. Increases in calcium and phosphorous concentrations were also found in the post-acid exposure pellicle in the 300 second HCL group (Figure 2 & Figure 3), suggesting that damage caused to the enamel surface by prolonged HCL exposure continues to affect the pellicle after the pellicle is reformed and the acid is removed. This would also indicate the pellicle has a limited role as a barrier during HCl acid challenges.

Previous studies have demonstrated the importance of the protein composition of the AEP. Distinct protein profiles have been observed between GERD patients with and without erosive tooth wear [Martini et al., 2019]. Furthermore, statherin is reduced in the AEP in eroded teeth compared to healthy teeth in the same individual [Mutahar et al., 2017b]. Interestingly, statherin is resistant to removal from the AEP by HCl although only to short, 10 second exposures [Taira et al., 2018]. However, the reduced protein in the AEP measured in this study has not been attributed to reduction in specific protein/s. Further work is required to determine whether specific AEP proteins are affected differently by rinsing with strong or weak acids and whether loss of certain proteins is responsible for reduction in protein concentration observed in this study.

The reduction in pellicle TPC following HCl destruction of the surface does not appear to be reflective of a change in surface roughness, as a decrease was observed after all acid challenges. This indicates that the decrease in TPC observed after 300 seconds HCl exposure is not a result of physical changes on the enamel surface i.e. a lower surface area for proteins to bind too. We hypothesise that the chemical alterations to the surface may interfere with protein binding, perhaps due to the permeation of acid into the enamel surface as the water content of the enamel is sufficient for acids to dissolve in [Shellis et al., 2012].

A different pattern was observed with citric acid exposure. The pellicle TPC decreased statistically after exposure to 300 seconds of citric acid but then recovered towards baseline levels after re-immersion in saliva (Figure 1). The initial decrease after acid exposure is likely due to the solubilisation of the outer globular layer of proteins in the pellicle in the acid solution while the basal layer remains intact [Hannig, and Balz, 1999]. The return to baseline levels may be due to lack of damage to the enamel surface, as indicated by non-significant changes to calcium and phosphorous in the pellicle. These findings indicate that citric acid has a greater impact on the pellicle than the enamel surface, but also suggests that the salivary pellicle acts as a barrier against citric acid, in contrast to HCl acid.

Few studies have researched natural, unpolished, human enamel and natural whole mouth saliva and this is a positive factor in this study. However, this meant that standard deviations for protein concentrations and surface roughness measurements were large. Although there was significant variation in the baseline mean TPC between groups, assessment of the reproducibility of the baseline pellicle showed that the mean TPC within each group was reproducible. This suggests that although there is variation between samples, within sample measurements are reproducible. The salivary pellicle was formed according to previously published protocols [Mutahar et al., 2017a]. However, the salivary freezing, defrosting and pellicle formation process may have resulted in protein degradation as studies have reported that the use of saliva over prolonged experimental regimes can affect protein [Esser et al., 2008; De Jong et al., 2011]. This may in particular affect the low molecular weight proteins such as the statherins and histatins, of which statherin in particular has been observed to have a role in the protection against tooth wear [Mutahar et al., 2017b]. The times of acid exposure were chosen to reflect minimal acid exposures, one after 30 seconds of contact with the pellicle to ensure minimal interaction with the enamel tissue was and the more prolonged 300 second challenge where the acid would contact the enamel but would not produce bulk enamel tissue loss [Mylonas et al., 2018]. Studies have shown that the pH can remain low for periods of up to 15 minutes in dietary exposure cases [Johansson et al., 2004]. This is particularly true with behaviours such as sipping an acid challenge slowly where there is constant replenishment of H+ ions. In the case of gastric reflux, the number of episodes lasting greater than 5 minutes is included in the DeMeester scoring system for assessing GORD activity [Moazzez et al., 2005].

The concentration of the acids used in this study were designed to reflect intrinsic and extrinsic acids individuals will be exposed to in the diet or during episodes of gastroesophageal reflux. The lower pH of HCl may be the causative factor in the greater enamel surface damage observed in this study but it also differs from citric acid by it being a strong acid. Furthermore, the pattern of total protein concentration changes within the pellicle differs between the two acids. If concentration were the only factor, then one would expect the difference between the acids to be one of magnitude. This suggests different mechanisms may be involved. Further studies are needed to elucidate the different mechanisms in which weak, organic acids and strong, inorganic acids interact with the AEP and the enamel surface.

The main limitation in this study, alongside most in situ or in vivo pellicle collection studies, is the inability to standardise the surface area collected. We followed previously published protocols and attempted to standardise the collection as much as possible [Carlén et al., 1998; Carpenter et al., 2014; Siqueira et al., 2012; Mutahar et al., 2017a]. Previously published protocols have verified 0.5% SDS being the optimal concentration for removal on protein from the enamel surface [Svendsen 2008]. Although we did not verify whether any protein remained on the enamel surface after 0.5% SDS treatment with protein staining the use of the method has been used in previously published studies [Carpenter et al. 2014]. A single sized filter paper (21x3mm) was used by a single investigator, collection was always performed in the same location the centre point of the 3mm collection diameter at the most bulbous point of the buccal surface. However, altered surface morphology could lead to differences in the surface area and therefore differences in the baseline total protein concentration as observed in the results. For this reason, each result was compared to its own baseline and percentage change rather than absolute change was measured. The same saliva was used for all groups which would have contained the same amount of Ca and P. Levels would not be reliant upon adhesion to the enamel surface and therefore you would not expect any differences in levels unless exposed to acid. The surface roughness data is limited in its ability to assess erosive tooth wear. This is due to non-linear fluctuations of surface roughness after an initial reduction during the erosive process [Mullan et al., 2018a]. This study would have benefited from another form of metrological assessment of erosion assessment. The study design meant that there would be insufficient profilometric loss of tooth tissue to be detected from a profilometer and microhardness testing is difficult on curved enamel surfaces.

Other methods such as measuring surface loss using OCT and micro CT imaging would have required a more advanced erosive challenge. Previous work within our lab has shown that an initial 24h collected pellicle resulted in significantly different levels of protection which is why the initial pellicle was formed for 24 hours [Mutahar et al., 2017a; Hara et al., 2006]. However others have shown that protection is conferred after 2 hours with no significant differences between a 2 hour pellicle and 24 hour pellicle. To prevent further protein degradation, saliva was replenished for this 2 hour period. Future protocols could investigate the effect on a 2 hour formed pellicle, followed by a 2 hour replenishment and a further 2 hour pellicle. Furthermore, we did not investigate what the Ca or P is bound to in the pellicle. Although it has been demonstrated that salivary proteins such as statherin, bind to calcium (G.B. Proctor, S. Hamdan, G.H. Carpenter, P. Wilde A statherin and calcium enriched layer at the air interface of human parotid saliva Biochem J, 389 (2005), pp. 111-116), this requires further investigation. We also do not know if the stability of the pellicle is affected by proteins bound to Ca or P. The possible implications of this also require further investigation.

These findings have implications in the understanding of the mediation process the pellicle provides during erosive challenges. It also may explain why there is large variation in both the clinical and laboratory data. This is the first study to differentiate that the protein concentration of the pellicle is altered as a result of damage to the enamel surface by HCl, whereas it appears that citric acid alters the protein concentration in the pellicle prior to engaging with the surface. This novel finding may indicate that prevention research for cases of intrinsic tooth wear should be targeted at improving native enamel resistance rather than improving salivary conditions. Significant levels of protein remained on the enamel surface, although whether specific proteins have been lost was not studied, for both acids. This reinforces studies which have found the pellicle does not completely inhibit enamel erosion [Hannig et al., 2004; Hara et al., 2006] and layers of the pellicle are permeable to protons [Hannig, and Balz, 1999]. Further research is required to test when the pellicle and the surface recovers after the acid insult and whether the process has rendered the surface more susceptible to further acid damage.

Conclusion

Longer exposures of HCl results in reduced TPC of the reformed AEP. This may be because of chemical changes to the enamel surface due to permeation of the acid. This suggests that prevention against HCl exposure should focus on enhancing natural enamel resistance. Citric acid caused limited enamel surface destruction despite an initial reduction in AEP protein concentration. This may suggest that the pellicle may be a good focus for both diagnosis and preventive strategies against dietary tooth wear. Further research is needed to determine whether the altered physical and chemical changes to the enamel increases susceptibility to future wear.

Statement of Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Figure Legends

Figure 1. Comparison between mean (±SD) total protein concentration in the AEP before (baseline), immediately after acid rinsing (acid exposed) or after reimmersion in saliva (reformed). Two acids were used in rinsing: citric acid (pH 3.2) or HCl (pH 2.4) for either 30 or 300 seconds. Only significant multiple comparisons within the same acid and rinse length shown (n = 10). ** = $P \leq 0.01$, * = $P \leq 0.05$.

Figure 2. Comparison between mean (±SD) calcium concentration in the AEP before (baseline), immediately after acid rinsing (acid exposed) or after reimmersion in saliva (reformed). Two acids were used in rinsing: citric acid (pH 3.2) or HCl (pH 2.4) for either 30 or 300 seconds (n = 5). **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$.

Figure 3. Comparison between mean (±SD) phosphorous concentration in the AEP before (baseline), immediately after acid rinsing (acid exposed) or after reimmersion in saliva (reformed). Two acids were used in rinsing: citric acid (pH 3.2) or HCl (pH 2.4) for either 30 or 300 seconds (n = 5). **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$.

Figure 4. a) Mean (±SD) enamel surface roughness pre- and post- citric acid or HCl acid exposure for 30 or 300 seconds.

Figure 5. Tandem scanning microscopy images enamel surface prior to acid exposure on the left and after acid exposure on the right. a-b) after 30 seconds of citric acid exposure there was no significant change on the enamel surface. c-d) after 300 seconds exposure to citric acid, changes can be seen but the prisms are still intact. e-f) after 30 seconds of HCl acid exposure there is evidence of the surface being affected with a uniform honeycomb appearance. g-h) after 300 seconds of exposure to HCl the surface is dull and inter-prismatic enamel has been eroded.