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The dynamic interplay of dietary acid pH and concentration during early-stage human enamel and dentine erosion

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Dental erosion continues to be a significant global health concern affecting nearly 30% of adults world-wide. With increasing soft drink consumption predominantly driving its prevalence, strategies for prevention and control are often implemented when erosion is severe, or rates are high in the populace. While factors affecting dental erosion such as pH on enamel has received much attention, the effect of dietary acid concentration when factored out to a commercially available pH has yet to be determined. Furthermore, understanding these effects on dentine, which is known to be more susceptible to erosion than enamel can unravel structure property relationships between acid characteristics and hard tissue types. This study aimed to develop structure-property relationships between dietary acid concentration, and pH, on the nano-textural and nano-mechanical properties of human enamel and dentine during short-term simulated drinking. To achieve this, a novel sample preparation methodology and analysis approach was developed by applying atomic force microscopy (AFM) in quantitative imaging mode. This enabled simultaneous measurement of enamel and dentine morphology and mechanical properties. Flow-cells were used to simulate drinking, exposing polished and smear layer free human enamel and dentine to 30 s repeated cycles of unbuffered citric acid 6% (pH = 1.88) and 1% (w/v) (pH = 2.55) and commercially available buffered pH = 3.8 states, for up to 180 s. The same 50 μm × 50 μm area of specimen morphology was analysed using in-house developed nanotextural analysis using the bearing area curve (BAC) with a focus on roughness (R_a), normalised peak (PA) and valley areas (VA). Mechanical properties were simultaneously measured for stiffness (N/m) after each 30 s. While all studies agree pH is a major factor in the erosion of enamel, here its dominance over the treatment time varied, with concentration surpassing the importance of pH after initial acid contact. Conversely, dentine erosion showed concentration-dependent changes in morpho-mechanical properties only. These results not only highlight the dynamic process of erosion, but how the interplay between acid characteristics and dental tissue type impact the progression of very early-stage erosion.

KEYWORDS

tooth erosion, enamel, dentine, *in vitro*, atomic force microscopy

Introduction

Dental erosion continues to be a significant global health concern with up to 29% of young adults (18–35-year-olds) exhibiting some erosion, of which 3% show severe erosion (1–4). While the causes of erosion are multifactorial, the consumption of fruit juices and carbonated soft drinks is particularly significant due their acidic pH; this leads to demineralisation of first the enamel, and eventually the dentine (5). Many laboratory studies have been conducted with the aim of better understanding the effect of acids on these mineralised tissues, but these studies tend to focus observations on either enamel (6–16) or dentine in isolation (17–26), with very few focussing on erosion of both tissues under the same conditions (27–32).

The application of remineralising agents such as fluoride in toothpastes (33–35) and in water sources (36, 37) have provided a moderate reduction in erosive wear in populations (38–40); Government guidelines even provide public health (41) advice on the condition and method of drinking, such as reducing the temperature of erosive drinks, and encouraging the use of straws (42). However, rates remain high. Identifying structure-property relationships between dietary acid characteristics and erosive wear has received much attention. The erosive severity of dietary acids based on; pH (29, 43–45), titratable acidity (44, 46), buffering capacity (15, 47), undissociated acid concentration (48, 49), acid dissociation constants (50–52), acid concentration (7), and phosphate/carbonate contents (45, 53) have highlighted their dynamic impact on dental erosion. Reduction in acid pH and increases in acid concentration have increased erosion on dental hard tissues (7, 15, 27, 29, 31, 44–46, 49–52) while improving buffering properties have shown a significant reduction in erosive severity (43, 45, 47–49, 54). While these studies have shown the impact of varying solution properties on erosion studies have applied varying degrees of erosive severity, experimental approaches, and have not approached their research questions from a dietary perspective. For example, the impact of drink properties such as concentration and pH of those found in commercially available products.

From the literature, four major problems in understanding the effect of dietary acid erosion on dental hard tissues have been identified. Firstly, as off-the-shelf products tend to have various acid concentrations and pH (45, 53, 55), the effects of pH and concentration on erosion are often convoluted. These can be deconvoluted by analysing the effect of unbuffered and buffered acids of similar concentrations. Secondly, a better understanding can be achieved by focusing on the dental erosion at the nanoscale, in combination with time-resolved, *in-situ* acid exposure, as very early changes can be detected, sequentially, on the same area. Thirdly, traditional measurements are based on roughness (13, 56–62), step-height changes (63–65) and hardness (54, 66–69), all of which are limited by the detection method and do not translate well for dentine compared to enamel (70). Utilising more complex surface analysis methods designed for multi-stratified surfaces can provide a more meaningful measure of surface features, such as the bearing area curve analysis (71–73). Finally, with enamel being the focus of dental erosion studies as it is normally the first structure to be attacked by acids, the morpho-mechanical analysis of dentine erosion has received less attention. Most studies that have focused on dentine erosion have approached experiments from an endodontic perspective (74–78), in which root dentine specimens are exposed to high concentrations of various acids or chelates, revealing the extremes of eroded morphology to enhance bonding procedures (79–81).

To overcome these problems, this study aimed to deconvolute the effect of dietary acid pH and concentration on human enamel and dentine erosion. This was conducted to identify the effect of acid concentration when pH is factored out to a typical, commercially available pH (pH = 3.8) (82), highlighting the impact of these 2 variables on erosive severity. To understand this effect in greater detail, novel advanced nanoscale techniques with complex textural surface and mechanical analysis, combined with a simulated drinking model during short-term repeated exposures have been applied.

Methods

Sample preparation

Enamel and dentine specimens ($n = 25$ per dental tissue type) were cut from healthy enamel and dentine taken from 40 erupted human 3rd molars in the occlusal plane, using a water-cooled diamond saw (Skilldent, Skillbond UK), to produce 0.5 mm^3 sections. This was conducted in accordance with local and national rules dictated by Newcastle University Biomedicine Biobank, and fully anonymised in compliance with the ethical and legal framework of the Human Tissue act (HTA) of 2004. Each section was embedded in polyester resin (Sigma-Aldrich, UK) and was cut out using the water-cooled diamond saw to produce 0.75 mm^3 embedded sections for polishing. Specimens were then polished perpendicular to the buccal surface using 1,200 grit silicon carbide paper (Norton, Northants, UK) to reach the embedded mineralised surface. This was then followed by 1 µm, 0.3 µm and 0.05 µm aluminium oxide solutions (BUEHLER®, Illinois, USA) in sequence (BUEHLER® Metaserv Twin Polisher, Illinois, USA). This enabled approximately 0.05 mm to be removed from the initial enamel and dentine surface providing consistency in section depth, allowing a 0.5 mm^2 2-dimensional hard tissue surface for acid exposure. Each section was then

ultrasonicated (Lanford electronic Ltd, UK) in Chloramine-T (1% w/v) (Sigma-Aldrich, UK) at 4°C and transferred to fresh Chloramine-T (1% w/v) solutions and stored at 4°C before use.

Dietary acid solutions

Citric acid solutions of concentrations 1% (w/v) and 6% (w/v) were produced by diluting citric acid (Sigma-Aldrich, UK) in distilled water. The solution pH was measured using a pH meter (Orion 4 Star, Thermo electronics Ltd, Massachusetts, USA) at room temperature (22°C). Next these solutions were divided in half and one half was buffered to pH = 3.8 by adding 1 M NaOH (Sigma) to the solution. All acidic solutions were freshly made on the day of analysis. The polished enamel and dentine sections were then randomly divided into five study groups containing five specimens each for each acid solution. Group 1 and 2 specimens were exposed to citric acid 6% (w/v) pH = 1.88 and citric acid 1% (w/v) pH = 2.55. Group 3 and 4 specimens were exposed to citric acid 6% (w/v) and citric acid 1% (w/v) both at pH = 3.8. Group 5 specimens were exposed to a control solution consisting of Dulbecco's phosphate buffered saline (PBS) (pH 7.20)(Lonza, Biowhittaker®).

In-vitro Simulated Drinking Model

Specimens were attached to a circular glass coverslip (AGL46R13-1, Agar Scientific, Essex, UK) using 2-part curable adhesive (Araldite Instant, Huntsman Advanced Materials, UK) and placed individually into an AFM flow-cell (JPK Instruments, Bruker, FR). 1 ml of PBS solution was injected into the flow-cell for baseline analysis. 1 ml of dietary acid solutions was then injected, left for 30 s, and then eluted using a 1 ml syringe (Terumo HT-SLWC-0R2W 1 ml Syringe, Tokyo, JP), washed with twice with fresh PBS and further replaced with PBS for analysis to stop the erosive process. This continued, performing quantitative imaging analysis after each 30 s interval under PBS hydrated conditions until each specimen experienced a total of 180 s acid exposure.

AFM imaging, textural and mechanical analysis

AFM imaging and mechanical analysis was performed using a Nanowizard 3 AFM (JPK Instruments, Bruker, FR), operating in quantitative imaging mode (QI™), using gold coated non-conductive silicon nitride cantilevers (NP-10, Bruker, Camarillo CA, USA). AFM cantilever spring constant calibration was performed using the in-built software calibration procedure for all probes providing an average spring constant of 0.204 ± 0.107 N/m. All quantitative images were obtained at a resolution of 256 × 256 and scans were performed over the same 50 μm × 50 μm areas after each acid exposure in PBS. Average roughness (R_a) was measured using integrated JPK software. Bearing area curves (BAC) were calculated by extracting two-dimensional line traces at 12.5 μm, 25 μm and 37.5 μm in both the *x*- and *y*-direction from each topographic image and importing them into using in house developed Visual Basic® code. From this code the BAC parameters peak area (PA) (area occupied by material peaks) and valley area (VA) (area occupied by material voids) were calculated within defined regions each BAC (67). Stiffness (N/m) was extracted from resulting force distance curves from each pixel in the 256 × 256 quantitative image using dedicated software (JPK Data Processing Software).

Statistical analysis

Average roughness (R_a), peak area (PA), valley area (VA) and stiffness were all found to be non-normally distributed, so non-parametric statistics were used for analysis. Statistically significant differences in all analysed parameters for each acid sub-group and exposure time were conducted by Kruskal-Wallis test ($P < 0.001$). A post-hoc Tukey's test was used to identify significant differences between acid pH, concentration, any interaction between pH and concentration, and the control specimens. Medians with interquartile ranges were used to identify statistically significant differences at a 95% confidence level. As the specimens were derived from different human donors there were some baseline variations in the PA, VA and stiffness. Consequently, we normalised these data to the value measured for each specimen before they were exposed to an acid solution, $t = 0$ s.

Results

Typical topography images for enamel specimens treated with each solution are shown in **Figure 1**, with the stiffness maps for these enamel specimens in **Figure 2**. Likewise, typical topography and stiffness images for dentine specimens treated with each solution are shown in **Figures 3**, **4**, respectively. Quantitative roughness, normalised peak and valley area and normalised stiffness data are plotted as a function of exposure time with enamel shown in **Figures 5A, C,E,G** and dentine shown in **Figures 5B,D,F,H** respectively.

Enamel erosion

For enamel specimens treated with PBS there were no obvious changes in morphology over the 180 s of exposure

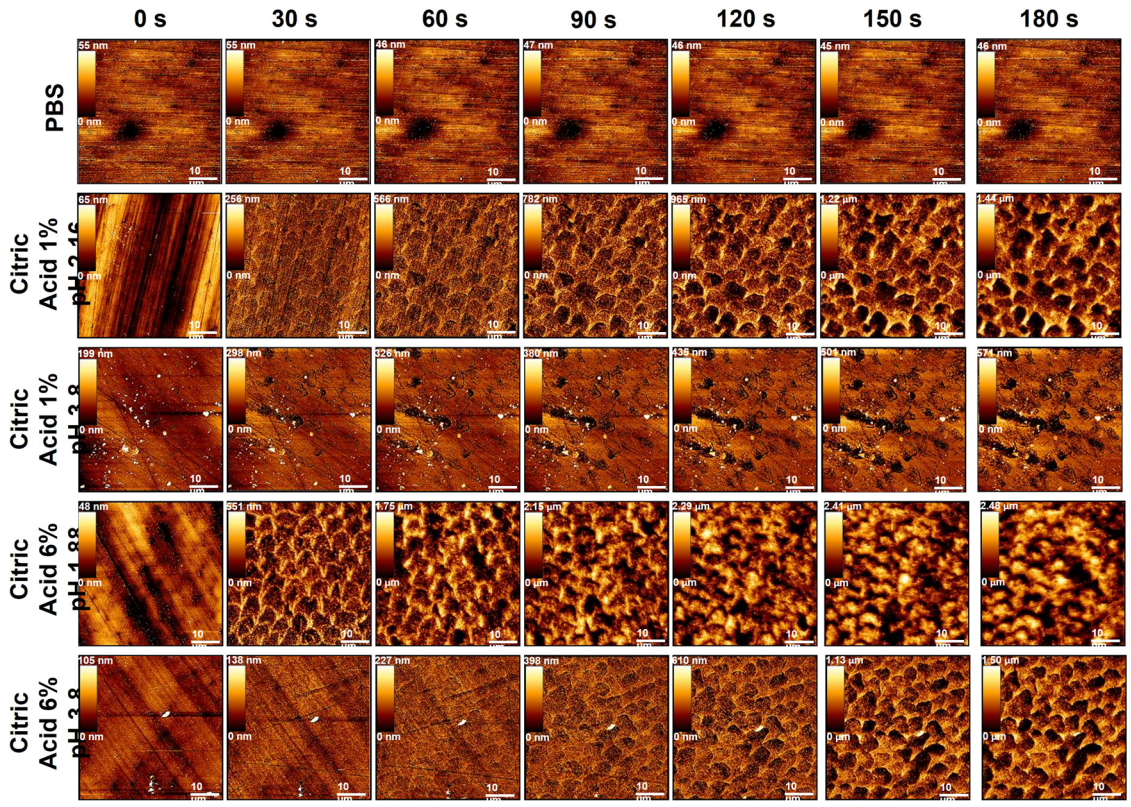

FIGURE 1
Showing example topographic AFM imaging of human enamel exposed to all dietary solutions (top) throughout the exposure regime starting from 0 s (top) to 180 s (bottom) at 30 s intervals.

while there are clear changes evident for all acid treated samples shown in Figure 1. When comparing the morphological changes with respect to acid concentration and pH, surface changes occur faster with enamel prisms becoming obvious sooner as acid concentration increases, and pH of the solution decreases.

The solution pH significantly affected roughness at each time point ($P < 0.001$) with the 6% pH = 1.88 solution producing the greatest increase in roughness, significantly so when compared to PBS treatment ($P < 0.05$), Figure 5A. Acid concentration also significantly affected roughness, with the 6% solutions always producing greater roughening than the 1% solutions ($P < 0.001$) at either pH. There was significant interaction between pH and concentration on the increase in roughness due to exposure to the solutions ($P < 0.001$).

The PA and VA results further clarify the effect of acid pH and concentration on enamel erosion, shown in Figures 5C,E. Specimens treated with either citric acid 6% pH = 1.88 and 1% pH = 2.55 showed a step rise in PA up to 60 s, after which the PA for the 6% pH = 1.88 plateaued while the 1% pH = 2.55 continued to rise, to a higher value, until 90 s of exposure. No significant rise in PA was seen for the 1% pH = 3.8 treated specimens, which corresponds to the lack of distinct prismatic structure visible in the topography images shown in Figure 1. However, 6% pH = 3.8 citric acid treated specimens showed a different PA behaviour, with a rise in PA, corresponding with the appearance of enamel prisms, seen after 90 s exposure. Similar behaviour was seen for the VA data, with the 6% citric acid pH = 1.88 producing the greatest increase in this parameter up to 90 s, after which there was a decrease. For the 1% pH = 2.55 and the 6% pH = 3.8 acid treated specimens VA increased over the full 180 s, while the 1% pH = 3.8 acid led to a plateau in VA after 60 s of exposure. As PA and VA both show how the enamel erosion results in the formation of peaks and valleys within the prisms, by using these BAC-derived textural parameters it is possible to elucidate the more complex relationships in enamel erosion between acid concentration and pH than using R_a alone.

Treatment with PBS resulted in no change in stiffness shown in Figures 2, 5D ($P > 0.05$), while clear changes were obvious when any of the acid solutions were used. The stiffness images showed that the changes in stiffness were associated with the appearance of the enamel prisms. Treatment with citric acid 6% pH = 1.88 and 1% pH = 2.55 acid solutions led to a similar reduction in stiffness, with an initial rapid decrease found after 30 s exposure, followed by a much slower decrease for the remaining 180 s, shown in

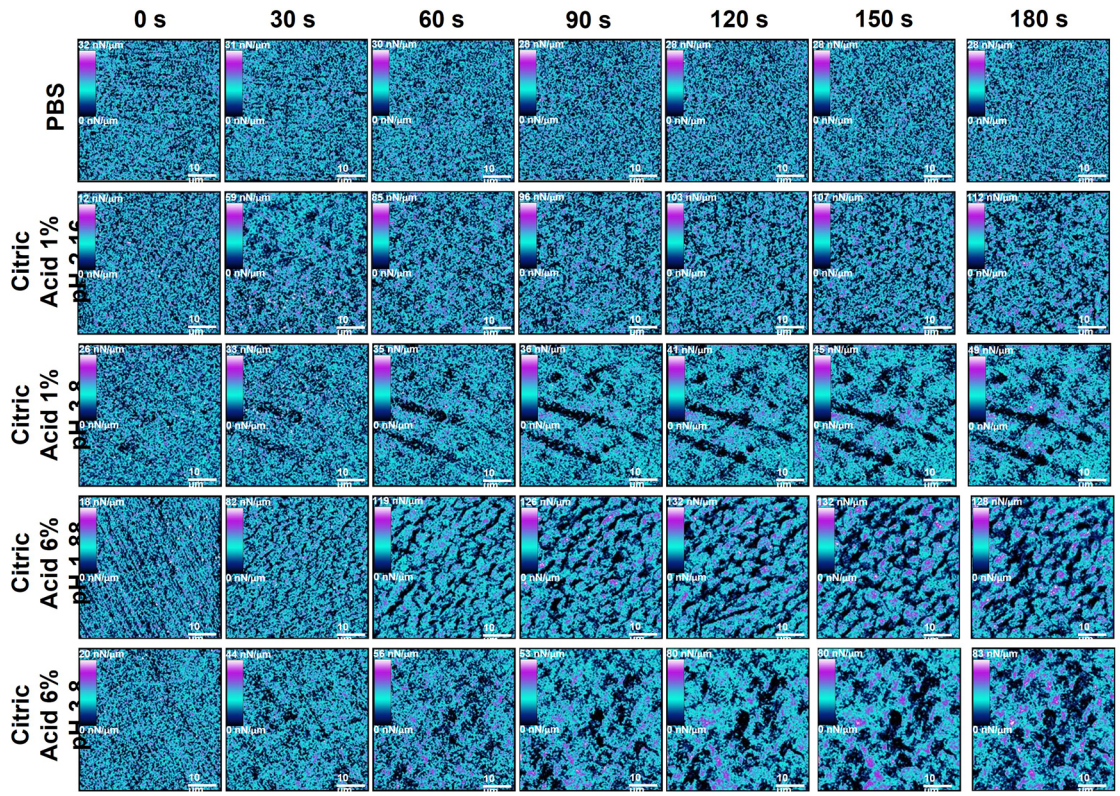

FIGURE 2
Showing example stiffness AFM mapping of human enamel exposed to all dietary solutions (top) throughout the exposure regime starting from 0 s (top) to 180 s (bottom) at 30 s intervals.

Figure 5G. Interestingly, a greater reduction in stiffness was observed on specimens exposed to 1% buffered acid when compared to 6% buffered acid.

Dentine erosion

Like that found for enamel there was no obvious effect on the dentine specimens due to exposure to PBS, shown in Figures 3, 4. While the specimen preparation method resulted in exposed dentinal tubules, confirming we were not testing enamel in the early time periods of this experiment, the overall appearance and stiffness of the PBS treated specimens remained the same. In contrast, treating the dentine specimens with any of the acidic solutions for 30 s led to appreciable tubule widening compared to the baseline, $t = 0$ s, images. After 30 s of exposure no clear differences in the topography image was seen for any of the acid solution groups.

The tubular structure of the dentine surface made the roughness measurement insensitive to any differences in erosion due to either pH or concentration. In general, the overall behaviour for each acid solution was the same with an increase in R_a up to 90 s followed by a plateau, with no significant effect due to differences in pH ($P > 0.05$) or concertation ($P = 0.166$).

No significant change in PA was measured for specimens exposed to any of the acidic solutions, shown in Figure 5D. VA analysis shown Figure 5F, showed that dentine exposed to citric acid 6% pH = 1.88 and 1% pH = 2.55 exhibited the greatest change while specimens exposed to the pH = 3.8 acids showed a more progressive increase in normalised VA across the treatment regime compared to pH = 1.88 and pH = 2.55 exposed specimens, also increasing with concentration. The fact that roughness and PA were insensitive to the effects of acid exposure while VA showed some differences in behaviour highlights the difference in behaviour seen between enamel and dentine. Acid exposure resulted in the exposure of the enamel prisms which eroded more towards the prism centre than the edge, certainly at early exposure times, meaning that roughness data was composed of both material peaks and valleys. Dentine eroded differently, with only tubule widening seen, which corresponded to the roughness data only comprising material valleys.

The change in dentine stiffness due to exposure to the different acid solutions shown in Figure 5H, clearly demonstrated that both pH and concentration were significant

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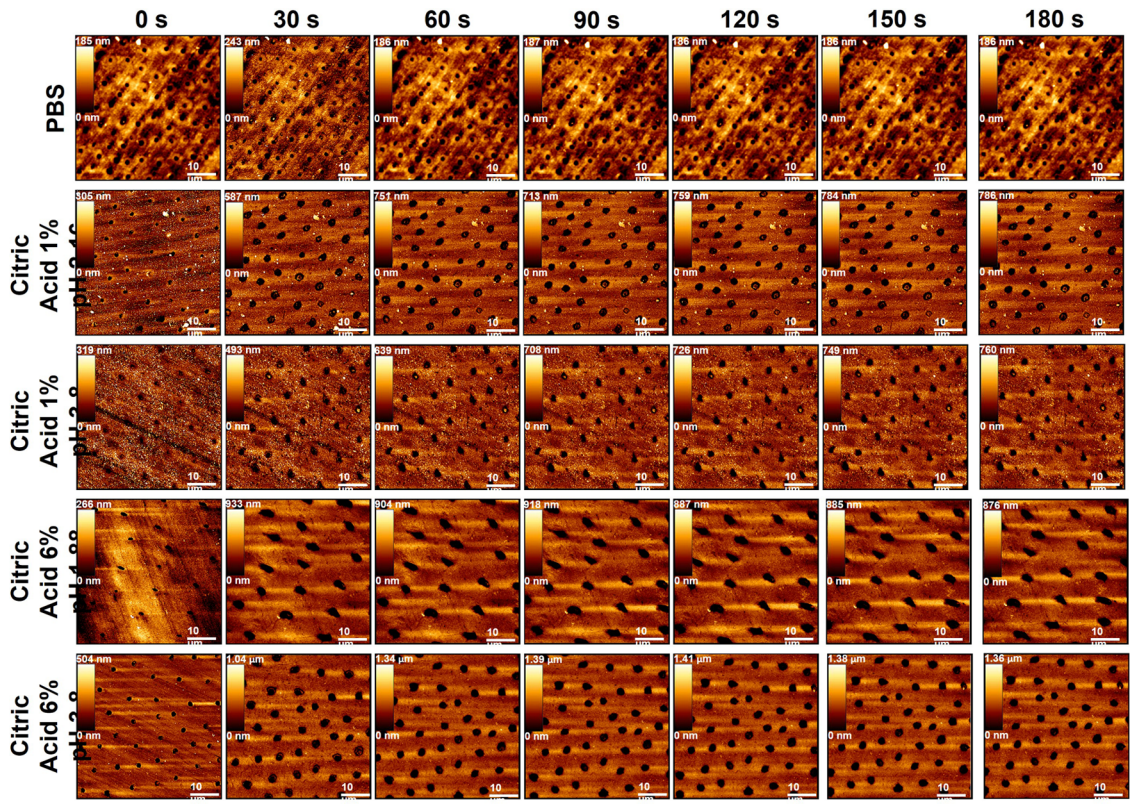

FIGURE 3
Showing example topographic AFM imaging of human dentine exposed to all dietary solutions (top) throughout the exposure regime starting from 0 s (top) to 180 s (bottom) at 30 s intervals.

factors. Treatment with the citric acid 1% pH = 3.8 solution resulted in the lowest reduction in stiffness after 180 s, while the 6% solution, at the same pH, caused significantly more reduction in stiffness ($P < 0.05$) at times after 120 s of exposure. Both citric acid 6% pH = 1.88 and 1% pH = 2.55 acid solutions, despite having different pHs, produced identical behaviour in the stiffness, both producing significantly more reduction in stiffness than either pH = 3.8 acid solution.

Discussion

Applying nanoscale textural and mechanical analysis enabled the effect of dietary acid pH and concentration on human enamel and dentine during short-term dietary acid exposure to be explored. While all studies agree pH is a major factor in the erosion of enamel (7, 29, 44, 45, 83–92), here its dominance over the treatment time varied, with acid concentration surpassing the importance of pH after initial acid contact. Conversely, dentine erosion showed concentration-dependent changes in morpho-mechanical properties only. These results not only highlight the dynamic process of erosion, but how the interplay between acid characteristics and dental tissue type impact the progression of very early-stage erosion.

Dietary acids are predominantly carboxylic and once in solution their dissociated ionic components play different roles in the erosive process (29). Mineral destabilisation of biological hydroxyapatite (bHAP) occurs through protonation (H^+) of carbonate and phosphate groups of the crystal lattice, termed direct acid attack (93). This weakens the coordination of the surrounding calcium ion (49, 94) where the anionic component ($R\text{-}COO^-$) subsequently adheres and decalcifies bHAP by chelation (50, 94, 95) through the adhesion-decalcification concept (93). For citric acid both mechanisms of erosion are known to occur on separate territories of bHAP at the Nernst layer (48, 49) i.e., the acid – enamel or dentine interface, which is static, and diffusion controlled (94). Additionally, undissociated acid (inactive acid) can diffuse into bHAP, acting as a mobile carrier for dissociation in enamel and dentine's water content (93). Further direct acid attack and chelation occur here in the subsurface as a high concentration of dissociated ions at the mineralisation front are maintained, termed subsurface erosion (48, 93). Buffering dietary acids to match the typical pH of commercially

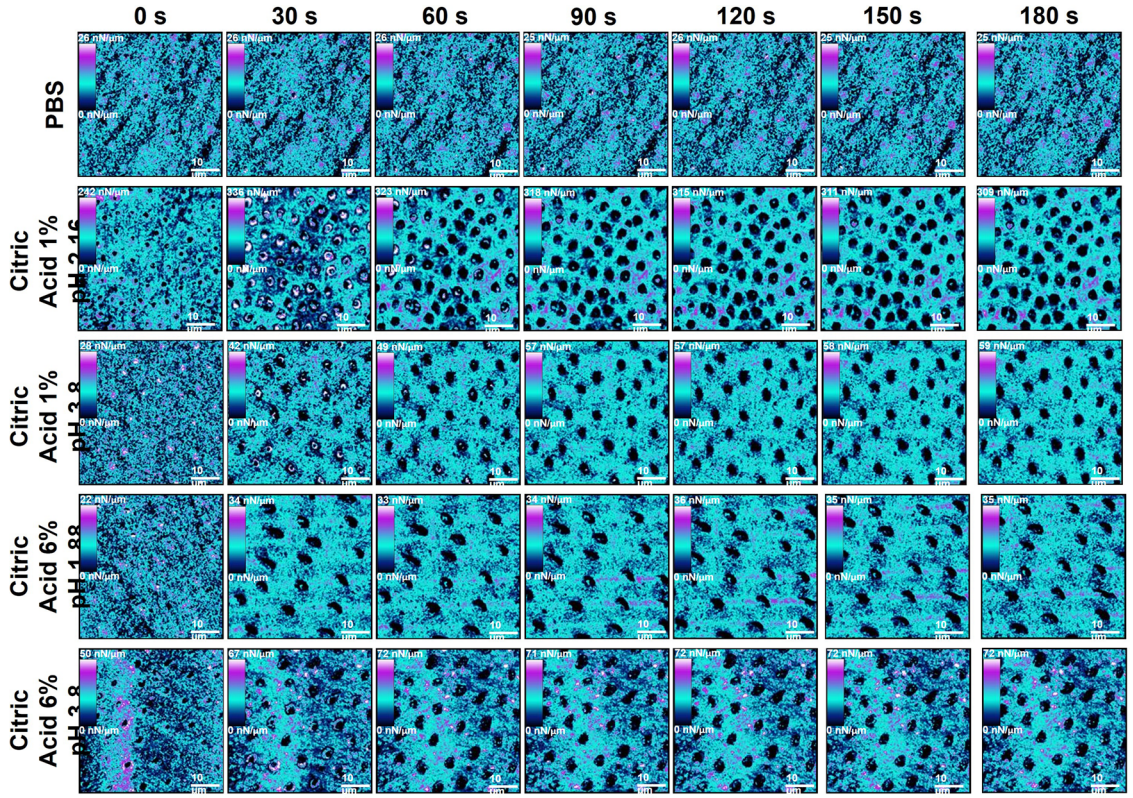

FIGURE 4
Showing example stiffness AFM mapping of human dentine exposed to all dietary solutions (top) throughout the exposure regime starting from 0 s (top) to 180 s (bottom) at 30 s intervals.

available fruit juices (pH = 3.8) reduces phosphate and carbonate group dissociation on the bHAP crystal lattice *via* direct acid attack (49) creating a higher diffusion gradient for subsurface erosion at the Nernst layer (49). As buffered citric acid approaches its pKa value (96), subsurface erosion is postulated to dominate the erosive activity.

Previous enamel erosion investigations have shown concentration dependent mineral loss (27, 97, 98) with before and after dietary acid challenges showing increases in the prevalence of key-hole structures (61, 99–101), roughness (53, 56, 58, 102, 103), BAC parameters (73, 103), and reduction in mechanical properties (49, 69, 94, 104–106). Only one study has monitored increases in roughness during consecutive citric acid exposure of enamel on the same area using AFM (61). The study used relatively long-term treatment times (90–250 min) with no mechanical analysis or simulated drinking. Profilometric studies into short-term dietary acid enamel erosion have determined increases in BAC parameters (73), although again, the effects of pH and concentration were not deconvoluted.

Buffering dietary acids to a commercially relevant pH = 3.8 still enables dissolution of enamel surfaces (105) as it is far lower than the critical dissolution pH. For enamel, we propose buffering dietary acids to pH = 3.8 while maintaining the same concentration causes subsurface erosion to dominate the erosive process, as opposed to pH-driven direct acid attack. This is evident in pH = 3.8 acid exposed enamels delayed increases in roughness, BAC derived normalised PA, VA, and normalised stiffness reduction, compared to citric 6% pH = 1.88 and 1% pH = 2.55 acid exposed enamel, at the same concentration, shown in **Figures 1**, **5A,C,E,G**. Citric 6% pH = 1.88 and 1% pH = 2.55 acid exposed enamel erosion was dominated by pH during initial exposure (30–90 s), causing saturation at the Nernst layer thereafter (94). At later exposure times (90–180 s) subsurface erosion dominates the morpho-mechanical changes of enamel.

Most research into dentine erosion has been applied from an endodontic perspective (74, 77, 107–111) using high concentrations of citric (92, 97), phosphoric (97), nitric (112) and lactic acids (92, 113), generating a rough surface to enhance bonding procedures. Here, dentine exhibited a very different trend in morpho-mechanical properties during erosion compared to enamel. The effect of tissue structure, organic components, ratio and microphase separation of the

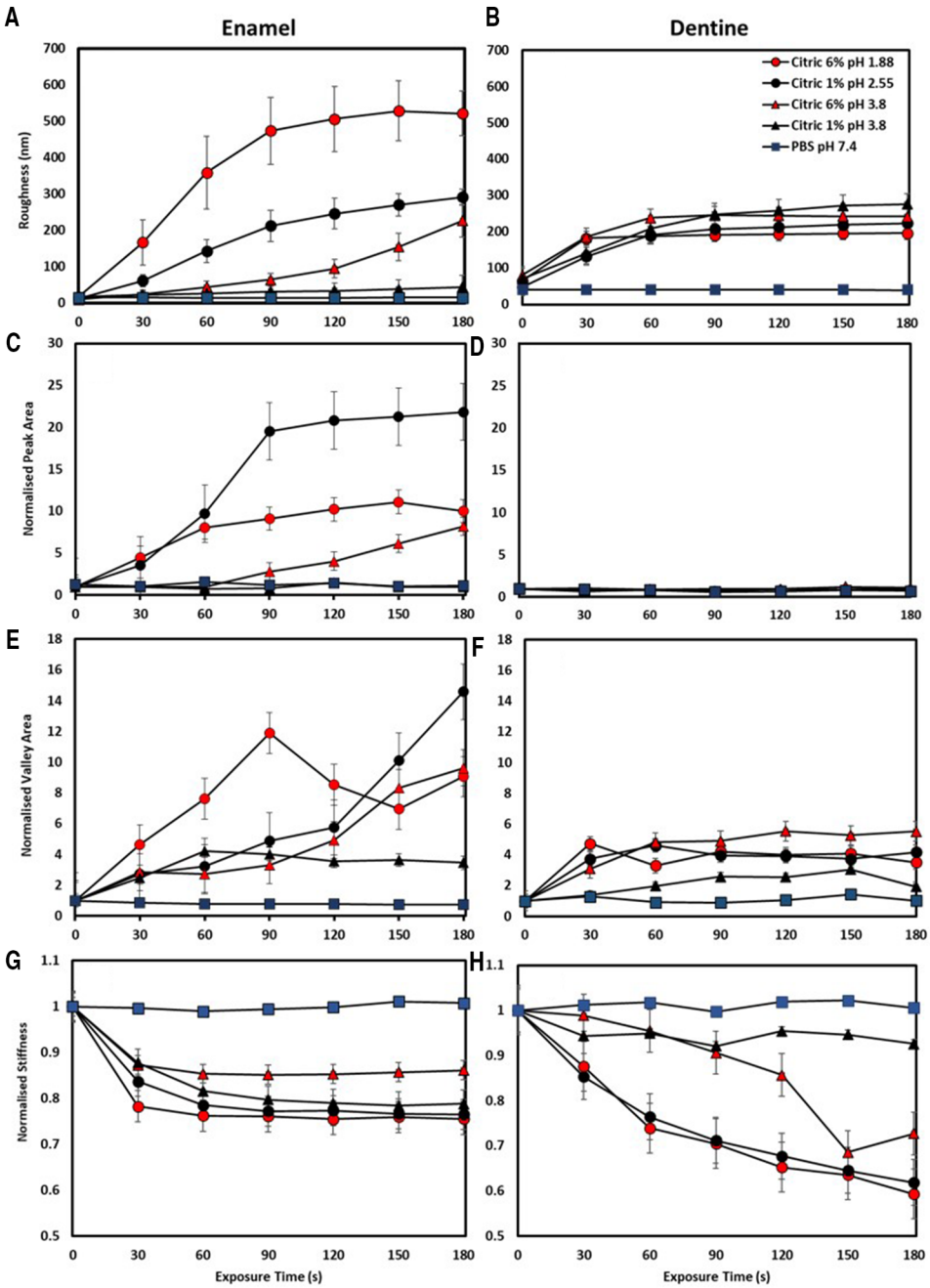

FIGURE 5
Showing median and interquartile ranges of n = 25 human enamel (left) and *n* = 25 dentine (right) exposed to all dietary solutions with (A,B) showing roughness (nm), (C,D) normalised peak area, (E,F) normalised valley area and (G,H) normalised stiffness. A post-hoc Tukeys test was used to identify statistically significant differences ($P < 0.05$).

mineral phases (intratubular and peritubular dentine) must be considered when comparing the erosive characteristics of enamel and dentine. These structures have enabled them to function for specific roles, with enamel providing a hard surface for mastication (114) and dentine withstanding compression (115). While both substrates can be regarded as polymer reinforced composites, their degrees of mineral and organic constituents vary according to their associated roles. Historically, dentine has been regarded as more susceptible to erosion than enamel, as dissolution of its mineral component occurs at a higher pH (pH = 6.3 compared to enamel's pH = 5.5) (116). Moreover, dentine's carbonate content is greater in

peritubular compared to intertubular dentine (117) increasing its susceptibility to direct acid attack (92, 112). This is evident by the immediate increase in observable tubule diameter and quantitative valley area analysis for all acid exposed dentine specimens shown in **Figures 3**, **5E** respectively. After a certain depth of mineral is removed by demineralisation, mineral loss has been shown to significantly decrease (17, 19) and, in the absences of a collagen matrix, the rate of dissolution is markedly higher (17, 118). The surrounding liquid phase protects the mineral components even further, as collagen provides a buffering effect to increase the local pH (119, 120). De-bound mineral is prevented from immediate removal, increasing calcium and phosphate saturation at the Nernst-layer (48). This protective effect of collagen, preventing substantial erosion is a vital aspect in overall tooth protection from acid erosion. Here, increased acid concentration was found to remove peritubular dentine and cause exposure and subsequent collapse of the extra cellular collagen matrix faster than that of 1% citric acid solutions. This subsequently limited the changes of roughness, BAC derived normalised PA and VA and normalised stiffness measurements thereafter.

The collapse of the collagen matrix was found to play a significant role in the prevention of further changes in roughness and BAC derived parameters after the erosion of peritubular dentine. While erosion of peritubular dentine created significant changes in morphological parameters after initial exposure treatment, plateaux's were obtained after complete peritubular dentine removal. Intertubular dentine was postulated to protect any further demineralisation, as no changes in roughness and BACs were obtained. This occurred at a shorter exposure time for 6% citric acid exposed specimens (30 s), while 1% acid exposed specimens exhibited this at 60–90 s treatment in either pH = 2.55 or pH = 3.8 states. The stiffness of dentine erosion was shown to reflect a similar trend to that of the morphological parameters. Increased acid concentration exposed specimens yielded the largest reductions in stiffness over the exposure time. Again, the collapse of the collagen matrix was found to play a significant role in the prevention of further changes in stiffness after the erosion of peritubular dentine. This occurred at 30 s for 6% acid exposed dentine, while 1% exposed specimens plateaued at 60–90 s, previously unreported.

Fundamental differences in enamel and dentine's mineral phase (106, 121–125) and proteinaceous material (121, 122, 126–128) give rise to these qualitative and quantitative differences in morphological and mechanical properties upon demineralisation. While carbonate substitution exhibited by bHAP increases the susceptibility of enamel to erosion compared to HAP (50, 119, 129, 130), increased carbonate substitution occurs in dentine compared to enamel (5%–6% compared to 3% respectively) (119), making dentine even more susceptible to erosion. Here, dietary acid pH i.e., direct acid attack dominated the erosive capacity of acids in very early-stage erosion of enamel, while concentration i.e., subsurface erosion surpassed the importance of pH after this. For dentine, we postulate the evolutionary role collagen plays in enabling bulk compression compliance also plays an effective and efficient role in protecting further morpho-mechanical changes during low pH dietary acid erosion.

If dietary acid solutions were buffered to a far lower pH = 2.3 to match the pH of cola drinks (131), we would expect pH driven direct acid attack to dominate the erosive process far more compared to concentration driven subsurface erosion. This is due to the greater presence of dissociated H^+ ions in solution for pH 2.3 dietary acid solutions and a reduction in undissociated acid for diffusion and subsequent dissociation into the subsurface. Enamel's delayed reduction in morpho-mechanical properties exposed to citric 6% pH = 3.8 solutions shown here would occur much faster in the exposure regime for those exposed to pH = 2.3. For dentine, we would expect concentration driven subsurface erosion to dominate the erosive process at later exposure times with pH driven direct acid attack dominating the erosive process during initial acid contact. This would erode peritubular dentine almost instantly with the buffering properties of the collagenous network taking longer to reduce direct acid attack.

AFM is a powerful tool to image and quantify morpho-mechanical changes in dental hard and soft tissues subject to dynamic processes such as erosion (61) and oral biofilm development (132, 133). Operating in QI™ mode enabled careful control of probe-surface contact force, allowing no surface damage during imaging while simultaneously measuring sample stiffness. While previous studies have utilised this technique on dry dental specimens (134), here, for the first time, it was used on PBS hydrated specimens, after each acid exposure, using a simulated drinking model.

AFM cantilevers with low spring constants on relatively hard tissues such as enamel and dentine were chosen to minimise sample damage during analysis as it was expected that eroded specimens would become increasingly softer after each acid exposure. This meant that there was considerable variability between sample stiffness values measured between different specimens with different probes. Only once the data were normalised to the initial median stiffness prior to acid exposure did the overall trend become apparent. Since the same nominal force was applied across all surfaces and exposure times, enamel and dentine's resistance to deformation decreased during erosion, exhibited by the normalised reduction shown in **Figures 5G,H**, characteristic of surface softening.

This study has potential limitations. Improving the sample size by applying a power analysis could enhance statistical analysis between tissue type, dietary acid variables and analysed parameters. Due to the limited number of intact dental specimens that could be obtained for each specimen

cohort $n = 5$ per group was sufficient. This enabled significant differences in analysed parameters to be obtained. Future investigators may wish to apply a power analysis in their study with a ready supply of intact dental specimens. Other limitations such as improving simulated drinking and the *in-vitro* model to more reflect that of the oral environment could be made. Utilising this novel platform investigators could reduce dietary acid exposure times to reflect sipping and apply artificial or pooled human saliva with varying mucin types and concentrations. Moreover, other dental hard tissue structures can be assessed such as prismatic or aprismatic enamel, enamel-dentine junctions, mantle, globular, sclerotic, primary, secondary, and tertiary dentine by sectioning at different depths. The effect of diseases could also be assessed enabling structure-property relationships to be developed on varying types amelogenesis imperfecta e.g., hypocalcification, hypoplasia, porphyria as well as fluorosis and celiac disease. While pH and concentration were the only variables assessed in this study, investigators may wish to understand the effect of other dietary acid properties such as titratable acidity, buffering capacity, undissociated acid concentration, acid dissociation constants and phosphate/carbonate contents, unravelling other structure property relationships.

Future studies can now utilise this novel sample preparation methodology and nano-scale analysis protocol to determine the effect of other dietary acids on a variety of dental hard tissue types. Including, utilising the novel platform to determine the effect of agents to prevent, arrest or even remineralise eroded tissue structures.

Conclusion

This investigation sought to uncover the effect dietary acid concentration and pH play in the erosion of human enamel and dentine during very early-stage exposure. By developing novel approaches to sample preparation and simulated drinking, simultaneous nano- textural and nano-mechanical analysis of enamel and dentine were shown to exhibit very different responses to dietary acid erosion. Interestingly, for enamel, pH-driven direct acid attack was shown to dominate the erosive capacity of acids in during initial exposure, while concentration driven subsurface erosion was shown to dominate thereafter. For dentine, concentration was shown to dominate the erosive capacity of dietary acids. Moreover, while dentine has a higher critical pH of bHAP dissolution compared to enamel, here we have shown dentine to be less susceptible to dietary acid erosion, due to the protective effects of the collagen matrix.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JP, JF, PJW and MJG contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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