The Influence of Hardness and Chemical Composition on Enamel Demineralization and Subsequent Remineralization

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Abstract

Objectives: The objectives were to investigate the hardness and chemical composition of sound, demineralized and pH-cycled bovine enamel and determine their influence on demineralization and remineralization behavior. **Methods:** Ninety-four, 5×5×2-mm bovine enamel specimens were demineralized using three different times [(24h (n=33), 48h (n=30), 96h (n=31)]. The specimens were then pH-cycled using either 367ppm F sodium fluoride or deionized water. Knoop hardness (HK) and energy-dispersive X-ray spectroscopy (measured elements: Ca, P, F, C, Mg, N) were performed at three stages (sound, after demineralization, after pH-cycling) and transverse microradiography was performed after demineralization and pH-cycling. Comparisons were determined by ANOVA. **Results:** Results showed that HK, integrated mineral loss and lesion depth were significantly different between stages, demineralization times and treatments. The weight% of F at the surface was significantly affected by treatment, irrespective of demineralization time, while the Ca:P ratio of the enamel remained stable even after de- and remineralization protocols. The F in fluoride groups and the artificial saliva in non-fluoride groups were both able to induce enamel remineralization, indicating the protective effect of salivary pellicle against demineralization even in the absence of fluoride. **Conclusions:** Harder specimens and those with greater surface F weight% were less susceptible to demineralization and were more likely to remineralize. However, the amount of surface Ca and P did not influence de- or remineralization behavior.

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Clinical Significance

This *in vitro* study can help clinicians better understand the caries process and the impact of the physical and chemical characteristics of enamel on its behavior during de- and remineralization. The over-the-counter fluoride toothpaste containing 1100 ppm-F was used, and was able to produce a mineralized enamel surface layer.

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Introduction

Tooth enamel is composed of 96 mass% inorganic material and 4 mass% organic material and water [1]. The inorganic material is mainly composed of the mineral hydroxyapatite (HAP), a crystalline structure comprising calcium (Ca) and phosphate (P). Stoichiometrically, HAP does not completely correspond to the chemical formula $Ca_{10}(PO_4)_6(OH)_2$, as it contains many impurities including carbonate (C), magnesium (Mg), sodium (Na) and chloride (Cl). Carbonate, in particular, along with magnesium, cause major disturbances to HAP crystals [2]. The carbonate ions can substitute phosphate ions or hydroxyl groups and increase apatite solubility, while the magnesium ions can substitute calcium ions and inhibit crystal growth. On the other hand, fluoride ions can substitute hydroxyl groups and decrease apatite solubility [3]. Dental caries is a dynamic process that involves alternating demineralization and remineralization cycles. Several studies have reported that baseline physical and chemical characteristics of enamel greatly influence its behavior in such de- and subsequent remineralization challenges [4,5,6].

Several attempts have been made to correlate the characteristics of enamel with its response to demineralization. Enamel specimens from primary teeth responded to demineralization by producing lesions of varying depths; deeper lesions were found to have higher amounts of carbon and nitrogen and lower amounts of calcium and phosphorus [7]. The hardness of dental enamel also had a strong correlation with its chemical content [8]. Areas with higher concentration of calcium and phosphorus were shown to have the highest nanohardness values. On the other hand, areas with higher sodium and magnesium concentrations showed the opposite trend. Lower microhardness values with concurrently lower calcium and phosphorus contents have been demonstrated by several others [9,10,11].

As for remineralization of enamel, it is well established that fluoride enhances this process [12,13], and that the greater the amount of fluoride, the less the amount of demineralization, or the smaller the lesion depth [14,15]. Moreover, an increase in lesion size seems to be associated with an increase in the remineralization rate [4]. Lesions with higher R values, which is calculated as the ratio of integrated mineral loss (ΔZ) to lesion depth (L), tend to remineralize, whereas those with lower R values further demineralize [16]. Therefore, baseline lesion characteristics have a profound impact on subsequent remineralization behavior. There is an increasing tendency towards net remineralization and a decrease in further mineral loss with increasing integrated mineral loss at baseline (ΔZ_{base}) [5,17]. Lesion depth also plays a role, as deeper, more porous lesions have a higher tendency to remineralize than shallower, less porous lesions [4,15]. In the shallower lesions, the more soluble materials are more readily accessed by bacterial acids than in the deeper lesions.

Although enamel response to demineralization and remineralization challenges has been investigated in the aforementioned studies, it has not been investigated throughout all three stages of enamel behavior (sound, after demineralization and after pH-cycling) nor has it related results from multiple methods, such as hardness, chemical composition and transverse microradiography. Hence, combining these techniques could better assess changes in both physical and chemical enamel characteristics.

Therefore, the aims of this study were to investigate the hardness and chemical composition of sound, demineralized and pH-cycled enamel and their influence on demineralization and remineralization challenges. The null hypothesis is that there is no correlation between enamel hardness and mineral content and between susceptibility to de- and remineralization.

Materials and Methods

Study Design

This *in vitro* study was performed on bovine enamel specimens. Incipient subsurface caries lesions were formed at three distinct severities (24, 48 or 96 h of demineralization). After that, the specimens were subjected to an established pH-cycling model during which they were exposed either to a diluted fluoride solution to promote remineralization, or deionized water. Knoop hardness (HK) and chemical analysis [energy-dispersive X-Ray spectroscopy (EDS)] were performed and compared among the sound, demineralized and pH-cycled specimens. Transverse microradiography (TMR) was performed to compare between demineralized and pH-cycled specimens. A total of 94 specimens were included in the study, with six experimental groups (three demineralization times and two treatment regimens), which were randomly divided based on the sound HK of each specimen. Four portions of the specimens (Fig. 1) were designated as follows: Section A was used for HK for all three stages, Section B was the sound stage for EDS, Section C was after demineralization for both EDS and TMR, and Section D was after pH-cycling for both EDS and TMR.

Specimen Preparation

Extracted bovine incisor teeth were obtained (Tri State Beef, Cincinnati, OH, USA). The crowns were cut into 5×5 mm blocks from the buccal surfaces only using a low speed saw (Isomet, Buehler, Lake Bluff, IL, USA). The teeth were stored in deionized water saturated with thymol during the sample preparation process. The dentin side of the blocks was first ground flat under deionized water with 500-grit silicon carbide grinding paper (Struers, Cleveland, PA, USA). The enamel side of the blocks was then ground under deionized water in a series of 1200-, 2400-, and 4000-grit paper. The enamel side was then polished using a 1-

μm diamond polishing suspension on a polishing cloth. This procedure helped ensure the removal of approximately 200-300 μm of surface enamel (depending on the natural curvature of the enamel surface of the specimen) to remove surface irregularities and create a flat enamel surface. The resulting specimens had a thickness range of 1.7 – 2.2 mm. Specimens with cracks, hypomineralized (white spot) areas, or other surface flaws were excluded. Specimens were covered with acid-resistant nail varnish except the polished enamel surface. The prepared specimens were then stored in 100% relative humidity at 4 °C until further use.

Demineralization

In vitro incipient caries lesions were created using approximately 40 mL of demineralization solution (0.1 mol/L lactic acid, 4.1 mmol/L $CaCl_2$ • 2 H_2O, 8.0 mmol/L KH_2PO_4) per specimen at 37 °C [16]. 0.2 *%w/v* Carbopol C907 (BF Goodrich, Cleveland, OH, USA), a synthetic high molecular weight polymer, was added to the demineralization solution as a surface-protective agent to preserve the surface enamel and help create subsurface lesions. The pH of the demineralizing solution was adjusted to 5.0 using potassium hydroxide (KOH). Two groups (24 h/a and 24 h/b) were demineralized for 24 hours, two groups (48 h/a and 48 h/b) were demineralized for 48 hours, and two groups (96 h/a and 96 h/b) were demineralized for 96 hours. The demineralization solution was not replaced during the entire demineralization period of each specimen. After demineralization, the specimens were rinsed under a steady stream of deionized water. The prepared specimens were then stored in 100% relative humidity at 4 °C until further use.

pH-Cycling

All specimens were pH-cycled for 10 days using an established pH-cycling model based on White [18], however, a daily 4-hour rather than 2-hour acid challenge in the

demineralization solution was used. Treatments were performed with either a sodium fluoride solution (367 ppm F simulating a 1100 ppm F dentifrice after 1:2 dilution - groups 24 h/a, 48 h/a and 96 h/a) or deionized water (groups 24 h/b, 48 h/b and 96 h/b), with storage in artificial saliva (2.20 g/L gastric mucin, 1.45 mmol/L $CaCl_2$ • 2 H_2O, 5.42 mmol/L KH_2PO_4, 6.50 mmol/L NaCl, 14.94 mmol/L KCl) all other times. The pH of the artificial saliva was adjusted to 7.0 using KOH [19].

The pH-cycling phase was conducted at room temperature and without stirring. Fresh artificial saliva and acid were used each day and fresh treatment solution was used prior to each treatment. The daily pH-cycling regimen is shown in table 1. Before the first treatment on day one, the specimens were placed in artificial saliva for 30 min to allow an artificial pellicle-like layer to form. After each treatment, the specimens were rinsed briefly under running deionized water. After the last treatment after ten days of pH-cycling, the specimens were placed in artificial saliva for 30 min before being rinsed under a steady stream of deionized water. The prepared specimens were then stored in 100% relative humidity at 4 °C until further use.

Measurements/Analyses

Surface Hardness

The specimens were mounted individually on 1-inch acrylic blocks using sticky wax. The center portion, approximately 5×3 mm, of the specimens was used to measure the HK (Fig. 1). At the sound stage, a total of five baseline indentations were made using the Knoop diamond indenter (2100 HT; Wilson Instruments, Norwood, MA, USA) with a 50-gram load along a line parallel to the external surface of the specimen approximately 100 μm apart from each other, and a dwell time of 11 seconds. The Knoop hardness number (KHN) for each

specimen was derived by calculating the mean of the length of the long diagonal of the five indentations. Specimens were then randomly divided into six groups based on the sound enamel KHN ensuring equal distribution of the specimens with low (<354), medium (354-375), and high KHN (>375) between the groups.

After demineralization and pH-cycling, a second and third set of five indentations were made to the left and right of the sound enamel indentations, respectively. The indentations were made approximately 100 μm apart from each other and approximately 200 μm from the sound enamel indentations. The extent of re-hardening, referred to as SMH recovery (%SMHr), was then calculated using the formula by Gelhard et al. [20]:

$$\%SMHr = \frac{D - R}{D - B} \times 100$$

Where *B* is the mean indentation length (μm) of the sound enamel specimen at baseline, *D* is the mean indentation length (μm) after demineralization, and *R* is the mean indentation length (μm) after pH-cycling.

Energy-Dispersive X-Ray Spectroscopy

Three portions of the specimens (Fig. 1) were cut off at the sound stage (Section B), after demineralization (Section C) and after pH-cycling (Section D) using a hard tissue microtome (Silverstone-Taylor Hard Tissue Microtome, Scientific Fabrications Laboratories, Lafayette, CO, USA) approximately 100 μm in thickness. Any section thicker than 120 μm was hand-polished using 2400-grit silicon carbide paper to the required thickness.

The sections were subjected to energy-dispersive X-ray spectroscopy (EDS, JEOL 7800F; JEOL, Peabody, MA, USA). Analysis was done using the EDS detector (EDAX,

Octane Super Detector) operating at 10 kV accelerating voltage to measure the content of calcium (Ca), phosphorus (P), fluorine (F), carbonate (C), magnesium (Mg) and nitrate (N) in weight percent at the surface of the enamel. A horizontal line scan was made at the surface of each specimen section, measuring 100 μm in length, and the mean weight percent of the chemical elements along that line was calculated.

Transverse Microradiography

Following EDS analysis, sections C (demineralized) and D (pH-cycled after demineralization) of the specimens were mounted with an aluminum step wedge on high-resolution glass plates type I A (Microchrome Technology, San Jose, CA, USA). The sections were placed in the TMR-D system and radiographed at 45 kV and 45 mA at a fixed distance for 12 seconds. The digital images were analyzed using the TMR software v.3.0.0.18 (Inspektor Research Systems, Amsterdam, The Netherlands). A window approximately 400 × 400 μm representing the entire lesion and not containing any cracks, debris, or other alterations was selected for analysis.

The following variables were recorded for each specimen: lesion depth (L), integrated mineral loss (ΔZ), and the maximum mineral content of the surface layer (SZ_{max}). The % mineral profile of each enamel specimen's demineralized and remineralized lesion was compared with the mean sound enamel % mineral profile [21]. The difference between the areas under the densitometric profile of the demineralized lesion and the mean sound enamel is represented by $\Delta Z_{d.}$ The difference between the areas under the densitometric profile of the remineralized lesion and the mean sound enamel is represented by ΔZ_r. These parameters were then converted to % change values after remineralization, as such, % remineralization (%R) represents the % change in ΔZ values:

$$\%R = \frac{\Delta Z_d - \Delta Z_r}{\Delta Z_d} \times 100$$

Statistical Analysis

The mean and standard deviations of KHN, L, ΔZ, SZ_{max}, and surface weight% of Ca, P, F, C, Mg and N were obtained and analyzed using three-way ANOVA, with factors for stage (sound, demineralized, and pH-cycled), demineralization time (24, 48 or 96 hours) and treatment (fluoride solution or deionized water), as well as all two-way and three-way interactions among the factors. The mean and standard deviations of %SMHr and %R were also obtained and analyzed using two-way ANOVA with factors for demineralization time and treatment. A repeated effect for stage was added to the model. The normality of the data in ANOVA's analysis was validated. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%. Pearson correlation coefficients were used to evaluate the associations among KHN, L, ΔZ, SZ_{max}, and surface weight% of Ca, P, F, C, Mg and N at the sound stage, after demineralization and after pH-cycling. Data was analyzed using software SAS Version 9.4 (SAS Institute).

Results

Surface Hardness

Table 2 provides the data for KHN and %SMHr for all groups. The KHN was significantly different between stages, demineralization times and treatments ($p<0.0001$). The two-way interactions between stage and demineralization time, as well as between stage and treatment were significant ($p<0.0001$). The three-way interaction among stage, demineralization time and treatment was also significant ($p=0.0001$). The %SMHr demonstrates that all groups re-hardened following pH-cycling, but the re-hardening was

significantly different between treatments ($p<0.0001$), being greater in fluoride than non-fluoride groups.

Transverse Microradiography

Table 3 provides the data for L, ΔZ, SZ_{max} and %R. L was significantly different between stages ($p=0.003$), demineralization times ($p<0.0001$) and treatments ($p=0.007$). ΔZ was also significantly different between stages, demineralization times and treatments ($p<0.0001$). L and ΔZ were significantly greater after demineralization than after pH-cycling in all groups, irrespective of the treatment received, except group 24 h/b (demineralized for 24h and received deionized water treatment), in which there was no significant difference after demineralization and after pH-cycling. SZ_{max} was significantly different between stages and treatments ($p<0.0001$), but not between demineralization times ($p=0.201$). Specimens had significantly higher surface zone mineralization after pH-cycling than after demineralization in all groups, irrespective of the treatment received. Within treatments, specimens that received fluoride had significantly higher mineral density of the surface zone than those that did not. The positive values for %R indicate that all groups remineralized following pH-cycling, irrespective of the treatment received, except group 24 h/b, which further demineralized.

Energy-Dispersive X-Ray Spectroscopy

The surface weight% of Ca, P, F, C, Mg and N for all groups at three stages (sound, after demineralization and after pH-cycling) is shown in Figure 2. The surface weight% of Ca, P, C, Mg and N was not significantly affected by demineralization time or treatment. Figure 2 also demonstrates the Ca:P ratio for all groups at three stages. The surface weight% of F was significantly affected by treatment, as specimens that received fluoride had higher surface

fluorine weight% than specimens that did not ($p<0.0001$), irrespective of demineralization time, as shown in Figure 3.

Correlations Between Variables

A weak positive correlation was found between sound KHN and KHN after demineralization ($r=0.31$, $p=0.002$), however, there was no correlation between sound KHN and KHN after pH-cycling ($r=0.07$, $p=0.49$).

After demineralization, there was a statistically significant but weak negative correlation between KHN and L ($r=-0.25$, $p=0.017$), between KHN and ΔZ ($r=-0.32$, $p=0.002$) and between the surface weight% of F and SZ_{max} ($r=0.24$, $p=0.019$). Furthermore, a weak positive correlation was found between KHN after demineralization and after pH-cycling ($r=0.35$, $p<0.0001$). A strong positive correlation was only found between L and ΔZ after demineralization ($r=0.91$, $p<0.0001$).

After pH-cycling, there was a statistically significant moderate correlation between KHN and L ($r=-0.35$, $p<0.0001$), between KHN and ΔZ ($r=-0.49$, $p<0.0001$), and between KHN and SZ_{max} ($r=0.58$, $p<0.0001$). The KHN after pH-cycling was found to be greater when the surface weight% of F was greater ($r=0.32$, $p=0.002$), although this correlation was weak. Moreover, a moderate correlation was found between the surface weight% of F and SZ_{max} ($r=0.43$, $p<0.0001$). Additionally, SZ_{max} showed a moderate negative correlation with both L ($r=-0.42$, $p<0.0001$) and ΔZ ($r=-0.55$, $p<0.0001$). A strong positive correlation was also found between L and ΔZ after pH-cycling ($r=0.91$, $p<0.0001$).

Discussion

Our study is the first investigation throughout all three stages of enamel behavior (sound, after demineralization and after pH-cycling) using multiple methods, such as hardness, chemical composition and contact microradiography. The null hypothesis was partially rejected; a correlation was found between the hardness and fluorine content and between the susceptibility to de- and remineralization, but no correlation was found for any of the other chemical elements examined.

Microhardness tests provide information on the physical property of surface enamel in response to de- and remineralization protocols. Microhardness testing has been proven to be a valid method to measure alterations in dental hard tissue [22,23]. In this current study, enamel hardness decreased as demineralization time increased, although there was no significant difference in KHN between 48 and 96 hours of demineralization. Conversely, KHN increased following pH-cycling in both fluoride and non-fluoride groups, with fluoride groups showing a greater increase. The extent of re-hardening, namely the %SMHr, showed that lesions that were demineralized longer were less able to re-harden following pH-cycling, irrespective of treatment received. This behavior is in accordance with other studies [11,24,25]. %SMHr results also demonstrated that fluoride was able to cause significantly greater enamel re-hardening than non-fluoride groups. The role of fluoride in reducing enamel demineralization and enhancing remineralization [9,14] as well as increasing the rate of enamel re-hardening in vitro [9,26] have been previously established. However, even in the absence of fluoride, salivary pellicle seems to offer a protective effect against demineralization of enamel by providing some, although not much, re-hardening of the enamel surface [27,28,29].

Transverse microradiographs of the lesions produced in this study showed a tendency for greater mineral loss and lesion depth with increased demineralization time. This relationship has been shown in several studies [25,30,31]. On the other hand, following pH-cycling, all groups demonstrated a decrease in lesion depth and gain in mineralization, irrespective of treatment received. This is further confirmed by the positive values of % remineralization. However, this was not true for specimens that were demineralized for 24 hours and did not receive fluoride. This group conversely demonstrated no net remineralization and instead further demineralized. This can be explained by the behavior of smaller lesions during dissolution. Smaller lesions are thought to have greater solubility than larger ones, or those that are demineralized for longer, and thus have a greater tendency to demineralize further [16,32]. This is likely caused by a decrease in the solubility of the lesions with continued demineralization as a result of modification in the chemical composition. As specimens are placed in demineralization solutions, the more soluble material in the lesion (i.e. magnesium and carbon) is removed and the HAP is re-precipitated without the magnesium and carbonate, thereby reducing lesion solubility [5]. Conversely, larger lesions have a greater ability to remineralize. Possible reasons include their greater porosity allowing more diffusion of remineralizing solutions, and greater enamel area per unit volume of remineralizing solution. Alternatively, smaller lesions reach SZ_{max} faster than larger lesions, thereby allowing larger lesions greater time to remineralize [15]. This is confirmed in the present study by the %R, which shows that larger, more demineralized lesions exhibited more remineralization after pH-cycling.

Fluoride, even in concentrations available in over-the-counter toothpastes, can enhance remineralization of lesions and form a highly mineralized surface layer in initially demineralized enamel and dentin specimens [14,32-34]. The maximum mineral density of the

surface layer in the tested groups exhibited greater mineralization in the presence of fluoride than in its absence [35]. In the presence of fluoride, further dissolution is prevented as a result of re-precipitation of the dissolved minerals in the form of a fluoride-rich surface layer. Nonetheless, this surface layer was present even in the non-fluoride groups. Salivary pellicle has been shown to have a protective effect on the surface of enamel [29], and can prevent demineralization of the surface layer even in the absence of fluoride [36]. Likewise, microradiograms of both fluoridated and non-fluoridated enamel samples were able to show a distinct mineralized surface zone [37].

The results of the energy-dispersive x-ray spectroscopy showed that the chemical composition of the surface enamel did not change significantly for any of the chemical elements examined following demineralization. It has been previously demonstrated that when bovine enamel was demineralized for up to 8 days at a pH of 5, the calcium at the surface only changed a few percent in weight compared to that of the sound specimens [38]. The microradiographic analysis performed in this study confirmed mineral loss following demineralization and mineral gain following pH-cycling. This suggests that the minerals may have been lost and gained at a fixed ratio. This can be seen in the Ca:P ratio at the three stages; which remained relatively stable irrespective of demineralization time or treatment. Several studies have shown that the composition of enamel does not seem to differ between sound and carious teeth [40], and that the Ca:P ratio remains stable at various mineralization stages, which may indicate the stoichiometric dissolution and redeposition behavior of minerals in bovine enamel [9,39]. On the other hand, Sabel et al. [7] found significantly lower amounts of calcium and phosphorus parallel to greater amounts of carbon and nitrogen in lesions compared with sound enamel. However, this study was performed on primary human enamel, which is of greater porosity and has a higher tendency for dissolution [41,42].

Furthermore, the demineralization protocol used by Sabel et al. was done using methylcellulose gel for 30 days. Research regarding the appropriateness of replacing bovine for human teeth has shown that subtle morphological differences do exist between the two substrates, as both tissues behave similarly but not necessarily identically [25,43]. Bovine enamel was found to be more porous, and have higher carbonate [24] but lower fluoride contents [44]. However, the two behave similar enough to provide an acceptable alternative with the advantage of reduced variability of the hard tissue substrate [45].

In this study, the weight% of fluorine showed a significant positive correlation with the surface mineralization, which became stronger following fluoride treatment during pH-cycling. Additionally, a negative moderate correlation was found between the mineralization of the surface layer and both the lesion depth and integrated mineral loss of the specimens. This indicates that the presence of fluoride facilitated the incorporation of minerals into the lesion thereby decreasing susceptibility to further demineralization. This effect of fluoride in reducing enamel demineralization in a dose-dependent manner was previously established [14], as the authors were able to show that mineral loss decreased as fluoride concentrations increased from 70 to 280 ppm F. The existence of this relatively intact surface layer also functions to distinguish the subsurface caries lesions created in this study from the chemical etching of enamel [30].

Regarding the correlations between Knoop hardness numbers at various stages, it can be seen that specimens with greater hardness at the sound stage and following demineralization also had greater hardness after demineralization and pH-cycling, respectively, although these correlations were weak. If hardness is considered a measurement of the presence, mineralization, or thickness of the surface layer, as well as a measurement of the subsurface

demineralization, then these results may indicate that when a mineralized surface layer was present, the specimens maintained their structural integrity throughout de- and remineralization challenges. However, due to the weak correlation, this data should not be over-interpreted. Comparably, previous research did not find significant correlations between the indentation length of sound specimens and the change in indentation length after demineralization of the specimens for up to 48 hours, using either Knoop or Vickers indenters [25].

Following demineralization and pH-cycling, the susceptibility of enamel to remineralization was influenced by the hardness of the specimen after demineralization, and not its hardness at the sound stage. In other words, no correlation was found between the sound hardness of enamel and its de- or remineralization potential. Therefore, susceptibility of enamel to remineralization may be more dependent on its demineralized characteristics than sound baseline values. In this study, the hardness correlated weakly to moderately with the mineral loss and lesion depth determined by transverse microradiography. Previous studies have either shown similar [32,46], or conflicting [24,25,47] results. One possible explanation for the difference in results could be the protocol used for demineralization. Weaker correlations for Carbopol lesions, as used in this study, were found compared to the other demineralization protocols [32]. Additionally, deeper lesions with greater subsurface mineral loss, such as those produced with Carbopol in comparison to methylcellulose (MeC) or hydroxyethylcellulose (HeC) lesions, showed weaker relationships between hardness and TMR data [32,46]. Furthermore, the linearity between indentation length and lesion depth is strongly load dependent; and as such is much weaker for 50-gram loads, as used in this study, than 500-gram loads [47]. Interestingly, a significant correlation between hardness and surface zone mineralization could only be found after pH-cycling, which stresses the role of

fluoride in creating a highly mineralized surface layer which has re-hardened as a result of remineralization [48].

In the future, focus should be on studying the physical and chemical structure of natural white spot lesions. The similarity between human and bovine enamel does not eliminate the fact that bovine enamel is more porous and has higher carbon content that human enamel. Furthermore, lesions produced by different systems and with distinctive mineral distributions may influence the de- and remineralization characteristics.

In summary, the results from KHN showed that harder lesions after demineralization and after pH-cycling were less susceptible to demineralization, as they showed less lesion depth and integrated mineral loss, and greater surface zone mineralization. The demineralization potential of enamel after demineralization and pH-cycling was dependent on its hardness after demineralization and pH-cycling, respectively, and not on the sound characteristics of enamel. Regarding fluorine, its increase correlated with the increase in both hardness and surface zone mineralization. The increase in surface zone mineralization, in turn, made lesions less susceptible to demineralization, as they showed less lesion depth and integrated mineral loss measured by transverse microradiography. Following pH-cycling, fluoride and, to a lesser extent, non-fluoride groups were both able to remineralize. In the non-fluoride groups, shallower lesions had a greater tendency to further demineralize, while deeper lesions remineralized. The artificial saliva used in this study played a role in remineralization of the enamel in the deeper lesions, i.e. those that were demineralized longer, as evident by the increase in hardness, decrease in lesion depth, and the formation of a mineralized surface zone.

In conclusion, harder specimens and those with greater surface F weight% were less susceptible to demineralization and were more likely to remineralize. However, the amount of surface Ca and P did not influence de- or remineralization behavior.

Declaration of Interest

The authors declare that there is no conflict of interest.

Figure captions

Figure 1. Sections of each specimen. Section A was used for HK and shows the location of the first sound enamel indentation. Section B was used for EDS analysis of the sound specimen. Section C was used for EDS and TMR analysis of the demineralized specimen. Section D was used for EDS and TMR analysis of the pH-cycled specimen.

Figure 2. Mean weight% of chemical elements at the surface in six groups at different stages (error bars omitted for better clarity).

Figure 3. Mean weight% of fluorine at the surface in six groups at different stages (* indicate statistically significant differences between treatments). Error bar represents 1 SD.

Table 1. Daily pH-cycling treatment regimen.

Table 2. Means and standard deviations for KHN at different stages + least square means and standard error of the least square means for %SMHr

Table 3. Means and standard deviations for L, ΔZ and SZ_{max} at different stages + least square means and standard error of the least square means for %R

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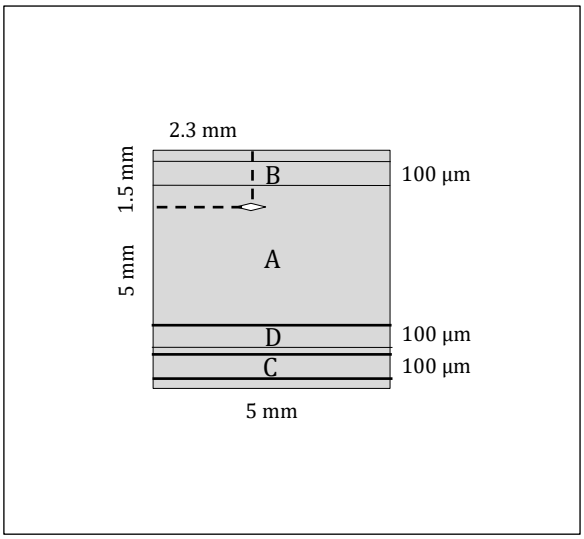
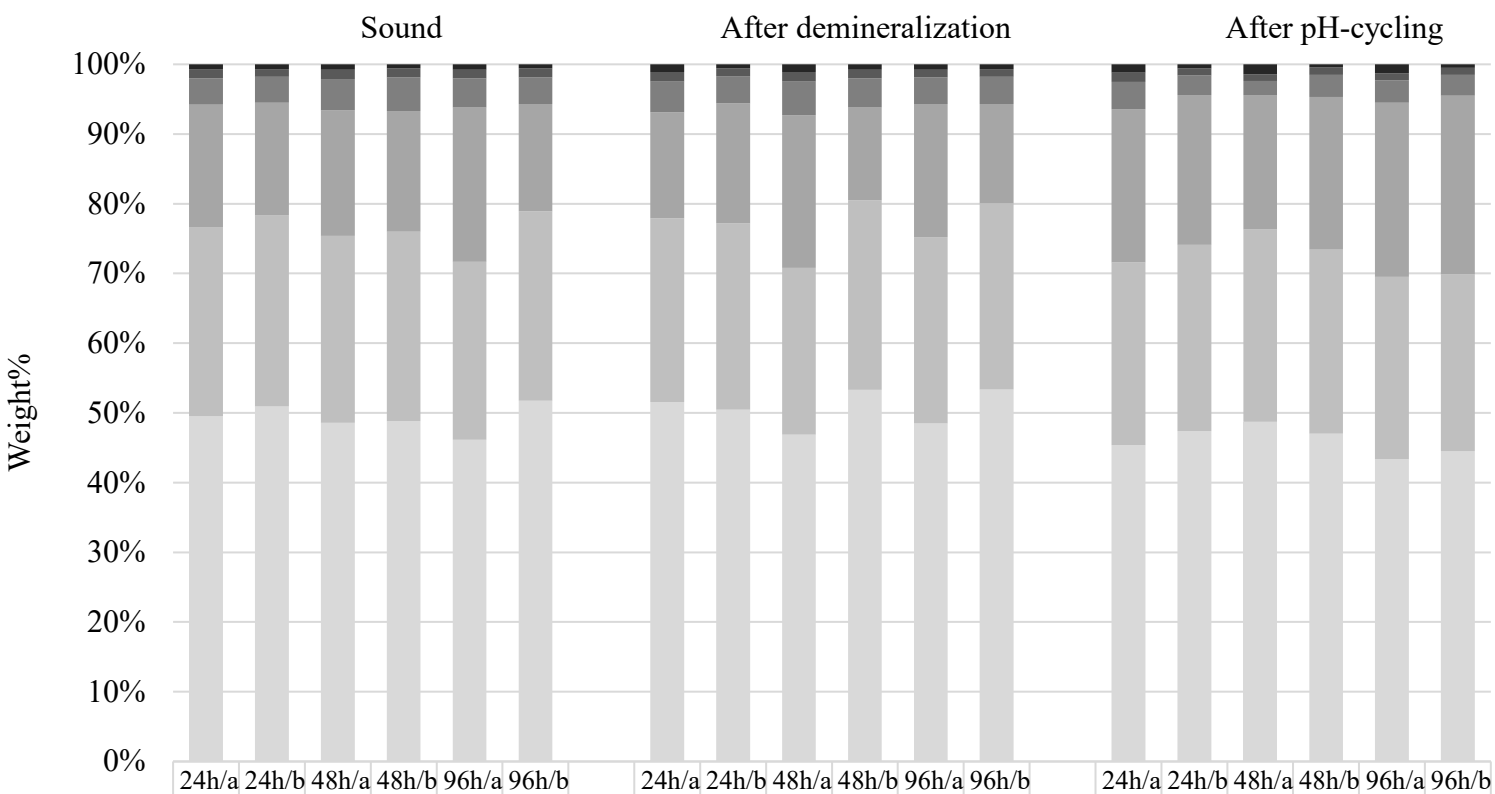


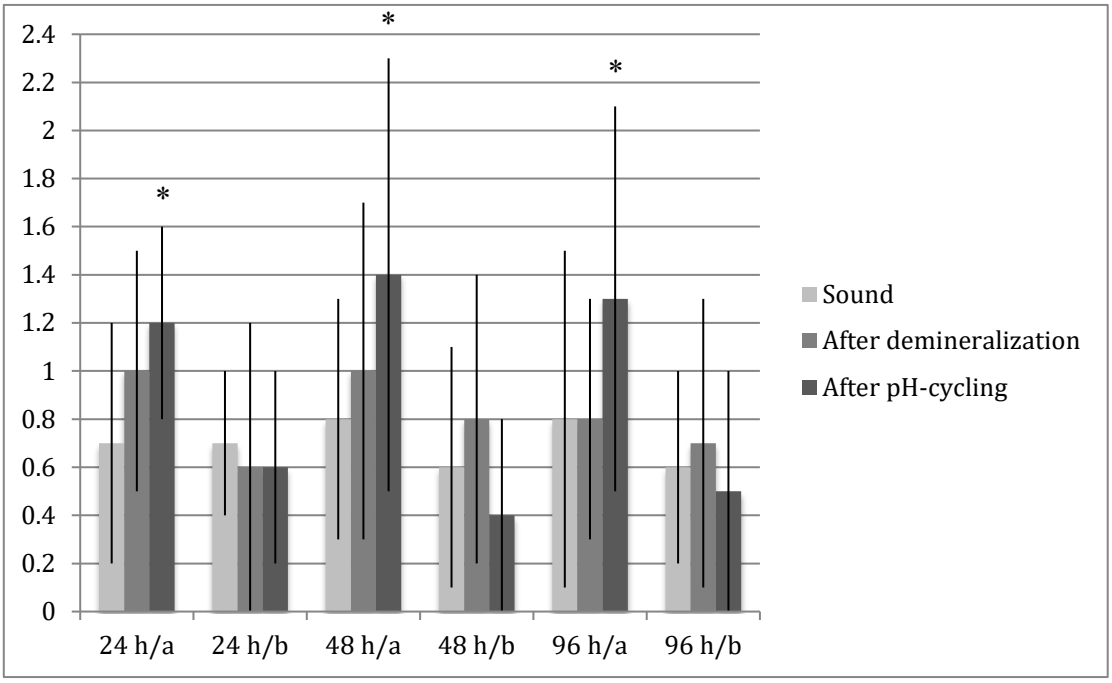
Fig. 1. Sections of each specimen. Section A was used for HK and shows the location of the first sound enamel indentation. Section B was used for EDS analysis of the sound specimen. Section C was used for EDS and TMR analysis of the demineralized specimen. Section D was used for EDS and TMR analysis of the pH-cycled specimen.



	Sound						After demineralization						After pH-cycling					
	24h/a	24h/b	48h/a	48h/b	96h/a	96h/b	24h/a	24h/b	48h/a	48h/b	96h/a	96h/b	24h/a	24h/b	48h/a	48h/b	96h/a	96h/b
Fluorine	0.7	0.7	0.8	0.6	0.8	0.6	1.1	0.6	1.1	0.8	0.8	0.7	1.2	0.6	1.4	0.4	1.3	0.5
Magnesium	1.3	1.1	1.3	1.3	1.2	1.3	1.4	1.1	1.4	1.2	1.1	1.1	1.3	1	1	1.1	1	1
Nitrate	3.8	3.7	4.5	4.8	4.2	3.8	4.4	3.9	4.8	4.2	3.8	3.9	3.9	2.8	2	3.2	3.2	3
Carbonate	17.6	16.1	18	17.3	22.1	15.4	15.2	17.2	21.9	13.3	19.1	14.2	22	21.5	19.3	21.9	25	25.6
Phosphorus	27	27.5	26.8	27.2	25.5	27.2	26.4	26.7	23.9	27.2	26.7	26.7	26.2	26.7	27.6	26.4	26.2	25.4
Calcium	49.5	50.9	48.6	48.8	46.1	51.8	51.6	50.4	46.9	53.3	48.5	53.3	45.3	47.4	48.7	47	43.4	44.5
Ca:P ratio	**1.8**	**1.9**	**1.8**	**1.8**	**1.8**	**1.9**	**1.9**	**1.9**	**1.9**	**1.9**	**1.8**	**1.9**	**1.7**	**1.8**	**1.8**	**1.8**	**1.7**	**1.8**

a: fluoride treatment b: DI water treatment

Fig. 2. Mean weight% of chemical elements at the surface in six groups at different stages (error bars omitted for better clarity).



a: fluoride treatment b: DI water treatment

Fig. 3. Mean weight% of fluorine at the surface in six groups at different stages (* indicate statistically significant differences between treatments). Error bar represents 1 SD.

Table 1. Daily pH-cycling treatment regimen

Time frame	Specimen treatment
8:00-8:01 a.m.	Treatment
8:01-9:00 a.m.	Artificial saliva
9:00-9:01 a.m.	Treatment
9:01-10:00 a.m.	Artificial saliva
10:00 a.m.-2:00 p.m.	Acid challenge
2:00-3:00 p.m.	Artificial saliva
3:00-3:01 p.m.	Treatment
3:01-4:00 p.m.	Artificial saliva
4:00-4:01 p.m.	Treatment
4:01 p.m.-8:00 a.m. (following day)	Artificial saliva

Table 2. Means and standard deviations for KHN at different stages + least square means and standard error of the least square means for %SMHr

Demineralization time	Treatment	n	KHN_{sound}	$KHN_{post\ demin}$	$KHN_{post\ pH\text{-}cycling}$	%SMHr, %
24 h	Fluoride	15	364.7±15.7 A	74.5±12.3 Ca	205.6±13.7 Ba#	72.6±2.6 #
	DI water	18	365.0±24.5 A	76.3±19.7 Ca	107.6±23.1 Ba	28.6±2.4
48 h	Fluoride	15	357.5±23.1 A	50.5±21.2 Cb	178.2±35.3 Bb#	75.0±2.6 #
	DI water	15	361.3±26.0 A	60.2±10.5 Cb	96.2±13.7 Bab	34.6±2.6
96 h	Fluoride	15	367.0±23.5 A	61.3±12.5 Cb	151.3±26.6 Bc#	61.1±2.6 #
	DI water	16	370.5±23.2 A	53.1±23.3 Cb	88.8±33.4 Bb	36.3±2.52

Uppercase letters indicate statistically significant differences in KHN between stages.

Lowercase letters indicate statistically significant differences in KHN between demineralization times.

indicate statistically significant differences in KHN and %SMHr between treatments within each demineralization time.

Table 3. Means and standard deviations for L, ΔZ and SZ_{max} at different stages + least square means and standard error of the least square means for %R

Demineralization time	Treatment	n	Stage	ΔZ, vol%min x µm	L, µm	SZ_{max}, vol%min	%R, %
24 h	Fluoride	15	After demineralization	737±325 A*	36±20 A*	64±8 A*	
			After pH-cycling	397±292 Ba#	27±18 Ba#	79±5 Ba#	44.3±13.6#
	DI water	18	After demineralization	718±341 A*	36±19 A*	62±8 A*	
			After pH-cycling	809±333 Aa	42±26 Aa	67±6 Ba	-33.1±12.4
48 h	Fluoride	15	After demineralization	947±411 A*	46±21 A*	65±8 A*	
			After pH-cycling	499±378 Ba#	34±22 Ba#	83±6 Bb#	37.6±13.6#
	DI water	15	After demineralization	1114±386 A*	52±15 A*	62±10 A*	
			After pH-cycling	859±294 Ba	41±9 Ba	70±6 Bb	14.9±13.6
96 h	Fluoride	15	After demineralization	1413±352 A	65±14 A	66±6 A*	
			After pH-cycling	839±688 Bb#	49±36 Bb#	81±6 Bb#	40.4±13.6#
	DI water	16	After demineralization	1724±493 A	79±26 A	62±7 A*	
			After pH-cycling	1363±653 Bb	68±28 Bb	71±6 Bb	20.7±13.2

Uppercase letters indicate statistically significant differences in L, ΔZ and SZ_{max} between stages.

* indicate statistically significant differences in L, ΔZ and SZ_{max} between demineralization times after demineralization.

Lowercase letters indicate statistically significant differences in L, ΔZ and SZ_{max} between demineralization times after pH cycling.

indicate statistically significant differences in L, ΔZ, SZ_{max} and %R between treatments within each demineralization time.