Comparison of cross-sectional hardness and transverse microradiography of artificial carious enamel lesions induced by different demineralising solutions and gels


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Abstract

The aims of this study were: (1) to correlate surface (SH) and cross-sectional hardness (CSH) with microradiographic parameters of artificial enamel lesions; (2) to compare lesions prepared by different protocols. Fifty bovine enamel specimens were allocated by stratified randomisation according to their initial SH values to five groups and lesions produced by different methods: MC gel (methylcellulose gel/lactic acid, pH 4.6, 14 days); PA gel (polyacrylic acid/lactic acid/hydroxyapatite, pH 4.8, 16 h); MHDP (undersaturated lactate buffer/methyl diphosphonate, pH 5.0, 6 days); buffer (undersaturated acetate buffer/fluoride, pH 5.0, 16 h), and pH cycling (7 days). SH of the lesions (SH(1)) was measured. The specimens were longitudinally sectioned and transverse microradiography (TMR) and CSH measured at 10- to 220-microm depth from the surface. Overall, there was a medium correlation but non-linear and variable relationship between mineral content and radical CSH. RadicalSH(1) was weakly to moderately correlated with surface layer properties, weakly correlated with lesion depth but uncorrelated with integrated mineral loss. MHDP lesions showed the highest subsurface mineral loss, followed by pH cycling, buffer, PA gel and MC gel lesions. The conclusions were: (1) CSH, as an alternative to TMR, does not estimate mineral content very accurately, but gives information about mechanical properties of lesions; (2) SH should not be used to analyse lesions; (3) artificial caries lesions produced by the protocols differ, especially considering the method of analysis.
Comparison of cross-sectional hardness and transverse microradiography of artificial carious enamel lesions induced by different demineralising solutions/gels

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Abstract
The aims of this study were: 1) to correlate the data of surface (SH) and cross-sectional hardness (CSH) versus mineral content, surface layer and lesion depth (TMR) and 2) to compare the artificial lesions prepared by different protocols. Fifty bovine enamel samples were allocated by stratified randomization according to their SH values into five groups produced by different methods: MC gel (8% methylcellulose gel + 0.1 M lactic acid, pH 4.6, 14days); PA gel (20 g/L polyacrylic acid + 0.1 M lactic acid, with 500 mg/L hydroxyapatite, pH 4.8, 16h); MHDP (50 mM lactic acid + calcium, phosphate and methyl diphosphonate, pH 5.0, 6days); Buffer (50 mM acetic acid + calcium, phosphate and fluoride, pH 5.0, 16h); and pH cycling. √SH₁ was calculated from the final surface hardness (SHᵢ). The samples were then longitudinally sectioned and sections were subjected to TMR and CSH at 10 to 220 µm depth from the surface. Overall, there was a medium correlation but non-linear relationship between mineral content and CSH or √CSH, except for MHDP and pH cycling. MHDP produced the highest subsurface mineral loss, followed by pH cycling, buffer, PA gel and MC gel. The conclusions were: 1) CSH, used as alternative to TMR, is not very accurate for estimating mineral content, but it gives information about the mechanical properties of lesions. However, SH should not be used to analyse lesions. 2) artificial caries lesions produced by the protocols differ, especially when considering the method of analysis.
Introduction
Artificially enamel caries lesions are commonly created to simulate in vivo caries development. In vitro models for producing enamel caries lesions are able to simulate the dynamics of mineral loss and gain, with the advantages of being standardized as well as fast and easy to perform. These models allow a better understanding of the interaction between de- and remineralisation processes and of factors affecting these processes (e.g. efficacy of fluorides) [White, 1995].

Enamel samples are usually exposed to demineralising solutions and gels composed of either acetic or lactic acid (pH between 4.5-5.0) undersaturated regarding apatite, in order to simulate the plaque fluid conditions and, consequently, allow the formation of initial enamel lesions [ten Cate and Duijsters, 1982; Edgar, 1983; Buskes et al., 1985; White, 1987; ten Cate et al., 1996; Queiroz et al., 2008; Kielbassa et al., 2005; Vieira et al., 2005; Lynch et al., 2007]. Differences among these solutions or gels, such as initial degree of saturation with respect to enamel minerals, fluoride concentration, kind of acid and viscosity can result in remarkable differences in physical and mechanical characteristics of the demineralised enamel, such as mineral distribution characteristics [Arends et al., 1987], chemical composition [Lynch and ten Cate, 2006] and hardness. Although de- and remineralisation of dental enamel have been extensively studied over the past 2–3 decades, relatively little work has been reported about the mineral content, depth and mechanical properties of artificial lesions produced by different demineralising procedures. As it is required that the demineralising procedures induce caries-like (subsurface lesion with a less-demineralised surface layer) rather than erosion-like lesions, a comparison of the different solutions or gels seems necessary. It is important to point out that the kind of lesion has influence on the effect of subsequent de- or remineralisation, as the surface layer, porosity and depth of a lesion can play an important role in mineral diffusion [Lynch et al., 2007].

Depth-related properties of artificial lesions can be described by mineral content and hardness profiles. Transverse microradiography demonstrates a quantitative measure of the amount of mineral, depth and surface layer. On the other hand, cross-sectional hardness reflects the mechanical resilience of enamel. To compare the properties of differently induced artificial caries lesions, preferably combined cross-sectional hardness measurements (mechanical test) and transverse microradiography profiles (mineral content) of the same lesions should be performed. Comparative data from microradiography and microhardness measurements are scarce but have shown some correlation [Featherstone et al., 1983; Kielbassa et al., 1999]. However, the equations for converting microhardness to mineral content seem to differ notably. This indicates that the calculation of the mineral content from cross-sectional microhardness data
may not be reliable. The relationship between the two measurements could be influenced by a variety of factors, and might differ between lesions created in situ [Kielbassa et al. 1999] and in vitro [Featherstone et al. 1983]. Additionally, surface hardness has been extensively used for quantifying dental caries lesion in vitro over years [White, 1988; Magalhães et al., 2008]. It was previously shown that indentation lengths reflect the demineralisation degree of lesion despite the presence of the surface layer [Arends et al., 1979], the mineral content of surface layer as well lesion depth [Arends et al., 1980]. However, depending on the surface softening, the penetration depth of the diamond into the lesion might be around 10 µm, thus, might not reflect deeper alterations. Therefore, it is not known if surface hardness analysis might reflect depth alterations of carious dental tissues and is able to detect differences among the lesions provoked by various acid solutions and gels.

Thus, the aims of this study were: 1) to correlate the data of surface (SH) and cross-sectional hardness (CSH) versus mineral content, surface layer and lesion depth (TMR) and 2) to compare the artificial lesions prepared by different protocols.

**Material and Methods**

**Specimen preparation**

Enamel specimens (4X4X2.5 mm) were prepared from 50 bovine incisors, which were freshly extracted and stored in 0.9% NaCl plus 0.1% thymol solution (pH 7.0). The teeth were cut using an ISOMET Low Speed Saw (Buehler Ltd. Lake Bluff, IL, USA) and two diamond disks (Extec Corp., Enfield, CT, USA), which were separated by a 4-mm wide spacer. The enamel surface of the samples was ground flat with water-cooled silicon carbide discs (320, 600 and 1200 grades papers; Buehler, Lake Bluff, IL, USA), and polished with felt paper wet using diamond spray (1 µm; Buehler), resulting in removal of about 100 µm of the outer enamel. This was controlled with a micrometer.

**Demineralisation procedures**

The samples were allocated to five groups (n = 10) by stratified randomisation according to their surface hardness (SH) means (368 ±0.18 KHN/group). SH determination is described below.

In the MC gel group, the samples were covered with 0.5 cm 8% methylcellulose gel which was left to set overnight at 4°C, then covered with a equal volume (1.5 mL) of 0.1 M lactic acid, pH adjusted to 4.6 with 1 M KOH and incubated for 14 days [ten Cate et al., 1996]. In the PA gel group, lesions were created using the demineralisation gel of White [1987] containing 20 g/l Carbopol 907 (polyacrylic acid, MW 450,000 D), 500
mg/l hydroxyapatite and 0.1 M lactic acid, pH 4.8 for 16 h. Each enamel sample was placed in 25 ml of demineralisation fluid gel [Iijima et al., 2004]. In the MHDP group, each sample was immersed in 30 mL of acid buffer containing 3 mM CaCl₂·2H₂O, 3 mM KH₂PO₄, 50 mM lactic acid, 6 µM methyl diphosphonate, KOH to adjust the initial pH to 5.0 and traces of thymol [Buskes et al., 1985], for 6 days. In the Buffer group, the enamel samples were immersed in 32 mL of 50 mM acetate buffer solution containing 1.28 mM Ca(NO₃)₂·4H₂O, 0.74 mM NaH₂PO₄·2H₂O and 0.03 ppm F at pH 5.0 for 16 h [Queiroz et al., 2008; Magalhães et al., 2008].

In the pH cycling group, the samples were subjected to pH-cycling for seven days according to Vieira et al. [2005]. During 5 days, the samples were immersed in demineralisation solution [2.0 mM Ca(NO₃)₂·4H₂O, 2.0 mM NaH₂PO₄·2H₂O, 0.075 mM acetate buffer, 0.02 ppm F at pH 4.7 using 30 mL/sample] for 6 h and in remineralisation solution [1.5 mM Ca(NO₃)₂·4H₂O, 0.9 mM NaH₂PO₄·2 H₂O, 150 mM KCl, 0.1 mol/L Tris buffer, 0.03 ppm F at pH 7.0 using 15 mL/sample] for 18 h. In the last 2 days, the samples were maintained only in remineralisation solution. In all groups, the samples were first protected by wax, exposing only the enamel surface (4x4 mm) and then separately immersed in unstirred solutions or gels at 37°C. Table 1 summarizes the degrees of saturation with respect to enamel minerals, pH and exposure time. The degree of saturation was calculated using a software program [Shellis, 1988].

**Hardness Measurement**

Initially, enamel surface hardness (SH) was measured using microhardness tester (HMV-2000; Shimadzu Corporation, Tokyo, Japan), using a Knoop diamond with a load of 25 g applied for 10 s. Five indentations, 100 µm apart, were made in the center of enamel samples (SH₀). After the treatments, final surface hardness measurement (SH₁) was performed. The square root of surface hardness (√SH₁), which is proportional to indentation length [White, 1988], was calculated to allow for comparison with the results of previous studies [Arends et al., 1979; Arends et al., 1980].

To perform cross-sectional hardness (CSH) tests, the samples were sectioned perpendicularly to the surface through the center. One half of each sample was embedded in acrylic resin and polished as described before, while the other half was used for TMR analysis. Three rows of 8 indentations each were made, one in the central region of the dental enamel exposed and the other two at 100 µm distance to both sides of the central row of indentations below and above using a 25 g load for 10 s. The indentations were made at 10, 30, 50, 70, 90, 110 and 220 µm from the outer
enamel surface. The mean values of all 3 measuring points at each distance from the surface were averaged.

**Transverse Microradiography (TMR)**

A section was cut with a diamond band saw perpendicularly to the exposed surface of the specimens and hand-polished plane-parallel from both cut sides with SiC (silicone carbide) paper up to FEPA P4000 under continuous water-cooling to a thickness of 138±7.6 µm. The sections were allowed to dry under ambient conditions. A microradiograph of each section together with an aluminum calibration step wedge with 14 steps was taken. High-speed holographic film (SO 253; Kodak AG, Stuttgart, Germany) were exposed with Ni-filtered quasi-monochromatic Cu Kα X-rays (λ = 0.154 nm) from a 1x10 mm focus X-ray tube (PW2233/20; Philips, Kassel, Germany) at 20 kV and 20 mA (PW 3830 generator; Philips) for 15 s. The film-focus distance was 40 cm. The developed film was analysed using a transmitted light microscope with x 20 objective (Axioplan; Zeiss, Oberkochen, Germany) with a CCD camera (XC-77CE, Sony, Tokyo, Japan) and a PC with framegrabber and data acquisition and calculation software (TMR 1.25e; Inspector Research BV, Amsterdam, The Netherlands). The analogue signal from the CCD camera was digitized with a framegrabber (Flashpoint 3D; Integral Technologies, Indianapolis, IN, USA). A detail of 400 x 315 µm of the original tooth section was displayed and imaged by using the described parameters.

The mineral content was calculated from the specimen grey levels using the formula of Angmar et al. [1963], assuming the density of the mineral to be 3.15 kg/l. The mineral content of sound enamel was assumed to be 87 vol% [Angmar et al., 1963; de Josselin de Jong et al., 1987]. In order to allow direct comparison of the TMR data with data from hardness, the mineral content was considered at steps of 20 µm from 10 to 220 µm distant from the specimen surface, which are the same depths where the indentations were placed [Buchalla et al., 2008]. The lesion depth was calculated using a threshold of 95% of the mineral content of sound enamel (82.7%). Integrated mineral loss (ΔZ), the average mineral loss over the depth of the lesion (R), the mean thickness of the “relatively intact” surface layer (SL) and the maximum mineral content of the surface layer (Zmax) were also calculated [Arends et al., 1987; Theuns et al., 1984a].

**Statistical analysis**

Means, standard deviations (SD) and coefficients of variation were calculated from cross-sectional hardness and mineral content at every depth. Equality of variances and normal distribution of the data were tested for all the variables using the Bartlett and
Kolmogorov-Smirnov tests, respectively (GraphPad Instat for Windows version 4.0, San Diego, CA, USA). All data showed equal variances and normal distribution.

To analyse a possible relationship between CSH and mineral content, the data for each lesion type and the combined data for all lesions were submitted first to quadratic regression and then to linear regression, (Statistica, Statsoft, Tulsa, Oklahoma, USA). Mineral content was regressed on both hardness and on its square root [Featherstone et al., 1983; Kielbassa et al., 1999]. Additionally, the CSH values were converted to mineral content using the formulas of both Featherstone et al. [1983] (mineral content = 4.3√CSH + 11.3) and Kielbassa et al. [1999] (mineral content = 3.66√CSH + 21.19) and the correlation between these values and mineral content determined directly TMR was examined (Pearson’s coefficient, GraphPad Instat for Windows version 4.0, San Diego, CA, USA).

The correlations between √SH, and surface layer thickness (SL), maximum mineral content of the surface layer (Z_{max}), lesion depth, integrated mineral loss (ΔZ) and average mineral loss (R) were also examined (Pearson’s coefficient).

To analyse possible differences among the lesions created by different protocols, two-way repeated measures ANOVA and Bonferroni post hoc test were used (GraphPad Prism 4 version 4.0 for Windows, Graph Pad Software, San Diego, CA, USA), considering the lesion type and the different enamel depths as variables, separately for the cross-sectional hardness and mineral content (TMR, Featherstone and Kielbassa formulas).

The data for the mean percentage surface hardness change (%SHC) or √SH1, mean lesion depth, mean surface layer thickness and integrated mineral loss (ΔZ) passed the normality test, but the variances were not homogeneous. Therefore, these data were compared using Kruskal-Wallis followed by Dunn’s multiple comparisons test. On the other hand, the average mineral loss (R) and maximum mineral content of the surface layer (Z_{max}) were compared by ordinary ANOVA followed by Tukey’s test (GraphPad Instat for Windows version 4.0, San Diego, CA, USA).

The level of significance for all tests was set at 5%.

Results

General
Cross-sectional hardness and mineral content profiles of the five types of lesion are given in Figures 1 and 2, respectively. Hardness was within a range of 42-347 KHN, and mineral content was within a range of 42-89%. Generally, the surface layer was visible in the mineral content profiles, but not in the hardness profiles. The mean
coefficient of variation was higher for hardness (25.5%) than for TMR values (6.3%) and the relative error in hardness was higher than that in TMR at each single measurement point (Figure 3a/b).

**Relationships between hardness and mineral content**

For quadratic regression, stronger relationships were found between mineral content and CSH for the total data and for MC gel, MHDP and pH-cycling lesions, while for PA gel and Buffer lesions a stronger relationship was seen for $\sqrt{\text{CSH}}$. For linear regression, the relationship was stronger for $\sqrt{\text{CSH}}$, except for pH-cycling, where the relationship was stronger for CSH. In Table 2 only the regressions for the X-variable showing the stronger relationships (greater $r^2$) are shown.

The quadratic and linear fits between CSH or $\sqrt{\text{CSH}}$ and mineral content varied from weak (MHDP: $r^2 = 0.20$) to strong (MC gel, PA gel and Buffer: $r^2 \sim 0.8$) (Table 2, Figure 4). For the combined lesions data only moderate relationships were found (quadratic $r^2 = 0.53$ for CSH; linear $r^2 = 0.48$ for $\sqrt{\text{CSH}}$). However, as, for all lesion types except for MHDP and pH cycling, the quadratic slope was significant, so it would not be valid to apply the linear regressions.

For the combined data for all lesions, mineral contents calculated by the formulas of both Featherstone et al. [1983] and Kielbassa et al. [1999], were moderately correlated with mineral content ($r = 0.69$, $p < 0.001$), although there was considerable scatter, which increased as mineral content decreased (Figure 5 a/b).

Overall, the correlation of surface hardness ($\sqrt{\text{SH}_1}$) with SL, R, depth and Z max ranged from low to medium ($p<0.05$) (Table 4). Surface hardness ($\sqrt{\text{SH}_1}$) and $\Delta Z$ presented a low and not significant correlation for the combined data of all lesions. Considering each group separately, the best correlation between surface hardness and radiographic parameters was seen for SL in MC gel group ($r=0.65$, $p=0.04$, Figure 6).

**Differences between types of lesion**

The protocols produced enamel lesions with significant differences in surface and subsurface hardness as shown in Table 3 and Figure 1. Taking into account the data of mineral content and cross-sectional hardness, the comparisons between the protocols were quite different, showing that CSH and TMR did not give the same result for the comparisons among the lesions (Figures 1 and 2).

Overall, subsurface lesions were produced with a mean depth between 35 and 52 µm, except for MHDP lesions (86 µm). The integrated mineral loss was higher for MHDP and pH cycling lesions than for MC gel lesions ($p=0.005$). However, higher average mineral losses (R) were found in buffer
and PA gel lesions; Buffer lesions differed significantly from MC gel, MHDP and pH cycling lesions (p = 0.007), similarly to surface hardness (p<0.0001).

On the other hand, MC gel and MHDP lesions had a thicker SL, differing only from Buffer lesions (p = 0.003). The MC gel, pH cycling and MHDP lesions presented the highest mean values of $Z_{\text{max}}$, differing only from Buffer lesions (p < 0.001).

**Discussion**

Differently from data of previous studies [Featherstone et al., 1983; Kielbassa et al., 1999, Buchalla et al., 2008], no linear regression between cross-sectional hardness or square root of cross-sectional hardness and mineral content could be detected in the present study considering the data of all groups. Groups MC gel, PA gel and buffer presented a good correlation between root of hardness and mineral content (for quadratic). This finding is agreement with Featherstone et al. [1983], who presented only a slightly better $r^2$ (0.84, linear plotting of $\sqrt{\text{CSH}}$) for buffer type lesions, while slope and intercept were different from the present results. Despite the high $r^2$ for MC gel, PA gel and buffer, the curves seem not valid for estimating mineral content from hardness as they show a non-linear relationship.

In contrast, groups MHDP and pH cycling presented a linear relationship between mineral content and cross-sectional hardness. Although the linear relationship might provide a valid basis for estimating one variable (mineral content) from the other (cross-sectional hardness), the low $r^2$-value detected indicated a high scattering of the values. Therefore, such a relationship, although valid, is not very useful.

These results show that any kind of linear or non-linear relationship might be highly dependent of the kind of lesion used for analysis. For a better understanding of the different lesions, the analysis of the elements and/or the type of mineral in each depth of the lesion might be helpful.

It is important to point out that the variability of hardness data was high compared to the mineral content, which may be partly attributed to the different volumes that were “probed” by the indenter compared to the specificity of the x-ray. The measure of hardness at each first depth (up to ~30 µm) of the demineralised enamel is quite imprecise due to the size of indentation, which in turn makes difficult the delimitation of the depth (the distance between each indentation should be at least 20 µm). On the other hand, TMR measures the mineral content every 2 µm and it inaccurate only at the first 10 µm. Another limitation of the hardness measurement is to define the threshold to the values corresponding to the sound enamel. Because of this, the integrated mineral loss was not calculated and the lesion depth could not be defined from hardness data.
Although cross-sectional hardness cannot be used to estimate mineral content reliably, this method can still be used to analyse dental caries lesions, since it gives important evidence regarding the mechanical resilience of the demineralised enamel in depth. On the other hand, surface hardness should not be used alone to evaluate dental caries lesions, even though some significant but low correlations with TMR parameters were shown overall groups. However, when the type of lesions was evaluated separately, no correlation between $\sqrt{SH_1}$ and surface layer, $Z_{max}$, lesion depth, $R$ and integrated mineral loss could be shown.

Only for MC gel, a correlation between surface hardness and surface layer thickness was found (Figure 6). This result is in disagreement with previous data that showed that indentation length is related to mineral content of surface layer [Arends et al., 1980; White, 1988] and to the lesion depth [Arends et al., 1980]. Again, one possible explanation for the different results are the protocols used for producing demineralisation, i.e. Arends et al. [1980] used an acid gel (pH 4-5) for 2-8 days.

Another interesting result of the present study was that both formulas to convert hardness to mineral volume [Featherstone et al., 1983; Kielbassa et al., 1999] presented a medium correlation with TMR data, using the combined data of all lesions. It is important to point out again that the lesions created by Kielbassa et al. [1999] and Featherstone et al. [1983] were either in situ or in vitro lesions, respectively, which might account for the medium correlation. Considering the data of Figure 5a/b, most of the data points are bunched up at high mineral content/hardness (>80%, sound enamel). In this region, estimates of $X$ from $Y$ might be good, because a regression line has to pass through the bivariate mean, and the confidence band is always narrowest in this region. However, for values lower than 80% mineral content, the confidence band gets wider, meaning that the error associated with an estimate gets bigger. Therefore, the conversion of the cross-sectional hardness to mineral volume seems not to be adequate using these formulas, especially in the body of the lesions.

Additionally, the conversion of hardness to mineral volume should not be used, since the formulas and the TMR data showed different results when they were used to compare the 5 lesions at each depth (data not shown). This finding is also shown by Figures 1 and 2, giving more support to the hypothesis that the results are dependent on the protocol used for creating artificial lesions.

Regarding the protocols to prepare artificial caries lesions, in the present study, MHDP generally showed higher subsurface mineral loss and lesion depth than the other protocols. It is important to point out that the demineralisation is determined by many factors such as the pH (pH 4.5-5.0) which influences predominantly the rate of demineralisation and consequently, the time of the experiment [Theuns et al., 1984b],
as well as the content of undissociated acid concentration, degree of saturation, presence of inhibitors of enamel dissolution (F\(^{-}\) and proteins) and temperature [Arends and Christoffersen, 1986; Amaechi et al., 1998]. In the case of MHDP lesions, the results might be explained by the degree of saturation and the higher exposure time compared to PA gel and buffer lesions as well as by the volume and viscosity of the preparative solution compared to MC gel. Therefore, the solution by Buskes et al. [1985] might be used to produce a deep lesion.

For creating a typical subsurface lesion it is necessary to preserve the surface layer, which is influenced by many factors, such as the presence of calcium and phosphate [Groot et al., 1986], fluoride in liquid phase [Theuns et al., 1984c; Arends and Christoffersen, 1986] and the time after an initial demineralisation [Theuns et al., 1983]. Initial lesions normally do not show a surface layer; the surface layer is formed over time and its thickness, once formed, appears to be roughly constant [Theuns et al., 1984a,c; Arends and Christoffersen, 1986]. The mineral saturation might be reached with time, depending on the volume and the viscosity of demineralisation solution relative to the area of enamel exposed to demineralising solution (as in the case of MC gel). In this sense, it is important to point out that in the case of MC gel some reduction in calcium activity occurred as was shown by Lynch et al. [2006], due to the Ca-binding activity of methyl cellulose. This activity might have enabled the great mineral precipitation on surface layer, which in turn might have reduced the deep penetration of the acid and the subsurface mineral loss.

An interesting finding in this study was that the lesions with thicker surface layers (MHDP) did not necessarily present the highest maximum surface-layer mineral content (which occurred in MC gel lesions). Additionally, the maximum mineral content in the surface layers did not reach 70%. According to Arends and Christoffersen [1986], the surface layer covering an enamel lesion is a porous but still mineral-rich area, with an expected mineral content higher than 70%.

Generally MC gel, PA gel and Buffer lesions were shallow, but of these Buffer lesions showed the highest average mineral loss (R). On the other hand, the mechanism of lesion formation is different in pH cycling since, unlike the other methods, it involves both de- and re-mineralisation. pH cycles are important to test the efficacy and dose-response of fluoride products [Vieira et al., 2005]. The lesion depth produced by pH cycling was similar to those of the other types of lesion, but mineral loss was second only to that in MHDP lesions.

The different physical and mechanical properties of the lesions produced by these five protocols might influence the results of subsequent demineralisation and
remineralisation (such as saliva and fluoride) protocols. Therefore, further studies need to be performed to prove if the differences found in properties of the lesions produced by different systems might influence de-remineralisation in vitro and in situ. Furthermore, it is necessary to analyse in further studies which of the lesions produced by the protocols of the present study are more similar to natural white-spot lesions as discussed previously by Lynch and ten Cate [2006] and Lynch et al. [2007].

Thus, from the results of the present study it can be concluded that: 1) CSH, used as alternative to TMR, is not very accurate for estimating the mineral content, but it gives some information regarding the mechanical (physical strength) properties of the lesions, which are not provided by TMR. Therefore, it should be advised to combine different methods to analyse enamel demineralisation, in order to get more information about the properties of the lesions. However, the SH should not be used, as it is not related to surface or deep alterations in enamel given by TMR, except for surface layer thickness for MC gel 2) the protocols for producing artificial caries lesion differ especially when considering the method of analysis. The impact of the different properties of the lesions produced by these 5 protocols in further de-remineralisation should be analysed.

Acknowledgements
The authors would like to thank The State of São Paulo Research Foundation-FAPESP for the concession of a scholarship to the second and third author (Proc 2009/03581-9 and 2008/01472-5), PD Dr. Hendrik Meyer-Lueckel for calculating the degree of saturation of the solutions/gels and Prof. Dr. José Roberto Pereira Lauris and Dr. Malgorzata Roos for the help with the statistical analysis.

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Table 1. Initial degree of saturation, pH and exposure time in each protocol at 37°C with $P_{CO_2} = 0$ atm.

Table 2. Quadratic and linear regression of mineral content on cross-sectional hardness (CSH) or its square root ($\sqrt{CSH}$) for different lesion types and for all lesions combined ('Total'). X-variables indicated are those which gave the higher value of $r^2$. NS: $p > 0.05$. (Statistician)

Table 3. Summary of $\sqrt{SH_1}$ and radiographic data for different lesion types. Mean ± SD.

Table 4. Correlation between $\sqrt{SH_1}$ and radiographic data for the combined data for all lesions ('Total')
Figure 1. Profiles of hardness (means) across the enamel lesion from the surface to deep enamel (220 µm) for all protocols.

Figure 2. Exemplary micrographic images and mineral content profiles in the different types of lesions: a. MC gel, b. PA gel, c. MHDP, d. Buffer, e. pH cycling

Figure 3. Coefficient of variation of hardness (a) and mineral content (b)

Figure 4. Plots of mineral content against square root of cross-sectional hardness for MC gel, PA gel and buffer (good correlation, but not linear relationship)

Figure 5. Correlation between mineral content estimated from √CSH by formulas of Featherstone (a) and Kielbassa (b) and mineral content measured directly by TMR

Figure 6. Correlation between SL (TMR) and √SH₁ for MC gel (r=0.65, p=0.04)
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Figure 3. Coefficient of variation of hardness (a) and mineral content (b)
MC gel

\[ y = 0.40x - 17.75 \]

PA gel

\[ y = 0.25x - 5.36 \]
Figure 4. Plots of mineral content against square root of cross-sectional hardness for MC gel, PA gel and buffer (good correlation, but not linear relationship, p<0.05)
Figure 5. Correlation between mineral content estimated from √CSH by formulas of Featherstone (a) and Kielbassa (b) and mineral content measured directly by TMR (r=0.69, p<0.001)
**Figure 6.** Correlation between SL (TMR) and $\sqrt{\text{SH}_1}$ for MC gel ($r=0.65$, $p=0.04$)
**Table 1.** Initial degree of saturation, pH and exposure time in each protocol at 37°C with $P_{CO_2} = 0$ atm.

<table>
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<th>Protocol</th>
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<td>MHDP</td>
<td>0.72</td>
<td>0.27</td>
<td>0.24</td>
<td>_____</td>
<td>5.0</td>
<td>6 d</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.35</td>
<td>0.13</td>
<td>0.09</td>
<td>1.11</td>
<td>5.0</td>
<td>16 h</td>
</tr>
<tr>
<td>pH cycling (De)</td>
<td>0.50</td>
<td>0.20</td>
<td>0.19</td>
<td>1.68</td>
<td>4.7</td>
<td>6h/day for 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>days then 2 days</td>
</tr>
<tr>
<td>pH cycling (Re)</td>
<td>8.26</td>
<td>1.46</td>
<td>0.53</td>
<td>15.7</td>
<td>7.0</td>
<td>18h/day for 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>days then 2 days</td>
</tr>
</tbody>
</table>

HAP: hydroxyapatite, OCP: octacalcium phosphate, DCPD: dicalcium phosphate dihydrate, FAP: fluorapatite. MC gel is infinitely undersaturated with respect to all calcium phosphates. The degree of saturation of PA gel was calculated based on its hydroxyapatite content, not considering that the concentrations might be slightly changed by the acid and at the gel-enamel interface.
Table 2. Quadratic and linear regression of cross-sectional hardness (CSH) or its square root (\(\sqrt{\text{CSH}}\)) on mineral content for different lesion types and for all lesions combined ('Total'). X-variables indicated are those which gave the higher value of \(r^2\). NS: \(p > 0.05\).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Parameter</th>
<th>MC gel</th>
<th>PA gel</th>
<th>MHDP</th>
<th>Buffer</th>
<th>pH-cycling</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quadratic</td>
<td>CSH</td>
<td></td>
<td>(\sqrt{\text{CSH}})</td>
<td>CSH</td>
<td>(\sqrt{\text{CSH}})</td>
<td>CSH</td>
<td>CSH</td>
</tr>
<tr>
<td>Linear slope</td>
<td></td>
<td>0.21</td>
<td>0.35</td>
<td>0.18</td>
<td>12.4</td>
<td>0.11</td>
<td>0.24</td>
</tr>
<tr>
<td>Quadratic slope</td>
<td></td>
<td>-0.0003</td>
<td>-0.0001</td>
<td>-0.0003 NS</td>
<td>-3.81</td>
<td>-0.0001 NS</td>
<td>-0.0004</td>
</tr>
<tr>
<td>Adjusted (r^2)</td>
<td></td>
<td>0.82</td>
<td>0.76</td>
<td>0.20</td>
<td>0.78</td>
<td>0.46</td>
<td>0.53</td>
</tr>
<tr>
<td>Linear</td>
<td>(\sqrt{\text{CSH}})</td>
<td></td>
<td>(\sqrt{\text{CSH}})</td>
<td>(\sqrt{\text{CSH}})</td>
<td>(\sqrt{\text{CSH}})</td>
<td>CSH</td>
<td>(\sqrt{\text{CSH}})</td>
</tr>
<tr>
<td>Intercept</td>
<td></td>
<td>-17.75</td>
<td>-5.36</td>
<td>5.71</td>
<td>-3.26</td>
<td>-300.02</td>
<td>-2.16</td>
</tr>
<tr>
<td>Slope</td>
<td></td>
<td>0.40</td>
<td>0.25</td>
<td>0.12</td>
<td>0.22</td>
<td>7.15</td>
<td>0.22</td>
</tr>
<tr>
<td>(r^2)</td>
<td></td>
<td>0.75</td>
<td>0.65</td>
<td>0.20</td>
<td>0.70</td>
<td>0.46</td>
<td>0.48</td>
</tr>
</tbody>
</table>
Table 3. Summary of surface hardness and radiographic data for different lesion types. Mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>MC gel</th>
<th>PA gel</th>
<th>MHDP</th>
<th>Buffer</th>
<th>pH cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>√SH1</td>
<td>11 ± 0.8</td>
<td>8 ± 0.5</td>
<td>13 ± 0.6</td>
<td>6 ± 1.3</td>
<td>13 ± 1.7</td>
</tr>
<tr>
<td>Surface layer-thickness (SL), μm</td>
<td>9 ± 4</td>
<td>6 ± 3</td>
<td>11 ± 6</td>
<td>4 ± 2</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>Maximum surface layer mineral content (Z_{max}), vol %</td>
<td>69 ± 5</td>
<td>57 ± 8</td>
<td>61 ± 12</td>
<td>48 ± 7</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>Lesion depth, μm</td>
<td>36 ± 3</td>
<td>49 ± 23</td>
<td>86 ± 50</td>
<td>43 ± 19</td>
<td>52 ± 14</td>
</tr>
<tr>
<td>Integrated mineral loss (ΔZ), vol%. μm</td>
<td>731 ± 141</td>
<td>989 ± 279</td>
<td>1519 ± 805</td>
<td>1108 ± 406</td>
<td>1211 ± 381</td>
</tr>
<tr>
<td>Average mineral loss over the lesion depth (R), vol%</td>
<td>21 ± 4</td>
<td>22 ± 5</td>
<td>21 ± 4</td>
<td>27 ± 4</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

Within rows, different superscript letters indicate significant differences between lesion types.
Table 4. Correlation between $\sqrt{SH_1}$ and radiographic parameters for the combined data for all lesions (‘Total’)

<table>
<thead>
<tr>
<th>TMR</th>
<th>$\sqrt{SH_1}$ (Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface layer-SL (µm)</td>
<td>$r=0.52$ (p=0.0001)</td>
</tr>
<tr>
<td>Z max (vol %)</td>
<td>$r=0.42$ (p=0.003)</td>
</tr>
<tr>
<td>Depth Lesion (µm)</td>
<td>$r=0.31$ (p=0.03)</td>
</tr>
<tr>
<td>R values (vol%)</td>
<td>$r=-0.40$ (p=0.004)</td>
</tr>
</tbody>
</table>