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Different protocols to produce artificial dentine carious lesions in vitro and in situ: hardness and mineral content correlation

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**Different protocols to produce artificial dentine carious lesion in vitro and in situ:
hardness and mineral content correlation**

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Short title: Methods to produce carious lesions in dentine

Key words: dental caries; dentine; demineralisation; hardness; mineral content

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25 **Declaration of Interests**

26 **No**

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Abstract

This study compared dentine demineralisation induced by in vitro and in situ models, and correlated dentine surface hardness(SH), cross-sectional hardness(CSH) and mineral content by transverse microradiography(TMR). Bovine dentine specimens (n=15/group) were demineralised in vitro with: MC GEL (6% carboxymethylcellulose gel and 0.1M lactic acid, pH 5.0, 14 days); BUFFER I (0.05M acetic acid solution with calcium, phosphate and fluoride, pH 4.5, 7 days); BUFFER II (0.05M acetic acid solution with calcium and phosphate, pH 5.0, 7 days) and TEMDP (0.05M lactic acid with calcium, phosphate and tetraethyl methyl diphosphonate, pH 5.0, 7 days). In an in situ study, 11 volunteers wore palatal appliances containing two bovine dentine specimens, protected with a plastic mesh to allow biofilm development. The volunteers dripped a 20% sucrose solution on each specimen, 4 times/day, for 14 days. In vitro and in situ lesions were analysed using TMR and statistically compared by ANOVA. TMR and CSH/SH were submitted to regression and correlation analysis ($p<0.05$). The in situ model produced a deep lesion with high R-value, but with a thin surface layer. Regarding the in vitro models, MC gel produced only a shallow lesion, while the BUFFERS I and II as well as TEMDP induced a pronounced subsurface lesion with deep demineralisation. The relationship between CSH and TMR was weak and not linear. The artificial dentine carious lesions induced by the different models differed significantly, which in turn might influence further de- and remineralisation processes. Hardness analysis should not be interpreted with respect to dentine mineral loss.

Introduction

The induction of artificial carious lesions in bovine dentine is an important tool to investigate strategies for prevention or treatment of dentine carious lesions [Okuyama et al., 2006; Zaura et al., 2007; Preston et al., 2008; Pavan et al., 2011], which is a common oral problem for patients suffering from periodontal recession [Ravald and Starkhammar Johansson, 2012].

In vitro models are particularly well suited to experiments whose objective is to test a single process in isolation, where a more complex situation with many variables may confound the data. The composition of the various demineralising systems (gels and solutions) has been developed in an attempt to simulate the conditions of cariogenic biofilm during sugar metabolism. However, it must be kept in mind that the concentrations of calcium and phosphate, and in some cases the pH values chosen in vitro are lower than in the natural intra-oral situation, in order to induce a faster demineralisation than occurs in vivo. Therefore, differences among these solutions or gels, such as initial degree of saturation with respect to tooth minerals, fluoride concentration, kind of acid and viscosity can result in remarkable differences in physical and mechanical characteristics of the demineralised substrate, such as mineral distribution characteristics [Arends et al., 1987; Mc Intyre et al., 2000], chemical composition [Lynch and ten Cate, 2006] and hardness [Magalhães et al., 2009; Marquezan et al., 2009].

On the other hand, in situ protocols for development of carious lesions are more close to the clinical situation, due to the presence of dental biofilm and the exposure to sucrose [Ögaard and Rølla, 1992]. However, to speed the demineralisation process, the cariogenic challenges applied in most in situ studies are also more aggressive than those that normally occur during the development of natural carious lesions [Hara et al., 2003; Aires et al., 2008].

Despite the large diversity of studies using different protocols to induce dentine carious lesions [Mc Intyre et al., 2000; Buchalla et al., 2003; Zaura et al., 2007; Marquezan et al., 2009], there is no study comparing in vitro to in situ models with respect to their potential to induce demineralisation. It is important to point out that the kind of lesion influences the behaviour to further de- or remineralisation, as the surface layer, porosity and lesion depth can play an important role in the mineral diffusion [ten Cate, 1994; Kawasaki et al., 2000; Preston et al., 2008; Bertassoni et al., 2010].

Depth-related properties of artificial caries lesions can be described by mineral content and hardness profiles. Transverse microradiography provides a quantitative measure of the mineral content, and has been widely used also to assess transverse mineral distribution of caries lesions in dentine [Inaba et al., 1997; Buchalla et al., 2003]. Therefore, this method is considered as the gold standard for the quantification of the mineral content of caries lesions in vitro. On the other hand, cross-sectional hardness reflects the mechanical resilience of the dental hard tissue. However, it is debated whether surface or cross-sectional hardness analysis might

reflect depth mineral alterations of carious dental tissues, or if it is able to detect differences among the lesions provoked by different protocols [Buchalla et al., 2008; Magalhães et al., 2009].

A previous work of our group has shown that CSH, as an alternative to TMR, is not a valid surrogate for mineral content of demineralised enamel [Magalhães et al., 2009]. Dentine caries is a diffusion-controlled process. The demineralisation involves not only chemical dissolution of the inorganic material, but also the exposure and degradation of the organic matrix, mainly collagen type I [Chaussain-Miller et al., 2006; van Strijp et al., 2003]. Therefore, it is expected that the high organic content of dentine could influence the measurement of mechanical properties [Herkströter et al., 1989; Balooch et al., 2008]. However, there is no study testing the correlation between hardness and mineral content of dentine demineralised by different laboratory protocols so far.

Therefore, the present study aimed 1) to compare different in vitro and in situ models proposed in the literature to induce artificial carious lesions in dentine and 2) to correlate the data of surface (SH) and cross-sectional hardness (CSH) with mineral content profiles using TMR.

Material and Methods

Ethical aspects

This study was approved by the local ethical research committee (FOB-USP, process nº 057/2009). For the in situ experiment, eleven adult volunteers took part after signing an informed consent. They fulfilled the inclusion criteria (physiological salivary flow rates: stimulated: >1 ml/min, non stimulated: >0.25 ml/min; good oral health: no frank cavities or significant gingivitis/periodontitis) without violating the exclusion criteria (systemic illness, pregnancy or breastfeeding, use of fixed or removable orthodontic appliances, use of fluoride mouth rinse or professional fluoride application in the last two months, hyposalivation).

Specimen preparation

Root dentine specimens (4 mm X 4 mm X 3 mm) were prepared from bovine incisors, which were freshly extracted and stored in water containing NaCl (0.9%) and thymol (0.1%) until used. 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 dentine surface of the samples was ground flat using water-cooled silicon carbide discs (320-, 600-, and 1200-grade papers, ANSI grit; Buehler, Lake Bluff, IL, USA), and polished using felt paper wet with diamond solution (1 µm; Buehler), resulting in removal of about 200 µm of the outer cement/dentine. This was controlled with a micrometer.

Prior to the experiment, the specimens were disinfected by dipping in 70% alcohol solution for 30 minutes in addition to the previous immersion in thymol solution [Schlueter et al., 2009]. Two third of the surface were covered with nail varnish in order to create control areas to both sides of a central band of exposed dentine ($\approx 1-1.5$ mm).

For the in vitro experiments, each $n = 15$ specimens were randomly allocated to each of the four groups. For the in situ experiment, $n = 22$ specimens were randomly allocated to 11 subjects ($n = 2/\text{subject}$). The randomization was done according to surface hardness (SH) means (29 ± 6 KHN/group or subject). SH determination is described below.

In situ experiment

Acrylic palatal appliances were made for each of 11 subjects with two positions for the specimens. In order to protect the dentine surface from mechanical disturbance and allow plaque accumulation, a plastic mesh with 1 x 1 mm apertures (Sanremo, Brazil) was fixed over the cavities containing the specimens, leaving a 1 mm space between mesh and specimen surface.

During 14 days, the appliances were only removed for the main meals (4 times a day, maximum 1 h each, interval between meals 2-3 h) and for the application of the sucrose solution (20% weight/volume, 1 drop/specimen) 4 times a day (each 5 min) [Hara et al., 2003; Aires et al., 2008]. Thereafter, the appliance was replaced into the mouth. The sucrose solution was renewed every 3 days of the experiment.

Seven days prior to and throughout the in situ phase, the subjects brushed their teeth with fluoride-free toothpaste, in order to avoid any residual effect of fluoride sources on the specimens. The specimens were not brushed to allow for plaque accumulation.

In vitro experiment

In the MC GEL group, the specimens were covered with 0.5 cm 6% carboxymethylcellulose gel that was left to set overnight at 4°C, in vials of 10 mL. Therefore, they were covered with an equal volume (1.5 mL) of 0.1 M lactic acid, pH adjusted to 5.0, and incubated for 14 days [Inaba et al., 1997]. In the BUFFER I group, the specimens were immersed in 30 mL of 50 mM acetate buffer solution containing 2.2 mM CaCl_2 , 2.2 mM KH_2PO_4 and 0.5 ppm F, at pH 4.5, for 7 days [ten Cate and Duijsters, 1983; Mc Intyre et al., 2000]. In the BUFFER II group, the specimens were immersed in 30 mL of 50 mM acetate buffer solution containing 2.2 mM CaCl_2 , 2.2 mM KH_2PO_4 , at pH 5.0, for 7 days [ten Cate and Duijsters, 1982; Damen et al., 1998]. In the TEMDP group, the specimens were immersed in 30 mL of 50 mM lactate buffer containing 3 mM CaCl_2 , 3 mM KH_2PO_4 , 6 μM tetraethyl methyl diphosphonate and traces of thymol, at pH 5.0, for 7 days [Buskes et al., 1985; Buchalla et al., 2003].

In all in vitro models, the specimens were separately immersed in unstirred solutions or gel at 37°C. Table 1 summarizes the degrees of saturation with respect to dentine minerals, pH and exposure time. The degree of saturation was calculated using a software program [Shellis, 1988].

The specimens were immersed in deionized water to avoid shrinkage of the dentine before and after the experiment.

Hardness Measurement

Dentine surface hardness (SH) was measured using a microhardness tester (HMV-2, Shimadzu Corporation, Tokyo, Japan) and a Knoop diamond, with a load of 10 g applied for 10 s. Five indentations, 100 µm apart, were made in the center of dentine specimens at baseline (SH₀) and at the end of the experiment (SH₁).

To perform cross-sectional hardness (CSH) tests, the specimens were sectioned once with a diamond band saw, perpendicularly to the surface and the protected areas through the center. One half of each sample was embedded in acrylic resin and polished as described before, while the other half was prepared further for TMR analysis. The specimens were maintained in deionized water until the analysis. For CSH determination the water was removed from the surface using a paper, and three rows of 7 indentations each were made, one in the central region of exposed area and the other two at 100 µm distance to both sides of the central row, using a 10 g load for 10 s. The indentations were made at 10, 30, 50, 70, 90, 110 and 220 µm from the outer dentine surface. The mean values of all 3 measuring points at each distance from the surface were averaged (KgF/mm²).

Transverse Microradiography (TMR)

The other half of the specimens was additionally cut and hand-polished plane-parallel from both cut sides with water-cooled silicon carbide discs (320-, 600-, and 1200-grade papers, ANSI grit; Buehler, Lake Bluff, IL, USA) to a thickness of 138 ± 7.6 µm. After immersion of the specimens in ethylene glycol (Sigma-Aldrich, Steinheim, Germany) for 24 h in order to avoid shrinkage during X-ray exposure due to desiccation [Buchalla et al. 2003], micrographs of each section together with an aluminum calibration step wedge with 14 steps were taken. High-speed holographic film (SO 253; Kodak AG, Stuttgart, Germany) was exposed with Ni-filtered quasi-monochromatic Cu K α X-rays ($\lambda = 0.154$ 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) equipped with a CCD camera (XC-77CE, Sony, Tokyo, Japan) and a PC with framegrabber, data acquisition and calculation software (TMR 1.25e; Inspektor Research BV, Amsterdam, The Netherlands). One

measurement was done for each microradiogram in the center of the demineralised window. Hereby, a field of 350 x 400 μm was analysed by averaging the grey value of pixel columns parallel to the outer surface of the specimen. The horizontal resolution initially was 2 μm .

The mineral content was calculated assuming that the mineral content of sound dentine is 50 vol% [Buchalla et al., 2003]. The lesion depth (ld) was calculated using a threshold of 95% of the mineral content of sound dentine (i.e. 47.5%). Integrated mineral loss (ΔZ), the average mineral loss over the lesion depth (R), the mean thickness of the “pseudo intact” surface layer (SL) and the maximum mineral content of the surface layer (Z_{max}) were also calculated.

Statistical analysis

Means and standard deviations (SD) were calculated for SH, CSH and TMR parameters (ΔZ , ld, SL, Z_{max} and R). 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).

To analyse a possible relationship between CSH and mineral content, the data (CSH and mineral content) for each lesion type at 10, 30, 50, 70, 90, 110 and 220 μm depth and the combined data from 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]; in this case, the highest r values using hardness or its square root was presented. The correlations between SH_1 , $\sqrt{\text{SH}_1}$, %SHC and surface layer thickness (SL), maximum mineral content of the surface layer (Z_{max}), lesion depth (ld), integrated mineral loss (ΔZ) and average mineral loss (R) were also examined (Pearson's coefficient).

For the comparison among the protocols, the data (Z_{max} , ld, SL and ΔZ) passed the normality test, but the variances were not homogeneous. Therefore, these data were compared using Kruskal-Wallis test followed by Dunn's multiple comparisons test. The R-values 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% (n=15 specimens).

Results

All 11 subjects included in this study were able to finish the in situ phase, but some specimens got lost. Thus, only 18 specimens from the in situ experiment could be analysed. In the in vitro experiment, CSH of 3 (BUFFER I) and 4 specimens (MC GEL) could not be measured due to the softening.

Relationships between hardness and mineral content

The quadratic and linear regression showed a weak relation between CSH or $\sqrt{\text{CSH}}$ and mineral content for each group and for all groups together (Table 2, $p < 0.05$). Generally, the coefficient of mineral content determination from hardness values was lower than 0.50. The same findings were shown when SH_1 , $\sqrt{\text{SH}_1}$ and %SHC were correlated to TMR parameters; there was a low correlation between the variables, and most of them were not statistically significant. The only significant correlations were found for BUFFER II ($\text{SH}_1 \times Z \text{ max}$, $r = 0.62$, $p = 0.01$) and TEMDP ($\text{SH}_1 \times \Delta Z$, $r = -0.70$, $p = 0.004$). Therefore, the other correlations ($p > 0.05$) were not presented in the Result section, because they were not statistically significant.

Differences among types of lesion

As the hardness showed no relation with the mineral content, the lesions were compared only using the TMR parameters.

Generally, the in situ model produced an intermediate lesion depth and ΔZ , with the highest R-value. The MC gel produced the shallowest and the least demineralised lesion. The BUFFERS I and II as well as TEMDP induced a subsurface and deep dentine demineralisation. BUFFER I additionally produced the deepest lesion with the highest ΔZ compared to the other groups. Table 3 shows an overview of all TMR parameters.

In respect to the surface layer (SL and Z_{max}), only BUFFER II and TEMDP produced a well-developed surface layer. For the in situ model and the in vitro models, MC GEL and BUFFER I, the surface layer was visible only in 2, 6 and 11 specimens, respectively. Figure 1 shows a representative image and mineral content profile for each group.

Discussion

In the present study, poor linear regression between cross-sectional hardness or square root of cross-sectional hardness and mineral content could be detected considering the data from each single model and the models overall. This finding is in accordance with previous studies focusing on enamel [Buchalla et al., 2008; Magalhães et al., 2009].

Furthermore, there was also only a low or even no correlation between the surface hardness and some TMR parameters. The statistical relationship between both methods was very weak for dentine compared to previous results from a similar study performed in enamel [Magalhães et al., 2009]. This was expected as the high organic content and thus, the elastic properties of the dentine [Herkströter et al., 1989] influences the hardness measurement. According to Marshall et al. [2001], the mechanical properties of dentine measured under hydrated conditions – as done in the present study – provides a more realistic estimation of the in vivo situation. Hardness even in sound dentine is not evenly distributed. The peritubular dentine is harder than the intertubular areas [Kinney et al., 1996], which cannot be distinguished

using microhardness testing. If hardness differences within the μm range are in focus, nanohardness testing would be required [Bertassoni et al., 2011].

As previously discussed, the variability of hardness (SH and CSH) data is high compared to the mineral content, which may be partly attributed to the different volumes that are “probed” by the indenter compared to the resolution of the x-ray. The hardness measurement at each first depth (especially at 10 and 30 μm depth) of the demineralised surface is not reliable due to the size of the indentation and because the edge of the specimen is very close to the indentation. Therefore, and because of the limited resolution, the exact depth of the lesion is also difficult to identify using hardness indentations 20 μm apart. Furthermore, in case of the dentine, the relationship between the organic compound and mineral, and the degree of humidity are factors that influence the mechanical testing. CSH of some samples in two models (BUFFER I and MC gel) could be not measured using Knoop indentation due to the high level of softening. On the other hand, TMR measures the mineral content at a much higher resolution (in this study every 2 μm depth). Its accuracy has some limitations only at the outermost 10 μm of the specimen [Magalhães et al., 2009].

Although the CSH gives important evidence regarding the mechanical resilience of the demineralised enamel [Magalhães et al., 2009], it cannot be used to estimate mineral content reliably, particularly not in case of the dentine. The same is valid for SH measurement, which showed only few significant correlations with TMR parameters in the models BUFFER II and TEMDP. This finding pointed out that the relationship between surface hardness and mineral content might also depend on the type of lesion, not being applicable in all cases.

Regarding the different models to prepare artificial caries lesions, BUFFER I generally showed higher subsurface mineral loss and lesion depth than the other models. It is important to keep in mind 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 dissolution inhibitors (fluoride, phosphate and some proteins) and temperature [Arends and Christoffersen, 1986; Amaechi et al., 1998]. In the case of BUFFER I lesions, the results might be explained by the lower degree of saturation regarding HAP, OCP and DCPD compared to the other models (Table 1). BUFFER I was saturated with respect to FAP, which might have influence on the formation of the “pseudo-intact” surface layer evident in most of specimens from this group. According to Damen et al. [1998], the addition of fluoride to demineralising solutions does not affect the lesion depth, but the preservation of a mineralised surface layer. The surface layer can also be formed by the re-precipitation of the minerals from the advancing front of lesion into the surface [Phankso et al., 1985]. Despite the presence of fluoride, only 11 specimens from BUFFER I presented a surface

layer, showing that presence of fluoride did not automatically ensure development of a surface layer under these severely demineralising conditions.

BUFFER II and TEMDP also produced a deep subsurface lesion with similar depth and integrated mineral loss. However, TEMDP produced a highly mineralized surface layer and, the mineral loss over the depth was lower compared to all groups, which might be explained by the presence of tetraethyl methyl diphosphonate, a dissolution inhibitor [Buskes et al., 1985; Arends and ten Bosch, 1992].

The preservation of the surface layer 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, Damen et al., 1998] and the time after an initial demineralisation [Theuns et al., 1983]. Dentine caries lesions initially do not show a surface layer as it was the case in the in situ model; the surface layer is formed over time and its thickness, once formed, appears to be roughly constant [Theuns et al., 1984a; Theuns et al., 1984c; Arends and Christoffersen, 1986].

Although the in situ model also produced a deep lesion with the highest mineral loss (high R value), the surface layer was not evident in most samples (only 2 specimens exhibited a surface layer). It can be speculated that the low level of fluoride in oral environment could be responsible for this finding associated with the severe cariogenic challenge in a short time period. Another possibility is the degradation of the demineralised organic matrix by collagenases from the host or microorganisms, impairing the formation of the surface layer and enhancing the demineralisation [Kleter et al., 1994; Tjäderhane et al., 1998; van Strijp et al., 2003]. This hypothesis was previously discussed by Marquezan et al. [2009]. The authors inferred that the lesions produced in vitro simulate the caries-affected dentine, while in the presence of microorganism (as it is expected in an in situ model) might create a lesion similar to caries-infected dentine. An interesting finding of our study was that the in situ protocol presented the highest r^2 value in the regression analysis. It might be speculated that the degradation of the demineralised organic matrix, to the same extent, could reduce the influence of collagen properties on the hardness measurement, improving the relationship between hardness and mineral content.

MC gel produced the shallowest lesion in accordance with a previous study performed in enamel [Magalhães et al., 2009]. The mineral saturation might be reached with time (MC gel presented the longest exposure time), depending on the volume (MC gel presented the lowest volume) and the viscosity of demineralisation solution/gel relative to the area of tooth exposed to demineralising solution/gel. Accordingly, in the case of MC gel, some reduction in calcium activity might have occurred [Lynch et al., 2006], due to the Ca-binding activity of methylcellulose. The MC gel method is the only in vitro method tested in this study that employs a diffusion barrier on top of the dentine surface, similar to what dental plaque would

be. Due to the gel-consistency diffusion processes are slowed down markedly as compared to buffer solutions.

Generally, our results are in agreement with other studies [Mc Intyre et al., 2000; Marquezan et al., 2009], in which the demineralisation was highest for BUFFERS followed by TEMDP and MC gel. Considering the formation of a subsurface lesion and the results of the present study, the BUFFER II and TEMDP should be appropriate models to be recommended for the laboratory preparation of dentine carious lesions. Generally, both models produced homogenous and deep lesions, in which a surface layer could be seen in all specimens. Furthermore, both methods are reliable and simple to perform.

The different physical and mechanical properties of the lesions produced by these five models might influence the results of subsequent demineralisation and remineralisation (such as saliva and fluoride) protocols [Mukai and ten Cate, 2002]. Therefore, further studies are needed to prove if the differences found in properties of the lesions might influence the results of de- and or remineralisation protocols in vitro and in situ.

Future studies should also analyse which kind of lesion created by the present models behaves most similar to natural lesions [Marquezan et al., 2009]. It has to be taken into consideration that the in vitro lesions are unable to simulate biological events such as bacterial penetration, collagen degradation, tubular occlusion and reactionary dentine [Shellis, 1994; Marquezan et al., 2009]. Also a point of interest is whether bovine dentine is an appropriate substitute for human dentine. In this respect, some studies have shown similarity in the mineral loss and lesion depth between both substrates when they were subjected to demineralisation [Mellberg, 1992; Hara et al., 2003].

Therefore, from the results of the present study it can be concluded that: 1) The models for producing artificial dentine caries lesion differ significantly. 2) CSH and SH used as alternative to TMR is not adequate for estimating the mineral content from dentine.

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Authors contribution: Conceived and designed the experiments: ACM. Performed the experiments: BMM, LPC, AW, HY. Analyzed the data: ACM, BMM. Wrote the paper: ACM, BMM, AW, MARB, WB

406

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517

LEGENDS**TABLES**

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

Table 2. Quadratic and linear regression of cross-sectional hardness (CSH) or its square root ($\sqrt{CSH}$) and mineral content for the different models and for all models combined ('Total').

Table 3. Summary and statistical comparisons for all TMR parameters. Mean $\pm$ SD.

FIGURE

Figure 1. TMR image and mineral content profile of a representative specimen from each model: **a.** MC gel, **b.** BUFFER I, **c.** BUFFER II, **d.** TEMDP and **e.** IN SITU.

TABLES

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

Protocol	HAP	OCP	DCPD	FAP	pH	Exposure time
MC GEL	_____	_____	_____	_____	5.0	14 d
BUFFER I	0.30	0.13	0.13	1.51	4.5	7 d
BUFFER II	0.66	0.25	0.27	_____	5.0	7 d
TEMDP	0.72	0.27	0.24	_____	5.0	7 d

HAP: hydroxyapatite, OCP: octacalcium phosphate, DCPD: dicalcium phosphate dihydrate, FAP: fluorapatite. MC gel is infinitely undersaturated with respect to all calcium phosphates [Shellis, 1988].

Table 2. Quadratic and linear regression of cross-sectional hardness (CSH) or its square root ($\sqrt{\text{CSH}}$) and mineral content for the different models and for all models combined ('Total').

Analysis	Parameter	MC GEL	BUFFER I	BUFFER II	TEM DP	IN SITU	TOTAL
Quadratic		$\sqrt{\text{CSH}}$	CSH	CSH	CSH	CSH	$\sqrt{\text{CSH}}$
	Intercept	0.02	20.49	15.63	9.69	2.14	1.16
	Linear slope	0.08	-0.55	0.08	0.31	0.63	0.16
	Quadratic slope	0.0001	0.0124	0.0014	-0.0005	-0.006	-0.0021
	Adjusted r^2	0.39	0.054	0.07	0.23	0.46	0.33
Linear		$\sqrt{\text{CSH}}$	CSH	CSH	CSH	CSH	$\sqrt{\text{CSH}}$
	Intercept	-0.07	15.00	14.51	10.22	3.09	2.05
	Slope	0.09	0.06	0.16	0.27	0.35	0.06
	r^2	0.39	0.01	0.07	0.23	0.45	0.26

*The Table shows the relation between mineral content and hardness at 10, 30, 50, 70, 90, 110 and 220 μm depth (X-variables indicated, CSH or its square root, are those that gave the highest r^2 value). $p < 0.05$ for all regression analysis.

563 **Table 3.** Summary and statistical comparisons for all TMR parameters. Mean $\pm$ SD.

	MC GEL	BUFFER I	BUFFER II	TEMDP	IN SITU
Surface layer-thickness (SL), μm	3 ± 4^a	14 ± 10^b	14 ± 7^b	12 ± 8^b	1 ± 3^a
Maximum surface layer mineral content (Z_{max}), vol %	6 ± 9^a	31 ± 22^b	37 ± 7^b	44 ± 7^b	2 ± 5^a
Lesion depth (ld), μm	87 ± 20^a	262 ± 25^c	163 ± 30^b	163 ± 30^b	137 ± 49^{ab}
Integrated mineral loss (ΔZ), vol%. μm	$1709 \pm$ 301^a	$7070 \pm$ 1071^d	$3065 \pm$ 772^{bc}	$2279 \pm$ 591^{ab}	$4406 \pm$ 1973^{cd}
Average mineral loss over the lesion depth (R), vol%	20 ± 4^b	27 ± 4^c	19 ± 4^b	14 ± 3^a	31 ± 5^d

564 Different superscript letters in the same line show significant differences among the models
565 (ANOVA for R values and Kruskal-Wallis for the other parameters, $p < 0.0001$). n=15/group for
566 the in vitro models and n=18 for the in situ model
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a

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b

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c

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d

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e

573 **Figure 1.** TMR image and mineral content profile of a representative specimen from each
574 model: **a.** MC gel, **b.** BUFFER I, **c.** BUFFER II, **d.** TEMDP and **e.** IN SITU.