Formation and assessment of enamel subsurface lesions in vitro

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Abstract: The present study compared two pH-cycling models designed to induce subsurface lesions (SLs) with a less demineralized surface layer on teeth, with the aim of developing new technologies for assessment of such lesions by examining the performance of confocal Raman microscopy for detection of white spot lesions (WSLs). Twelve sound premolars were exposed to two sets of model conditions (A, B) designed to induce SLs. Teeth on which white lesions had formed in vivo were used as positive controls. All specimens were inspected using an intraoral camera and Raman microscopy to detect small changes in the appearance and structure of the enamel. Changes in the natural color of the teeth during the treatment were recorded via the camera. Phosphate maps with their spectra were constructed from the phosphate peak at 960 cm⁻¹. The depth of lesions was measured on the basis of variations in phosphate peak intensity. Protocol B was reliable for reproducing SLs in a relatively short period. Both protocols had intrinsic limitations in not completely simulating the complex intraoral conditions leading to WSL formation with respect to lesion depth and preservation of an intact surface layer. Raman microscopy can be considered the gold standard for analysis of hard tissue mineralization.

Keywords: white spot lesion; pH cycling model; Raman microscopy.

Introduction

Dental caries is a multifactorial disease characterized by a dynamic disparity between alternating periods of dissolution and mineral loss (1). White spot lesions (WSLs) represent an early stage of caries formation involving subsurface enamel demineralization. WSL formation can be reproduced in the laboratory in a relatively short period using chemical systems (2). The pH cycling model is a chemical system involving exposure of dental enamel to combinations of de- and remineralization phases, thus mimicking the dynamics of mineral loss and gain operating during caries formation. This can be used to investigate possible approaches for caries prevention and/or remineralization and the planning of clinical trials (3).

The Soprolife light-induced fluorescence assessor system (Acteon Soprolife, La Ciotat, France) is a newly released apparatus for detection and assessment of incipient dental caries, based on imaging and auto-fluorescence of dental tissues (4).

Raman microscopy is a non-invasive spectroscopic method requiring minimal specimen preparation that provides details of the biochemistry and molecular structure of mineralized tissues. Its high spatial resolution (300 nm) makes it an optimal approach for analyzing calcified tissue components and detecting early changes in enamel composition (5).

The incremental prevalence of mild hypomineralization due to developmental defects on tooth surfaces poses a challenge for caries detection and prevention. The pH-cycling models allow subsurface lesions to develop in a relatively short period representing 6 to 12 months.
of actual clinical lesion progression (3). The present study was designed to compare two chemical models for their potential to induce artificial caries confined to enamel surfaces and investigate their ability to mimic the intra-oral conditions leading to WSL formation. It was anticipated that the data obtained would be useful for development of new techniques for assessment of subsurface lesions employing Raman microscopy for detection of incipient caries.

Materials and Methods
This study involved the use of sound premolars that had been extracted for orthodontic reasons from individuals aged 13 to 14 years. All specimens were collected with informed consent from the subjects, and approval for their use was obtained from the local ethical research committee (process No. 2017-2907).

The number of teeth required for this study was calculated using “BiostaTGV 2000”. By comparing two means that were observed during the preliminary studies, it was found that a minimum number of 4 teeth in each group was necessary. Four premolars on which WSLs had formed in vivo were used as a reference (positive control) group. Twelve sound premolars were divided randomly into 3 groups, each of which was exposed to two pH cycling models, A and B. The teeth were subjected to 7, 8 and 14 cycles, respectively. Each cycle lasted 24 h. Different numbers of cycles were used to produce different types of caries lesions with various lesion depths to measure the representative characteristics of early caries lesions such as fluorescence loss, color change and the amount of mineral loss using an intraoral camera and confocal microscopy (6). Preliminary studies had revealed that protocol A caused a slight change in enamel appearance after 8 cycles. Therefore, the substrates subjected to this protocol were controlled twice: once after 8 cycles and again at the end of the 14th cycle, as reported for the original protocol (Featherstone, 1986). A recently published study (7) had tested the effect of protocol B on enamel surface structure, and this had revealed that 7 cycles created the deepest artificial subsurface lesions in vitro along with preservation of an intact surface layer. On the other hand, 8 cycles of protocol B yielded a reduced lesion depth due to considerable loss of the enamel layer. Thus, a period of 8 cycles appeared to represent a transition point in the outcome of both chemical models.

The first model (A), as described by (Featherstone, 1986), was conducted for 14 days. The demineralizing solution was composed of 2.0 mM Ca(NO₃)₂•4H₂O, 0.9 mM KH₂PO₄, 130 mM KCl, 20 mM Na₂H₆AsO₂•3H₂O (pH 7).

The second model (B) (7) used a demineralizing solution composed of 0.075 M CH₃COOH, 1.0 mM CaCl₂, 2.0 mM KH₂PO₄ (pH 4.3), and the remineralizing solution contained 150 mM KCl, 1.5 mM Ca(NO₃)₂, 0.9 mM KH₂PO₄ (pH 7). All the chemical components were supplied by Sigma-Aldrich, Montpellier, France. Each tooth was immersed in 20 mL of demineralizing solution for 6 h, then removed and rinsed with running water before being immersed in 20 mL of remineralizing solution for 18 h. The temperature was maintained at 37°C (Featherstone, 1986). After treatment, the teeth were sectioned longitudinally into two halves to obtain cross-sections, and then embedded in self-curing acrylic resin so that the cross-sectioned surface of each sample faced the surface to be polished later.

Soprolife camera
This camera can be used to capture high-resolution images of caries lesions, thus facilitating clinical assessment (8). All teeth were imaged before and after the intervention to detect changes in enamel surface appearance. The camera provides anatomical images (daylight mode) and fluorescent images (diagnostic mode). The light is provided by white and blue light-emitting diodes (LEDs) with a wavelength of 450 nm.

Raman microscopy
Raman spectra were recorded using a Witec Confocal Raman Microscope System alpha 300R (Witec, Ulm, Germany). Excitation was achieved by a frequency-doubled Nd: YAG laser (Newport, Evry, France) at 532 nm. Enamel chemical mapping was started at the outer surface and ending before the dentin-enamel junction (DEJ). The phosphate (PO₄³⁻) ion has four vibrational modes. The peak at 960 cm⁻¹ is assigned to the ν₁ vibration peak of the phosphate group in enamel, and this was selected as the inner standard to observe changes in intensity of the strongest peak at 960 cm⁻¹. Using an indicative look-up table (LUT), yellow and dark brown hues were taken to indicate the highest and lowest phosphate intensity in a chosen area, respectively.

The overall mean and standard deviation (S.D.) of lesion depth was calculated. Statistical analysis was performed using one-way analysis of variance (ANOVA). Pairwise multiple comparison procedures (Tukey Test) were used to isolate groups that differed from the others. All statistical procedures were performed at an overall significance level of α = 0.05 using SigmaPlot.v11.0 (Systat Software, Inc., San Jose, CA, USA: www.systat-
Results

Image pairs were recorded using the intraoral camera as an additional method for visual inspection (Fig. 1A-D). A and B represent images in daylight mode before intervention. C and D represent images exhibiting changes in enamel surface appearance after 8 cycles of protocol A and 7 cycles of protocol B consecutively. The depth of artificial lesions was measured and compared to that of natural lesions based on phosphate peak intensity variations in each dental section. A statistically significant difference ($P < 0.05$) in depth was found between the positive control group and 8 cycles-B group. The difference between the 7 and 8 cycle lesions (protocol B) was also significant. Non-significant differences ($P > 0.05$) were detected between other groups (Fig. 1E).

Phosphate maps with their corresponding spectra presented in Fig. 2A-E were constructed from the phosphate peak intensity at 960 cm$^{-1}$. All examined samples exhibited an important phosphate signal at the outer enamel surface that indicated the presence of an intact surface layer (ISL). There was severe depletion of the $\text{PO}_4^{3-}$ peak in the area corresponding to the body of the lesion. At greater distances into the enamel, the intensity approached that of sound enamel, indicating the end of the lesion.

Discussion

The aim of the present study was to compare two pH-cycling models, focusing on their strengths, limitations and the period required to induce subsurface lesions. The chemical composition of subsurface lesions was determined using Raman microscopy, which can detect very small variations in phosphate intensity that reflects the mineral content in the target zone (9). Scan data comprising spectra graded into ten-thousandth units were used to reconstruct detailed images.

Natural WSLs are formed under complex intraoral conditions due to imbalances between pathological factors that cause demineralization and protective factors that drive remineralization (10,11). De/remineralization occurs in the mouth several times every day as a dynamic process stretching over time periods much longer than those in laboratory models (12). Therefore, WSLs are more subtle than in vitro subsurface lesions (SLs) in...
terms of the chemical composition of the enamel and lesion depth due to the presence of saliva and plaque in vivo. WSLs are quite variable; the intact surface layer is more likely present because of the strong likelihood of remineralization within the oral environment, although the amount of mineral loss from the subsurface layer varies widely (12).

The subsurface lesions produced by protocol A after 8 cycles were invisible after inspection with the camera (Fig. 1C) even after prolonged dryness due to low-grade of demineralization, and thus small changes in the chemical composition of the enamel represented by PO$_4^{3-}$ peak intensity (Fig. 2A). After 14 cycles, changes became more evident with the formation of a pronounced subsurface lesion accompanied by undermining of the ISL (Fig. 2B). However, protocol B was able to induce changes in the enamel that appeared after only 3 cycles. Severe whitening of the enamel became evident after only 7 cycles (Fig. 1D), reflecting the chemical map that showed a deep lesion with an intense decrease of phosphate in the body of the lesion and the presence of an ISL (Fig. 2C). Distinct dissolution of the outer surface was encountered after 8 cycles (Fig. 2D).

When light enters sound enamel, which shows only a low degree of light scattering, it travels an average distance of 0.1 mm before being scattered (13). When minerals are lost from enamel due to demineralization, they are partly replaced by water, leading to greater differences in refractive index (RI) between sound and demineralized enamel and thus greater scattering at the
enamel/air interface (14). When the lesion is dried, the water is replaced by air and the average refractive index declines even more, producing an even whiter lesion. Furthermore, the whiteness of the initial lesion is an optical phenomenon resulting from increased porosity within the crystalline enamel structure (13,15).

Thus, changes in enamel appearance can be detected only when a distinct subsurface lesion has developed, owing to interior opacity and a subsurface scattering effect (16). Chemical protocols have limitations in terms of lesion depth and ISL preservation due to their difficulty in completely simulating the complex intraoral conditions that lead to WSL development (17). Protocol A induced a slight change in the appearance of the enamel after 8 cycles, whereas protocol B produced deep subsurface lesions after only 7 cycles. The latter model is thus less time consuming and reliable for reproducing SLs in vitro in a relatively short period as long as the process is limited to seven cycles to ensure the presence of an ISL, which represents a characteristic feature of these lesions that complicates their non-invasive treatment. These subsurface lesions are essential in profile studies for rapid and low-cost testing of newly developed and recently marketed remineralizing dental products (3).

Raman microscopy can be considered the gold standard for hard tissue mineralization analyses (18,19). Further work is required to investigate the clinical application of this technique for detection of carious lesions at an early stage of development.

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Conflict of interest
The authors declare that they have no conflict of interest.

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