Use of Transverse Microradiography to Quantify Mineral Loss by Erosion in Bovine Enamel

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Artificial saliva
Dental erosion
Image analysis
Orange juice
Transverse microradiography

Abstract
The aim of the present study was to develop transverse microradiography (TMR) in order to quantitatively assess the influence of artificial saliva and orange juice on the erosion of bovine enamel. Bovine incisors were sectioned sagittally into two equal halves. Each half was coated with acid-resistant nail varnish except for a rectangular enamel window on the labial surface of the tooth. While both halves of each tooth were immersed in pure orange juice six times daily for a period of 5 min at each occasion making a total of 30 min per day, one half was stored in artificial saliva and the other half in deionized distilled water between exposures to orange juice and for the remaining 12 h overnight, for 24 days making a total of 12 h of exposure to orange juice. Sections bearing intact and eroded enamel surfaces were cut from each specimen and ground to 80 µm thickness, and TMR of the sections was made. Mineral loss was quantified from the microradiographs using two-step image analysis. Mineral loss was significantly greater in those specimens cycled in orange juice and de-ionized distilled water. TMR was used successfully to quantify the mineral loss by erosion in vitro, and it is envisaged that it will be useful for specimens used in intra-oral appliances during in situ trials. Artificial saliva saturated with respect to calcium and phosphate salts can possibly remineralize an erosive lesion and may reduce the degree of erosion.

It is well known that the rate of consumption of pure fruit juices and acidic beverages is increasing [Well, 1989; ten Cate and Imfeld, 1996] consequent to their easy and inexpensive availability in various prepackaged and canned forms. This increase in the rate of consumption is reflected by an increased prevalence of dental erosion [Nunn, 1996]. The question of the erosive effect of these drinks has recently assumed new importance and generated interest in the field of dental research. This area of research has however been limited by the lack of an accurate and reliable method for quantifying the loss of tooth mineral in an erosive lesion.

Grenby [1996] has recently made a detailed review of the various techniques, which have been developed and published since the 1940s, for the assessment of dental erosion. Restarshi et al. [1945] used a scoring system to evaluate the erosive lesion from acidic drinks and beverages. Many researchers have used this system [McCune and Ruszkic, 1946; Wynn and Halk; 1948] or its modifications [Reussner et al., 1973]. Erosion has also been evaluated by grading the severity of the lesion [Eccles, 1979]. Davis and Winter [1977] and Rytonaa et al. [1988] used surface profilometric analysis to evaluate the loss of tooth material by erosion caused by acidic drinks and other foodstuffs. A semiquantitative method was demonstrated by Sorvani and Kiviranta [1988]; drawings of the selected surfaces were produced by hand, and these were then analysed with a computer-cou-
plied analyser. Most of these methods have limitations and are subjective. The use of digital image analysis to evaluate the area and depth of an erosive lesion has recently been demonstrated by Mistry and Grenby [1993], and although this is a fast method, it is unable to quantify mineral loss. Quantification of the mineral loss was shown by the measurement of the amount of calcium and/or phosphorus liberated into the erosive solution [Grenby et al., 1989]. Although this system is quantitative, it does not exactly mirror the conditions in vivo [Grenby, 1996].

The use of transverse microradiography (TMR) in the field of cariology for the quantification of mineral loss or gain in de- and remineralization studies has been demonstrated [De Josselin de Jong et al., 1987]. The aim of the present study was to develop TMR in order to quantitatively assess the influence of artificial saliva and orange juice on the erosion of bovine enamel.

Materials and Methods

Erosive Agent and Artificial Saliva
Pure orange juice in a wax paper package was purchased from a supermarket (J. Sainsbury, batch No. X6171Q). Five aliquots of 30 ml each were taken from the same package and the pH of each sample was measured with an Alpha 500 pH meter (Aqua Scientific) with a combination pH electrode (Orion, Boston, Mass., USA). The mean pH of the orange juice was found to be 3.85 ± 0.05.

The artificial saliva used was similar to that described by McKnight-Hanes and Whitford [1992] but was modified by the exclusion of sorbitol. It contained (g/l): methyl-β-d-xylopyranoside, 2.0; sodium carboxymethyl cellulose, 100; KCl, 0.625; MgCl₂·6H₂O, 0.059; CuCl₂·2H₂O, 0.166; K₂HPO₄, 0.804; KH₂PO₄, 0.326. The first two components increased the viscosity of the saliva, simulating the mucin and protein content of natural saliva, while the other constituents provided the inorganic components at levels comparable to that of natural saliva. The pH was adjusted to 6.75 using concentrated KOH.

Preparation of Teeth
A total of 10 freshly extracted bovine incisors were cleaned, polished with pumice and then with a 1,200-grit abrasive sandpaper with water to remove organic contaminants. A carbon pencil was used to map out a horizontal rectangular window (8 x 2 mm) on the middle region of the labial surface of the enamel of each tooth. Each tooth was then sectioned sagittally into two equal halves using a water-cooled diamond wafering blade (Buehler, Warwick, UK). Each half was then air dried and coated with two layers of acid-resistant nail varnish (Max Factor®) except for a rectangular window (4 x 2 mm). The hollow cavities (pulp chamber) in the specimens were filled with blue in-lay wax.

Experimental Procedure
In a pilot study, a clearly observable lesion was produced after 12 h of continuous immersion of bovine incisors in pure orange juice. In an attempt to mimic more closely what happens in the in vivo situation, the teeth were subjected to a cyclical exposure to pure orange juice and either artificial saliva or de-ionized distilled water for 24 days, giving a total of 12 h of exposure to orange juice as follows:

The experiment was divided into two regimens (A and B) with each containing one half of each of the 10 teeth used. The teeth were immersed in continuously stirred pure orange juice (20 ml/specimen) at regular intervals 6 times per day for 5 min at each occasion. The immersion was carried out at room temperature (approx. 20°C). In between exposures to orange juice and for 12 h overnight, the teeth in regimen A were stored in artificial saliva (20 ml/specimen), while those in regimen B were stored in de-ionized distilled water (pH 6.80 ± 0.05), both on a rotary mixer (10 rpm) at room temperature. On each occasion, prior to immersion in orange juice, the teeth were taken out of the artificial saliva, rinsed with de-ionized distilled water (although a film of the viscous saliva was retained on the tooth surface which simulated the normal bathing of teeth in saliva in the mouth) and immersed into the orange juice. On withdrawal from orange juice after 5 min, the teeth in both regimens were rinsed with de-ionized distilled water and replaced into either saliva (regimen A) or distilled water (regimen B). The orange juice, artificial saliva and de-ionized distilled water were changed on a daily basis.

Preparation of Sections
At the end of the cycling period, the specimens were washed with de-ionized distilled water and air dried. The nail varnish was removed with acetone and 4 sections bearing intact sound enamel surfaces at both ends with the eroded area in between were cut from each specimen using a water-cooled diamond saw (Well, Walter Ebner, Le Locle, Switzerland). The sections, approximately 250 µm in thickness, were mounted on brass anvils with nail varnish allowed to harden overnight and polished to give plane-parallel specimens of 80 µm thickness using a diamond disc.

Each section was imbedded with wax and examined under polarized light using a Nikon, optiphoto® light microscope with rotating stage, polarizer and analyzer at a magnification of ×450 (Nikon, Tokyo, Japan). This was carried out as an initial screening for homogeneity of the lesions produced and for possible differences in the shape and depth of the lesions from the two experimental regimens.

Microradiography
The sections were mounted on a microradiographic plate-holder bearing an aluminium stepwedge (25-µm steps). The microradiographs were taken with a 20-min exposure on Kodak high-resolution plates (type 1A) using a Cu(Kα) X-ray source (Philips BV, Eindhoven, the Netherlands) operating at 25 kV and 10 mA at a focus-specimen distance of 30 cm. The plates were developed using standard techniques.

Two-Step Image Analysis
The microradiographs of the sections were subjected to analysis using a Leica Leitz DM1RB optical microscope (Leica, Wetzlar, Germany). The image was captured at a magnification of ×200.40 via a CCD video camera (Sony, Tokyo, Japan) connected to a computer (Vigen PC, London, UK). The integrated mineral loss (vol%) and the lesion depth (µm) were quantified using a two-step image analysis technique in a software package (TMRB v. 1.22, Inspektor Research Systems BV, Amsterdam, the Netherlands) based on the work described by De Josselin de Jong et al., [1987]. The integrated mineral loss and lesion depth were calculated under the following defined parameters: start of lesion 20 vol%, start of sound enamel 95 vol% (end of lesion), 87 vol% mineral for the sound enamel. From the TMR ra-
diographs, the mineral loss is defined as the difference in volume percent of mineral between sound and eroded/demineralized tissue integrated over the lesion [Raben and Arens, 1993].

**First Step:** The analysis of an erosive lesion necessitates the reconstruction of the sound enamel surface. This was possible by firstly capturing an image of an erosive lesion together with an adjacent area of sound enamel and positioning the analysis box over a wide area of the sound enamel only (fig. 1a). The area within the analysis box was then analysed by adjusting a computer-generated analysis bar over the screen, on which the mineral volume percentage distribution of the sound enamel tissue is shown, to highlight (1) the outside of the sample, (2) the sound tissue (mineral volume percentage values of sound tooth tissue), (3) the start of the sample (edge of sound tooth tissue) and (4) the end of the lesion (fig. 1b).

**Second Step:** This second step which was necessary to quantify mineral loss and lesion depth in the erosive lesion involved keeping the same captured image and moving the same size of analysis box to the area of erosion (fig. 2a). Keeping the analysis line indicating the start
Table 1. Mean values of mineral loss and lesion depth, from individual samples after cyclical exposure to orange juice and either artificial saliva or de-ionized distilled water

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>S (μm)</th>
<th>W (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.266</td>
<td>10.615</td>
</tr>
<tr>
<td>2</td>
<td>4.175</td>
<td>7.445</td>
</tr>
<tr>
<td>3</td>
<td>4.714</td>
<td>9.026</td>
</tr>
<tr>
<td>4</td>
<td>4.066</td>
<td>8.647</td>
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<td>5</td>
<td>7.934</td>
<td>10.978</td>
</tr>
<tr>
<td>6</td>
<td>4.578</td>
<td>8.881</td>
</tr>
<tr>
<td>7</td>
<td>5.039</td>
<td>9.230</td>
</tr>
<tr>
<td>8</td>
<td>5.492</td>
<td>10.735</td>
</tr>
<tr>
<td>Mean</td>
<td>5.283</td>
<td>9.447</td>
</tr>
<tr>
<td>SD</td>
<td>1.288</td>
<td>1.227</td>
</tr>
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<table>
<thead>
<tr>
<th>Sample No.</th>
<th>S (μm)</th>
<th>W (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81.5</td>
<td>148.3</td>
</tr>
<tr>
<td>2</td>
<td>74.1</td>
<td>119.6</td>
</tr>
<tr>
<td>3</td>
<td>77.4</td>
<td>141.4</td>
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<td>4</td>
<td>59.6</td>
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<td>84.7</td>
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<td>76.2</td>
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<td>142.6</td>
</tr>
<tr>
<td>SD</td>
<td>7.7</td>
<td>11.7</td>
</tr>
</tbody>
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The value for each sample is the average value of 4 sections from each sample. S = Orange juice and artificial saliva; W = Orange juice and de-ionized distilled water.

of the sample in the same position (this effectively reconstructed the sound enamel surface), the line indicating the end of the lesion is adjusted to the new end of lesion position (fig. 2b). This then gave quantitative data for the mineral loss (μm), the lesion depth (μm) and the ratio (vol%) of these two parameters.

Each section was scanned once, and the microradiograph was moved manually under the microscope so that the whole length of the lesion can be viewed with the video to examine for homogeneity of the lesion. A wide analysis box was used to cover as much area of the lesion as possible and was placed away from the sloping border of the sound and eroded enamel area in order to get the full depth of the lesion. The analysis was carried out by three independent operators (the authors) to test for reproducibility of the method. Only the data from one operator were presented since the data from the three operators were exactly the same.

Statistical Analysis
The data from the image analysis were analysed statistically using a 2-way analysis of variance (ANOVA) and paired Student t tests, with the level of significance prechosen at 0.05.

Results
The lesions produced were homogeneous, and there was no observable difference in the shape of the lesions produced in the two experimental regimens under both polarized light and optical microscopes used for the analysis of the microdiagrams.

The values of the integrated mineral loss and the lesion depth presented for each sample are an average of the values from the 4 sections from each sample. A significantly greater mineral loss (p<0.001) and lesion depth (p<0.001) were observed in specimens cycled in orange juice and de-ionized distilled water when compared with specimens cycled in orange juice and artificial saliva (table 1A and B).

Discussion
The use of TMR to analyse an erosive lesion in enamel is a new and reliable method of quantifying the mineral loss by erosion. It is a sensitive and accurate technique in contrast to the existing microquantitative [Sorvari and Kiviranta, 1988], scoring (Restarski et al., 1945; McClure and Ruzicka, 1946; Wynn and Hald, 1948; Reussner et al., 1975) and grading [Eccles, 1979] methods which are considered subjective and are of limited sensitivity [Greenby, 1996]. While TMR and digital image analysis described by Misty and Greenby [1995] are both computer-aided, quantitative and sensitive techniques, digital image analysis measures the area and depth of the erosive lesion whereas TMR evaluates the amount of mineral loss and the depth of the lesion. The analysis of erosion with TMR is a modification of the technique which is now widely recognized for the evaluation of mineral loss or gain in de-remineralization studies. Once the image has been captured, analysis can be initiated by a programmed step-by-step instruction displayed by the computer with a simultaneous data storage. It can also be used for dental lesions in which case the volume percent mineral content for the sound tissue should be redefined (87 vol% for enamel, 45 vol% for dentine). Erosion does not only create a crater on enamel surface but also leads to a slight subsurface mineral loss [Meurman and ten Ooote, 1996]. The le-
sion depth was assessed as the distance from the measured sound enamel surface to the location in the lesion at which the mineral content is larger than 95% of the mineral content in sound enamel. So the lesion depth measured in this study is 'the depth of the crater plus a certain depth below the bottom of the crater at which the mineral content is less than 95% of the mineral content of sound enamel'. It was not surprising that the same data were obtained by the independent operators since the guidelines and the landmarks for the placement of the analysis lines are computer aided and reproducible on each occasion. In a situation where the lesions are inhomogeneous, that is part of the lesion close to the sound tissue is not comparable to the centre of the lesion, measurements can be taken at both ends of the lesion and the average calculated. Wide analysis boxes would facilitate inclusion of the centre of the lesion in the measurement.

In this study, pure orange juice was able to produce appreciable erosion on bovine enamel. This result was not unexpected since it is now well established that fruit juices, especially those containing citric acid, have the highest buffering capacity among the acidic drinks and beverages [Hay et al., 1962; Grenby et al., 1989].

The significantly lower mineral loss and lesion depth observed in specimens stored in artificial saliva after orange juice exposure suggested a possible protective and/or remineralizing effect by the artificial saliva. It has been reported that an erosive lesion is associated with a layer of slight subsurface mineral loss or softened enamel which has been shown to be remineralizable [Mühlmann et al., 1994]. It is in this layer that the remineralization of the eroded lesions was envisaged to have occurred. And it is well established that mineral formed during remineralization is less soluble than the original tooth mineral [Ingram and Edgar, 1994]. Moreover, it has been reported by many researchers [Gellhard et al., 1983; Vissink et al., 1985; Joyston-Bechal and Kidd, 1987] that saliva substitutes containing calcium and phosphate ions have the potential of hardening artificially softened enamel.

The protective action of the artificial saliva may be a combination of the following factors: (1) the protective effect of CMC against enamel demineralization which has recently been demonstrated by Van der Reijden et al. [1997], who speculated that the formation of an adsorbed polymer layer on the enamel surface may be the mechanism for this protective effect; (2) the artificial saliva-juice mixture in immediate contact with the enamel surface may be saturated with respect to calcium and phosphate salts at the early stage. The presence of calcium and phosphate ions in sufficient concentration in acidic drinks and foods has been shown to reduce the erosive effect of these drinks and foods [Hay et al., 1962; Reussner et al., 1975]. The migration of these ions from the saliva into the juice would reduce the kinetics of enamel dissolution by decreasing the driving force (degree of saturation of the juice with respect to enamel) [Zahradnik et al., 1976].

The results suggest that cycling teeth in artificial saliva as opposed to water reduces the degree of erosion caused by pure orange juice in vitro. The technique of TMR to quantify mineral loss by erosion is appropriate for use in in vitro studies, and it is envisaged that it will be useful for enamel specimens used in intra-oral appliances during in situ erosion trials.

References


