In Situ Fluoride Response of Caries Lesions with Different Mineral Distributions at Baseline

F Lippert, R.J.M. Lynch, G.J. Eckert, S.A. Kelly, A.T. Hara, D.T. Zero

Department of Preventive and Community Dentistry, Oral Health Research Institute, Indiana University School of Dentistry, Indianapolis, Ind., USA

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Corresponding author:

Frank Lippert

Department of Preventive and Community Dentistry

Oral Health Research Institute, Indiana University School of Dentistry

415 Lansing Street, Indianapolis, IN 46202 (USA)

Tel. +1 317 274 3983, Fax +1 317 274 5425, E-Mail flippert@iupui.edu

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Declaration of Interests

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Abstract

The present in situ study investigated the fluoride response of caries lesions with similar mineral loss but two distinct mineral distributions (low- and high-“R” calculated as the ratio of mineral loss to lesion depth). Sixteen subjects wore eight gauze-covered enamel specimens with preformed lesions placed buccally on their mandibular partial dentures for periods up to four weeks. The participants brushed twice daily for 1 min with an 1100 ppm F (as NaF) dentifrice. After three and four weeks, specimens were retrieved and analyzed microradiographically (TMR) and by quantitative light fluorescence (QLF). TMR results revealed that low- and high-R lesions showed opposite behaviors – low-R lesions further demineralized, whereas high-R lesions exhibited some remineralization. In comparison, lesion depth increased in low-R, but remained unchanged in high-R lesions; R decreased in both, but more in high-R lesions; mineral density at the lesion surface remained unchanged in low-R, but increased in high-R lesions. Differences in mineral loss between lesion types increased further between three and four weeks. QLF did not mirror TMR results as low-R lesions were found to remineralize, whereas high-R lesions remained unchanged. It is likely that low-R lesions differ from high-R lesions chemically and microstructurally; therefore rendering low-R lesion more susceptible to further dissolution. During lesion formation, low-R in contrast to high-R lesions may not lose all of the solubility-determining impurities such as magnesium and carbonate, which can reprecipitate again in different mineral phases within the lesion. In conclusion, mineral distribution at baseline directly impacts in situ lesion response to fluoride.

Introduction

The overall effectiveness of fluoride dentifrices in the reduction of caries incidence in vivo is well documented [e.g. Marinho et al., 2009]. As clinical caries trials are not only time- but also resource-consuming, a vast range of in situ caries models have been developed as surrogate measures for fluoride efficacy, dating back to pioneer work conducted by Koulourides and Volker [1964]. Ideally, these models should serve as bridges between the natural uncontrolled clinical situation and the highly controlled laboratory situation [Zero, 1995]. Reviews [ten Cate, 1994; Zero, 1995] highlighted the importance of various model parameters from different aspects. The mineral distribution of caries lesions at baseline has received only limited attention in in situ caries research given the widely accepted importance of this parameter [Strang et al., 1987; Mellberg, 1992; Schäfer et al., 1992; ten Cate, 1992]. Typically, studies were either limited to artificial caries lesions created using one particular demineralization protocol or lesions with different severities but similar mineral distributions.

Arends et al. [1987] first introduced the concept of describing the average amount of mineral loss in caries lesions, and therefore mineral distribution characteristics, using the “R” value which can be calculated for each lesion as the ratio of mineral loss (ΔZ) to lesion depth (L). More recently, Lynch et al. [2007] were able to divide artificially created lesions into low- and high-R lesions, depending on the demineralization protocol being used. More importantly, it was shown on in vitro lesions with similar ΔZ_{base} that high-R lesions were more responsive to remineralizing solutions than low-R lesions, possibly driven by the greater porosity of high- vs. low-R lesions.

In caries research in general, the use of artificially created lesions is (for obvious reasons) preferred over natural white spot lesions as a study substrate. While this allows for better control, it is somewhat removed from the in vivo situation, especially since very little is known about the mineral distribution characteristics of naturally occurring white spot lesions. To the authors’ knowledge, only one study [Lynch et al., 2007] actually reported R values for these lesions ($R = 16 \text{ vol\%}$), whereas two other studies [Iijima et al., 1999; Iijima and Takagi, 2000] provided sufficient information that R values of 32 and 25 vol%, respectively, could be calculated. Several other studies were concerned with natural white spot lesions in general; however, the lack of information provided did not allow for R values to be retrieved. This information would be vital in designing more appropriate laboratory and clinical models for the study of caries and

consequently for the development of strategies to reduce caries prevalence. As considerable variations in R values were found in the aforementioned studies, it would be advantageous to build on a previous study [Lynch et al., 2007] by investigating the effects of fluoride on lesions with different mineral distributions simultaneously and under more dynamic conditions, with potential for periods of undersaturation with respect to enamel as well as supersaturation, typical of the caries process.

Therefore, the aim of the present study was to investigate the response of low- and high-R lesions to a twice daily fluoride application using an established in situ caries model.

Subjects and Methods

Ethical Aspects

The study protocol was reviewed and approved by the IUPUI Institutional Review Board, #1002-59. It was conducted at the Oral Health Research Institute of the Indiana University School of Dentistry. All subjects signed a written informed consent prior to screening. Seventeen adult volunteers met the inclusion criteria which included having stimulated and unstimulated salivary flow rates equal or greater than the minimum requirement of 0.8 and 0.2 ml/min, respectively.

Experimental Design

The study was a randomized, single-center, single-product study design. Seventeen subjects between the ages of 52 and 79 years undertook the study, with 16 completing the study. Two to three days following a dental cleaning, eight partially demineralized specimens with two different mineral distributions (four low-R and four high-R lesions) but similar lesion volume at baseline (ΔZ_{base}) were placed in the buccal flange areas of the subject's mandibular partial denture, four specimens with low-R lesions on one side and four with high-R lesions on the opposite side. The side position (i.e. left or right) of specimens with low- and high-R lesions was randomized among subjects. Specimens were wrapped in gauze in pairs of two to facilitate plaque growth. Wrapped specimen parcels were mounted side by side and flush with the denture surface. As four specimens (or two specimen parcels containing two specimens each) were

mounted on each side of the partial denture; one parcel had to be mounted in the mesial and one in the distal position. The parcel position, lesion type and treatment period were all randomized. During the four week test period, subjects brushed with a full ribbon of the study toothpaste (Crest Cavity Protection, Procter & Gamble, USA; 1100 ppm F as NaF, silica abrasive) twice daily for one timed minute. Subjects brushed their teeth with their partial denture in place, taking care to not brush the specimen sites. After brushing, subjects were instructed to expectorate the toothpaste slurry and rinse with 15 ml of water for 10 seconds and expectorate. Subjects wore their partial dentures 24 hours a day during the test period. At the end of three weeks, four specimens (two of each lesion type) were removed and analyzed. The remaining four specimens were removed after four weeks. During specimen removal from the partial denture, care was taken not to damage any part of the specimen, including the lesion area and the nail varnish-covered sound enamel reference areas. Immediately after specimen removal, any plaque residue was carefully removed from the specimens using a deionized water-moistened cotton bud, before the specimens were placed, individually, into Eppendorf vials containing a moist cotton pellet. Three and four week treatment periods were chosen to obtain information on potential lesion reversal or progression rates.

Changes in the mineral content of the experimental caries lesions were measured using transverse microradiography (TMR) and quantitative light fluorescence (QLF). The primary response variable was change in mineral content of the lesions from baseline ($\Delta\Delta Z$). Secondary response variables were change in lesion depth (ΔL), R value (ΔR), degree of surface zone mineralization ($\Delta SZ_{\max}$), and lesion fluorescence ($\Delta\Delta F$).

A washout period of two to three days was observed before starting the study. During this period subjects were instructed to brush their teeth with a standard non-fluoridated dentifrice (Silly Strawberry Fluoride Free Toothpaste, Tom's of Maine, USA).

Specimen Preparation

Enamel specimens were obtained from human permanent teeth. Tooth crowns were cut into 4×4 mm specimens using a Buehler Isomet low-speed saw. The teeth were stored in thymol during the sample preparation process. Specimens were ground and polished to create flat, planar parallel dentin and enamel surfaces using a Struers Rotapol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, Pa., USA). The dentin side of the specimens was ground flat to a

uniform thickness with 500-grit silicon carbide grinding paper. The enamel side of the specimen was serially ground using 1,200, 2,400 and 4,000 grit paper. The specimens were then polished using a 1 μ m diamond polishing suspension on a polishing cloth until the enamel surface had a minimum of a 2 $\times$ 4 mm highly polished facet across the specimen. Resulting specimens had a thickness range of 1.7 – 2.2 mm. The specimens were assessed under the Nikon SMZ 1500 stereomicroscope at 20 $\times$ magnifications for cracks, hypomineralized (white spots) areas or other flaws in the enamel surface that would exclude them from use in the study. An experimental window, measuring approximately 1.7 $\times$ 4 mm, was created on the specimens using acid-resistant, clear nail varnish (Sally Hansen Advanced Hard As Nails Nail Polish, Natural, USA), leaving sound enamel (reference) areas on either side. Prepared specimens were stored at 100% relative humidity at 4 °C until use.

Artificial Caries Lesion Creation

In vitro incipient caries lesions were prepared using two methods described in detail elsewhere. A modification of the method described by White [1987] was used to create low-R lesions, whereas a modification of the method used by Laboratory D as described by ten Cate et al. [1996] was used to create high-R lesions.

To create low-R lesions, sound enamel specimens were immersed in a demineralization solution containing 0.1 M lactic acid, 4.1 mM Ca (as $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$), 8 mM PO_4 (as KH_2PO_4) and 0.2 % w/v Carbopol 907 (BF Goodrich Co., USA), pH adjusted to 5.0 using KOH at 37 °C for 11 d. Specimens were demineralized in groups of 50 using 10 ml demineralization solution per specimen. The solution was not stirred or replaced during the demineralization period.

To create high-R lesions, sound enamel specimens were demineralized at pH 4.6 and 37 °C in 8% methylcellulose (aqueous, 1,500 cP, 63 kDa) covered with an equal volume of 0.1 M lactic acid, pH adjusted with KOH, for 7 d. Specimens were demineralized in groups of 50 using 10 g of gel and 10 g of demineralization solution per specimen. Neither the gel nor the demineralization solution was replaced during the demineralization period.

Sodium azide (3 mM) was added to all demineralization solutions as a bacteriostat. After demineralization, specimens were rinsed with deionized water. Specimens were then stored individually in Eppendorf vials containing a moist cotton pellet and at room temperature.

A total of 400 specimens, 200 with a low- and 200 with a high-R lesion, were prepared for this study. Before clinical use, all enamel specimens were sterilized by ethylene oxide gas.

Microradiography

Sections, approximately 100 μm in thickness, were cut from one side of the specimens and across the lesion window and sound enamel (reference) areas after lesion creation (lesion baseline) using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA). After sectioning, a colored nail varnish was used to cover the cut surface of the specimens, serving as a reference point. Specimens were wrapped in gauze in such a way that the colored sides were facing each other. Post-treatment, another section was cut from each specimen and from the same side the baseline section was cut from (i.e. the colored side). The sections were mounted, with an aluminum step wedge, on high resolution glass plate Type I A (Microchrome Technology Inc., San Jose, CA) and X-rayed at 20 kV and 30 mA at a distance of 42 cm for 65 min. The film was developed in Kodak d-19 developer for 3 min, placed in a stop bath (Kodak 146-4247) for 45 s, and then fixed (Kodak 146-4106) for 3 min. All plates were then rinsed in deionized water for 15 min and air-dried. Microradiographs were examined with a Zeiss EOM microscope in conjunction with the TMR software v.3.0.0.11. Sound enamel was assumed to be 87% v/v mineral.

Only lesions exhibiting an intact surface zone were included in the study. Further inclusion criteria were based on ΔZ_{base} [$\text{vol}\% \cdot \text{min} \cdot \mu\text{m}$]: $1900 \leq \Delta Z_{\text{base}} \leq 3000$ for low-R lesions, and $2200 \leq \Delta Z_{\text{base}} \leq 2800$ for high-R lesions, to achieve a similar average ΔZ_{base} for both lesions of approximately 2500. To allow for appropriate study comparisons, lesions were randomized among subjects based on ΔZ_{base} with the four low- and the four high-R lesions exhibiting the lowest ΔZ_{base} being allocated to subject 1; the next set of four low- and four high-R lesions exhibiting the second lowest ΔZ_{base} was allocated to subject 2 and so on. Furthermore, lesions were also randomized to achieve similar ΔZ_{base} for three and four week treatment periods, for left and right sides and for specimen parcels in distal and mesial positions for each lesion type.

QLF Measurements

All specimens were air-dried for at least 30 min before QLF measurements were performed using the QLF Clin System and the QLF Patient software v.3.0.0.35 (Inspektor Research,

Netherlands). The clear nail varnish used to protect sound enamel reference areas was not removed, renewed or otherwise altered prior to QLF measurements.

Acquired QLF images were analyzed using the QLF Analysis software v.2.00f. ΔF values were recorded and at a threshold level of 5 %, i.e. a minimum of 5 % fluorescence loss between sound and demineralized enamel. The distance between the camera and the surface of the specimen was kept constant throughout the experiment to facilitate repeat measurements. Lesion baseline and post-treatment measurements were performed before sectioning.

Study Variables

For better clarity, all reported variables are summarized below:

ΔZ – lesion volume (product of lesion depth and the mineral loss over that depth)

L – lesion depth (83% mineral; i.e. 95% of the mineral content of sound enamel)

R – ratio of lesion volume to lesion depth ($\Delta Z/L$)

$SZ_{\max}$ – maximum mineral density at the lesion surface zone

ΔF – fluorescence of lesion area in relation to sound enamel

Changes in variables were calculated as follows:

$$\Delta\Delta Z^* = \Delta Z_{\text{base}} - \Delta Z_{\text{post}} \quad \Delta L =$$

$$L_{\text{post}} - L_{\text{base}}$$

$$\Delta R = R_{\text{post}} - R_{\text{base}}$$

$$\Delta SZ_{\max} = SZ_{\max, \text{post}} - SZ_{\max, \text{base}}$$

$$\Delta\Delta F^* = \Delta F_{\text{post}} - \Delta F_{\text{base}}$$

* – indicative of remineralization if parameter > 0 , or further demineralization if < 0

Statistical Analysis

ANOVA was used to compare the effects of lesion type (low- and high-R) and treatment duration (three and four weeks) on $\Delta\Delta Z$, ΔL , ΔR , $\Delta SZ_{\max}$, and $\Delta\Delta F$. The ANOVA models included terms for lesion type, treatment duration, lesion type – by – treatment duration interaction, side of mouth, and location within side (mesial or distal). The models also included a random effect to correlate multiple measurements within a subject. Correlation coefficients (Pearson) were calculated to evaluate the associations among the measurements and calculated changes from baseline. Analyses were performed with SAS statistical software, version 9.1 (SAS

Institute Inc., Cary, N.C., USA), at a significance level of 5%, with no adjustment for multiple comparisons.

Results

Baseline findings

Figure 1 shows the average mineral distributions of low- and high-R lesions at baseline, whereas table 1 provides baseline data for all variables. All variables were found to be statistically significantly different between lesion types at baseline. Percentage differences in variables between low- and high-R lesions at baseline were smallest for ΔZ_{base} ($< 5\%$). No statistically significant difference in variables was observed between lesions assigned for the three or four week study duration for each lesion type (data not shown). ΔZ_{base} was found to only weakly correlate with ΔF_{base} ($r = 0.36$).

Subject effects

Individual, mean subject responses ($\Delta\Delta Z$) to the study treatment with respect to lesion type and treatment duration are shown in table 2. Several observations can be made. There was a trend for high-R lesions to remineralize, whereas low-R lesions tended to demineralize further. Low-R lesions exhibited greater variability than high-R lesions which was also observed at lesion baseline (data not shown). Subject 16 showed a very distinctive behavior which cannot be sufficiently explained by a protocol deviation associated with this subject. Therefore, these data were included in the analysis. For the interested reader, it is worth noting that exclusion of subject 16 would have yielded a statistically significant change in $\Delta\Delta Z$ for high-R lesions at both the three- and four-week time points. Other comparisons were unaffected by the in/exclusion of this subject; likewise, the use of nonparametric statistics to reduce the influence of potential outliers would have yielded similar results for all variables (data not shown).

Lesion type characterization

Figure 1 shows the average mineral distributions of low- and high-R lesions after the four-week study duration in comparison to baseline. In the low-R lesions, a widening of the surface

zone and some remineralization in the original lesion body can be seen in addition to demineralization beyond the original lesion. No changes in the maximum surface zone mineralization can be observed. Furthermore, about half of the lesions showed lamination, with some showing multiple laminations; a feature that was lost by showing average mineral profiles. Figure 2 shows a representative microradiographic image of a low-R lesion after the three-week study duration. The original lesion (OL) can be seen as well as the lamination (Lam), separating the original lesion from the demineralization zone beyond the original lesion. In contradiction to the results obtained on low-R lesions, remineralization occurred throughout the entire lesion and including the surface zone in high-R lesions. Demineralization beyond the original lesion can also be seen; however, this was predominantly due to the results obtained for subject 16. Laminations occurred in only about 20 % of the high-R lesions. For both lesion types, similar results were obtained after three weeks in comparison to the four week study duration. Thus, three week results were omitted from figure 1 for better clarity.

Lesion type vs. treatment duration

Table 1 highlights results for all study variables by treatment duration and lesion type. Statistically significant changes from baseline are highlighted. The lesion type – by – duration interaction was not statistically significant for any of the outcomes ($p = 0.13$ for $\Delta\Delta ZM$, $p = 0.20$ for ΔL , $p = 0.26$ for ΔR , $p = 0.36$ for ΔSZ_{max} , $p = 0.31$ for $\Delta\Delta F$) so the lesion type comparisons are valid for both durations, and the duration comparisons are valid for both lesion types.

Low- and high-R lesions had significantly different $\Delta\Delta ZM$ ($p = 0.0001$). The numerical difference in $\Delta\Delta ZM$ between low- and high-R lesions was 816 at the three-week study duration and increased further to 1227 after four weeks. High-R lesions had significantly less increase in L ($p = 0.0001$), more decrease in R ($p = 0.0001$), more increase in SZ_{max} ($p = 0.0001$), and less decrease in ΔF ($p = 0.0001$) than low-R lesions. Worth pointing out is the discrepancy between QLF and TMR measurements, as $\Delta\Delta F$ showed opposite results in relation to $\Delta M\Delta Z$. The three week duration had significantly less increase in L than the four week duration ($p = 0.0347$). However duration did not significantly affect $\Delta\Delta ZM$ ($p = 0.09$), ΔR ($p = 0.12$), ΔSZ_{max} ($p = 0.96$), or $\Delta\Delta F$ ($p = 0.11$).

Side of mouth effect

Side of the mouth and position of specimen parcels in either distal or mesial position were also examined to evaluate their influence on the study outcomes. The left side of the mouth had a significantly more negative $\Delta\Delta Z$ ($p = 0.0108$) and significantly less increase in ΔL ($p = 0.0024$) than the right side, while side did not significantly affect ΔR ($p = 0.13$), $\Delta SZ_{\max}$ ($p = 0.42$), or $\Delta\Delta F$ ($p = 0.53$). Position of specimen parcels in either distal or mesial position did not significantly affect any of the outcomes ($p = 0.08$ for $\Delta\Delta Z$, $p = 0.07$ for ΔL , $p = 0.83$ for ΔR , $p = 0.92$ for $\Delta SZ_{\max}$, and $p = 0.21$ for $\Delta\Delta F$).

Correlation tests

Only weak correlations were found between ΔZ_{base} and $\Delta\Delta Z$ for high- ($r = -0.236$) and low-R ($r = -0.30$) lesions, respectively. The only high correlation found was between $\Delta\Delta Z$ and ΔL ($r = 0.92$). While several other correlations were statistically significantly greater than zero, none of these correlations would be considered as indicating anything other than a weak association.

Discussion

The present in situ study aimed to gain a better understanding of the relative fluoride response of caries lesions with different mineral distributions at baseline under physiologically relevant conditions typical for the caries process. An established in situ caries model [Zero et al., 2004] was used in the present study, with the key features being the use of gauze-covered specimens to facilitate plaque growth and to simulate a caries prone stagnation area. Furthermore, no diet restrictions were imposed on the study subjects, and the twice-daily, one minute, 1100 ppm F dentifrice application resembled a typical oral hygiene regimen. Therefore, this model/study can be considered of high clinical relevance. Although model parameters have been refined over the years, leading to a better understanding of the caries process, the impact of mineral distribution of caries lesions at baseline remains poorly understood. Lynch et al. [2007] found in vitro that high-R lesions show greater ability to remineralize than low-R lesions, which can possibly be attributed to differences in porosity or $SZ_{\max}$ with low-R lesions reaching inhibitory (for further remineralization) $SZ_{\max}$.

values faster than high-R lesions. Another possible factor which was discussed is the difference in area of enamel per unit volume of remineralizing solution within the low- and high-R lesions. The present study evaluated the behavior of these lesions under more dynamic in situ conditions, and for their relative responsiveness to fluoride.

It must be mentioned that low- and high-R lesions were also found to differ in ΔZ_{base} ; therefore somewhat compromising on the validity of study comparisons, especially considering that ΔZ_{base} was shown to directly impact the tendency of lesions to either de- or remineralize in situ [Mellberg, 1991; Schäfer et al., 1992]. However, the difference in ΔZ_{base} between lesion types was relatively small ($< 5\%$) and can be considered negligible, especially bearing in mind an earlier reported measurement error for TMR alone of approximately 4% [de Jong and ten Bosch, 1985].

At similar ΔZ_{base} , low-R lesions were deeper than high-R lesions, and low-R lesions also showed a significantly greater surface zone mineralization in the present study. This was not surprising, as the use of a surface-protective polymer and partially saturated (with respect to hydroxyapatite) conditions during lesion creation allowed for better establishment of a surface layer. It is also worth noting that R values of low-R lesions in the present study were considerably greater compared to a previous, relevant study [Lynch et al., 2007] (consequently, differences in R values were smaller between low- and high-R lesions in the present study). Although very similar lesion creation protocols were used, the reason for this discrepancy is not clear. In fact, the system used for creation of low-R lesions in the present study was less undersaturated with respect to hydroxyapatite than the commonly used system [White, 1987] utilizing a “50% saturation with respect to hydroxyapatite (HA)” (data not shown). Therefore, lower R values were expected in the present study, considering our current understanding of artificial lesion creation. However, this was not observed to be the case. Lynch et al. [2007] used bovine enamel and created shallower lesions, thus making a comparison between studies not necessarily straightforward.

The results obtained on high-R lesions are comparable to previous in situ studies on this lesion type [e.g. ten Cate, 1993; Laheij et al., 2010]. Although the models used had inherent differences, there was a general trend for remineralization as a result of daily fluoride treatments. Almost identical post-treatment changes in mineral distribution of the lesions from baseline were

observed in the present (figure 1) compared to a very recent study [Laheij et al., 2010], highlighting perhaps the “robustness” of this (very commonly used) lesion type.

The results obtained on low-R lesions, and especially in comparison to the high-R lesions, were intriguing. Despite very similar ΔZ_{base} for both lesions and virtually identical conditions, both lesion types exhibited opposite behaviors – high-R lesions tended to remineralize, whereas low-R lesions further demineralized. This was evident after the three-week study duration and further pronounced after four weeks. The differences in structure and solubility between lesion types at baseline may provide an explanation for their contradictory behaviors. Two aspects need to be discussed: the inherent solubility of the lesions after lesion creation on one hand and differences in microstructure and the extent of demineralization in inter- and intra-prismatic enamel and resulting consequences on solubility on the other hand.

While net demineralization is the result of lesion formation, remineralization or reprecipitation can still occur within the lesion and potentially influence lesion characteristics during subsequent de- and remineralization cycles. Enamel mineral has been described as a solid solution with appreciable inhomogeneity [Shellis et al., 1993]. During lesion formation, there is preferential dissolution of the least stable fractions, especially those associated with the impurities Mg and CO_3 [LeGeros, 1990]. Within the developing lesion, this can lead to levels of supersaturation which in turn will allow (re)precipitation of mineral phases different to the original enamel mineral. Several different mineral phases have, therefore, been postulated or shown to be present in caries lesions. Although there is still considerable debate in the literature, brushite (BR or dicalcium phosphate dihydrate (DCPD) – $\text{CaHPO}_4 \times 2\text{H}_2\text{O}$) [Moreno and Zahradnik, 1974], monetite (MT – CaHPO_4) [Featherstone et al., 1978] and Mg-substituted whitlockite (WHM – $\text{Ca}_9\text{Mg}(\text{HPO}_4)(\text{PO}_4)_6$) [Shellis et al., 1997] have all been reported. This is not surprising, as BR and MT are more stable than HA under acidic conditions [Brown, 1973]; whereas the mineral phase associated with Mg in (sound) enamel was proposed to be WHM [Driessens and Verbeeck, 1982]. BR has also been strongly associated with one of the mineral phases (among HA and F-HA) present in the lesion surface layer [Moreno and Zahradnik, 1974; Margolis and Moreno, 1985]. Especially WHM has been associated with the caries process, as several investigators were able to detect this mineral phase in natural caries lesions [Aoba and Yagi, 1991; Kodaka et al., 1992], with the site of WHM formation being perhaps close to the advancing front as there is preferential loss of Mg [Hallsworth et al., 1972]. Little is known

about F effects of Mg incorporation in WHM and HA. The sole study [LeGeros et al., 1992] reported that F in small amounts caused less Mg incorporation in WHM, but greater Mg incorporation in HA was observed, and that Mg did not affect F incorporation in HA.

It can only be speculated that under the highly unsaturated conditions employed during high-R lesion creation, reducing the mineral content by more than half by volume in the lesion body and at the surface (figure 1), the vast majority of soluble fractions including enamel impurities, such as Mg, Na, CO₃, was removed. This would render the remaining lesion relatively more robust to further demineralization, as little F can be expected to have been removed during initial demineralization. The resulting lesion is therefore likely to show a higher F concentration than the original, sound enamel (if calculated as F concentration per weight). Furthermore, due to the initial infinite undersaturation present during lesion creation (at least initially) and the relatively low pH, very little (re)precipitation of mineral phases can be expected. This is also highlighted by the fact that the formed surface layer was not as pronounced as in the low-R lesions, where lesion creation conditions were considerably different. Here, the partial saturation and higher pH give rise to the assumptions that soluble fractions were only partially removed, or removed from deeper parts of the lesion and then reprecipitated again closer to the solution-lesion interface. This would result in lesions with considerably higher SZ_{max} and a more even mineral loss throughout the lesion – or in other terms, lesions with a lower R value. Low-R lesions present less or at least narrower “diffusion pores” than high-R lesions, suggesting they are less prone to further dissolution than high-R lesions, assuming the hypothesis for the differences in dissolution rates between (sound) deciduous and permanent enamel by Poole et al. [1981]. However, the mineral in low-R lesions can be assumed to be considerably more soluble than in high-R lesions and contain (more) non-apatitic mineral phases such as BR and WHM. This is further complicated because not only porosity and solubility, but also structure are important factors in the caries process, and these factors have been shown to be linked [Shellis, 1995].

Shellis and Wilson [2004] reported that enamel contained a very soluble fraction (approximately 14%), which is the fraction presumably lost in its entirety in high- but not necessarily in low-R lesions. Initial dissolution was proposed to occur at prism junctions and inner enamel [Shellis et al., 1993], with the solubility behavior thereafter controlled by the less soluble middle and outer intraprismatic enamel, which make up the bulk of the enamel [Shellis, 1996]. Due to the different

mineral distribution profiles between low- and high-R lesions (figure 1), differences in the extent of demineralization in intra- and perhaps also interprismatic enamel must exist between lesion types. Furthermore, mineral phases that may have reprecipitated during lesion formation in low-R lesions may have (artificially) increased the interprismatic fraction. Although the comparison to the present study is not exactly straightforward, relatively greater lesion depths observed in deciduous vs. permanent enamel were attributed to greater mean prism-junction density and mean volume fraction of interprismatic enamel in deciduous enamel [Shellis, 1984]. This may provide another explanation for the observed differences in the present study. Whether chemical and/or structural or other aspects were responsible for the contradictory behavior of low- and high-R lesions in the present study remains to be determined. The results obtained in the present study have shown that in situ caries lesion de- and remineralization are by no means straightforward and that with the current knowledge no definitive explanation of the results can be provided. Furthermore, more research should focus on de- and remineralization of established lesions and under more dynamic conditions, as most studies focus on only one aspect and are therefore of limited clinical relevance.

In situ models can be, although rather crudely, divided into net de- or remineralization with the present model been described as a net remineralization model [Zero, 1995]. In the present study, this discrimination is not possible as net de- and remineralization were observed for the sole study treatment. In this context, the results of the QLF analysis, which contradict the TMR findings (table 1) as no net demineralization was observed, must be discussed. The reason for this discrepancy is not clear, especially as previous, comparative studies [Ando et al., 2001; Fujikawa et al., 2008; Hafstrombjorkman et al., 1992] have shown good correlation between $\Delta(\Delta)F$ and $\Delta Z/\Delta\Delta Z$. These studies, however, were rather limited in their approach as only de- and/or remineralization were studied, not taking into account caries dynamics. As little is known about the sensitivity of QLF to lesion lamination or the impact of the R value on ΔF , further research is needed on this still relatively new de- and remineralization evaluation technique.

In the present study, differences in response to the study treatment were observed between left and right test sites of the mouth (regardless of in- or exclusion of subject 16), despite similar ΔZ_{base} between sites for both lesion types. These findings have been reported before [Mellberg et

al., 1992; Zero, 1995] and may be explained by chewing patterns and brushing preferences of the study subjects, which in turn can lead to side differences in fluoride delivery.

Perhaps the most important question remaining is which of the two lesions is more clinically relevant for the study of caries? Or can both lesions be used for the simultaneous assessment of treatment effects on caries progression and reversal? Studies on natural white spot lesions [Iijima et al., 1999; Iijima and Takagi, 2000; Lynch et al., 2007] indicated a relatively high degree of surface zone mineralization for these lesions which is more comparable to the low-R lesions employed in the present study. Likewise, the “pattern” of mineral distribution (but not necessarily the R value) was more comparable to low- rather than high-R lesions. Lynch et al. [2007] suggested that low-R lesions would be more appropriate where more physiologically relevant mineral distribution is required, whereas high-R lesions would be appropriate for studying inherent remineralization efficiency. It must be noted that natural white spot lesions form over a considerably longer period of time than “laboratory lesions” (assuming similar severity) and that they are subject to appreciably higher fluctuations in pH and F, Ca and Pi concentrations during their development where laboratory lesions are not. Especially the importance of lesion bound F deserves more attention as this may impact the inherent solubility of the remaining lesion mineral the most. Perhaps the choice of lesion type in in situ research is more determined by personal preference, as most investigators follow the same lesion creation protocol for their studies as this will allow comparison between studies. However, the impact of differences in mineral distribution at lesion baseline is therefore somewhat ignored. To expand on this, it would be advantageous to study treatment effects also on sound and very early, surface softened lesions in addition to the lesions used in the present study as this would provide information on a more complete spectrum of study substrates. This would aid in the development of treatment interventions specifically designed for lesions at different stages in the caries process. However, one should not forget that in situ models are “only” models of in vivo caries, and that observations in these models do not necessarily mirror the real life situation.

Further in situ studies on high- and low-R lesions should perhaps focus on determining the relative effectiveness of fluoride in a dose-response manner. Studies on lesions with different ΔZ_{base} and R values may further our understanding of the relative importance of ΔZ_{base} and R in determining their responsiveness to fluoride. In addition, potential chemical and structural

differences between lesion types need to be studied and in comparison to natural white spot lesions.

In conclusion, the present in situ study has shown that lesions of similar severity but different mineral distribution showed different responses to fluoride – low-R lesions further demineralized, whereas high-R lesions exhibited some remineralization.

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Table 1. Lesion parameters at baseline and after three and four weeks of study duration

		ΔZ [vol% min $\times \div \mu\text{m}$]	$\Delta\Delta Z$ [vol% min $\times \div \mu\text{m}$]	L [μm]	ΔL [μm]	R [vol% min]	ΔR [vol% min]	SZ _{max} [% min.]	Δ SZ _{max} [% min.]	ΔF [%]	$\Delta\Delta F$ [%]
Baseline	Low-R	2599 $\pm$ 54	-	95.5 $\pm$ 1.7	-	27.5 $\pm$ 0.4	-	58.8 $\pm$ 0.7	-	29.6 $\pm$ 0.8	-
	High-R	2477 $\pm$ 54	-	68.1 $\pm$ 1.7	-	36.7 $\pm$ 0.4	-	39.2 $\pm$ 0.7	-	25.9 $\pm$ 0.8	-
	High- vs. low-R difference ^a	-122 $\pm$ 54 ^a	-	-27.4 $\pm$ 1.7 ^a	-	9.2 $\pm$ 0.4 ^a	-	-19.6 $\pm$ 0.7 ^a	-	-3.7 $\pm$ 0.8 ^a	-
Three weeks post-treatment	Low-R	3137 $\pm$ 245	-525 $\pm$ 221 ^b	122.0 $\pm$ 8.6	26.7 $\pm$ 8.3 ^b	25.9 $\pm$ 0.9	-1.9 $\pm$ 0.9 ^b	60.3 $\pm$ 1.3	1.8 $\pm$ 1.5	21.9 $\pm$ 2.2	7.7 $\pm$ 2.2 ^b
	High-R	2187 $\pm$ 244	291 $\pm$ 220 ^c	71.6 $\pm$ 8.5	3.3 $\pm$ 8.2	31.7 $\pm$ 0.9	-4.8 $\pm$ 0.9 ^b	45.6 $\pm$ 1.3	6.7 $\pm$ 1.5 ^b	26.5 $\pm$ 2.2	0.3 $\pm$ 2.2
Four weeks post-treatment	Low-R	3553 $\pm$ 243	-958 $\pm$ 219 ^b	141.5 $\pm$ 8.5	45.0 $\pm$ 8.2 ^b	25.0 $\pm$ 0.8	-2.3 $\pm$ 0.9 ^b	60.1 $\pm$ 1.3	0.6 $\pm$ 1.5	17.6 $\pm$ 2.2	12.2 $\pm$ 2.3 ^b
	High-R	2203 $\pm$	269 $\pm$ 220 ^c	74.4 $\pm$	7.9 $\pm$	30.2 $\pm$ 0.9	-7.2 $\pm$ 0.9 ^b	46.9 $\pm$	8.1 $\pm$	23.8 $\pm$	1.3 $\pm$

244	8.5	8.2	1.3	1.5 ^b	2.2	2.2
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The results are means $\pm$ between-subject standard error.

^a calculated as variable [high-R lesion] minus variable [low-R lesion]. All paired comparisons between high- and low-R lesions at baseline were statistically significant ($p < 0.05$).

^b indicates a statistically significant change from baseline ($p < 0.05$)

^c indicates the only variable where the exclusion of subject 16 would have resulted in a statistically significant change from baseline

Table 2. Mean change in mineral loss ($\Delta\Delta Z$; vol%min $\times$ μm) by subject, lesion type and treatment duration

Subject	high-R lesions		low-R lesions	
	3 wk	4 wk	3 wk	4 wk
1	600	270	n.s. ^a	-225
2	540	740	-35	-255
3	540	120	-530	-1435
4	220	465	-140	15
5	340	195	-355	-1615
6	-110	140	-740	-725
7	655	475	-605	-650
8	370	990	165	205
9	810	1160	170	n.s.
10	535	975	-50	-1025
11	370	45	-200	-200
12	-160	270	-1335	-3915
13	435	710	-1060	-170
14	395	610	-830	-185
15	-1565	-3130	-2225	-3290
16	585	555	-670	-1345

^a specimens did not yield sections suitable for TMR analysis

Figure Legends

Figure 1. Average mineral distributions for low- and high-R lesions at baseline and after the four-week study duration.

Figure 2. Microradiographic image of a low-R caries lesion after the three-week study duration. The original lesion (OL) and a clear lamination (Lam) are visible.

OL Lam