Re- and Demineralization Characteristics of Enamel Depending on Baseline Mineral Loss and Lesion Depth in situ

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Abstract

Objectives: The aim of this double-blinded, randomized, cross-over in situ study was to evaluate the re- and demineralization characteristics of sound enamel as well as lowly and highly demineralized caries-like enamel lesions after the application of different fluoride compounds.

Methods: In each of three experimental legs of 4 weeks, 21 participants wore intraoral mandibular appliances containing 4 bovine enamel specimens (2 lowly and 2 highly demineralized). Each specimen included one sound enamel and either one lowly demineralized (7 days, pH 4.95) or one highly demineralized (21 days, pH 4.95) lesion, and was positioned 1 mm below the acrylic under a plastic mesh. The three randomly allocated treatments (application only) included the following dentifrices: (1) 1,100 ppm F as NaF, (2) 1,100 ppm F as SnF$_2$ and (3) 0 ppm F (fluoride-free) as negative control. Differences in integrated mineral loss ($\Delta\Delta Z$) and lesion depth ($\Delta L D$) were calculated between values before and after the in situ period using transversal microradiography.

Results: Of the 21 participants, 6 did not complete the study and 2 were excluded due to protocol violation. Irrespective of the treatment, higher baseline mineral loss and lesion depth led to a less pronounced change in mineral loss and lesion depth. Except for $\Delta\Delta Z$ of the dentifrice with 0 ppm F, sound surfaces showed significantly higher $\Delta\Delta Z$ and $\Delta L D$ values compared with lowly and highly demineralized lesions ($p<0.05$, t test).

Conclusion: Re- and demineralization characteristics of enamel depended directly on baseline mineral loss and lesion depth. Treatment groups should therefore be well balanced with respect to baseline mineral loss and lesion depth.

Since 1964 a wide range of in situ caries models have been developed using a variety of hard tissue substrates [Zero, 1995]. Sound surfaces, natural lesions as well as lesions formed in vitro, in situ or in vitro/in situ have been used [Mellberg, 1992]. Although several reviews highlighted the potentials and limitations of the different in situ models from different perspectives [Wefel, 1990; Manning and Edgar, 1992; Zero, 1995], only one of them discussed the influence of baseline mineral loss and lesion depth on re- or demineralization characteristics [ten Cate, 1994]. However, this review showed inconclusive results.
In fact, only two in situ studies, specifically analyzing the influence of baseline mineral loss and lesion depth on the re- and remineralization characteristics of enamel, could be found in the currently available literature [Strang et al., 1987; Lippert et al., 2011]. After the application of dentifrice containing 1,100 ppm F (as sodium fluoride, NaF), a linear relationship between both factors could be shown for predemineralized human enamel specimens, situated in the lingual position of the lower jaws [Strang et al., 1987]. Lesions with higher baseline mineral loss produced a more pronounced remineralization. The second study, also using dentifrice containing 1,100 ppm F (as NaF), revealed different reactions for lesions with different ‘R’ values (being the ratio of mineral loss, ΔZ, to lesion depth) [Lippert et al., 2011]. Highly demineralized lesions (high R value) tended to remineralize, whereas lowly demineralized lesions (low R value) further demineralized. Predemineralized human enamel specimens situated in the vestibular position of the lower jaws were brushed twice daily for 1 min. Additionally, two articles, summarizing data from different in situ studies, revealed similar linear relationships for specimens treated with fluoride dentifrices [Mellberg, 1991b; Schafer et al., 1992] but not for specimens treated with fluoride-free dentifrices [Mellberg, 1991b]. In the first article data from nine fluoride dentifrice groups (1,100 ppm F as NaF) showed an increase in mineral uptake with higher baseline mineral loss, whereas data from eleven fluoride-free dentifrice groups (0 ppm F) showed a loss in mineral content in all lesions except the largest ones, with the smallest lesions having the greatest absolute mineral loss [Mellberg, 1991b]. Furthermore, baseline mineral loss did not affect the percentage change when specimens were treated with fluoride dentifrices, whereas it strongly affected the percentage change for fluoride-free treatment. In the second paper data from four in situ studies revealed that small lesions acquired less mineral than large lesions and that baseline mineral loss was related to whether net demineralization or net remineralization occurred [Schafer et al., 1992]. In each of the four included studies human enamel specimens were brushed with dentifrices containing 1,100–1,450 ppm F (as sodium monofluorophosphate, SMFP, or NaF).

Although studies have shown an increase in mineral uptake for lesions with higher baseline mineral loss, when testing predemineralized human enamel specimens, none of the articles used bovine specimens or examined sound surfaces compared with predemineralized specimens. No fluoride compounds other than NaF or SMFP (without differentiating between them) have been investigated. Just one of the articles not only analyzed dentifrices containing 1,100–1,450 ppm F (as SMFP or NaF) but also fluoride-free dentifrices [Mellberg, 1991b].

Thus, the purpose of the present study was to investigate the relation between baseline mineral loss and baseline lesion depth on re- and remineralization characteristics of sound surfaces as well as lowly and highly demineralized caries-like enamel lesions using an established in situ caries model. The secondary purpose of this study was to investigate the influence of SnF₂, NaF and fluoride-free dentifrice on this performance. We hypothesized that regardless of the respective fluoride, sound surfaces and lowly demineralized lesions are significantly more prone to demineralization than highly demineralized ones.

Materials and Methods

Ethical Aspects

Ethical approval was given by the local institutional ethical committee (Medical Faculty, RWTH Aachen University; No. EK 136/13).

The number of participants was calculated on the basis of previously performed in situ studies [Dijkstra et al., 1990; Mellberg et al., 1992; Meyer-Lueckel et al., 2015a, b]. The α-error was set at 5%. Considering the differences between the toothpastes with 1,100 and 0 ppm F the statistical power calculated was 98% (mean difference of 879, SD = 910). The dropout rate was assumed not to exceed 20%. Approximately 20 participants should have been enrolled into the study for an expected completion of at least 16. However, at the end of the in situ periods the dropout rate was 38% (13 participants completed the study). Since the retrospective power analysis with 13 participants has still provided a power of at least 80%, no additional participant was involved in the study.

All participants (21 volunteers, 13 women and 8 men, aged from 21 to 55 years) lived in Aachen, Germany, used tap water with a fluoride concentration of approximately 0.2 mg/l and signed a written informed consent. They were all in good general health with no signs of active caries or periodontal disease. Exclusion criteria were as follows: pregnancy, current participation in another study, institutionalized patients, periodontal disease, active caries lesions, age <18 years, salivary flow rate <0.7 ml/min, no written informed consent and incapability of contracting.

Study Design

The study design was a double-blinded, randomized, crossover in situ trial with three treatment legs. After screening for general eligibility, dental impressions of the lower jaw were taken and appliances with bilateral flanges were prepared (fig. 1B) [Koulourides et al., 1974]. In each of both flanges 2 predemineralized bovine enamel specimens were inserted 1 mm below the acrylic under a plastic mesh (Perfect Splint® System; Hager and Werken, Duisburg, Germany) mimicking ‘plaque-retaining’ surfaces [Meyer-Lueckel et al., 2007; Schirrmeister et al., 2007; Meyer-Lueckel et al., 2015b]. Each specimen included one sound enamel and either one lowly demineralized (7-day, pH 4.95) or one highly demineralized lesion (21-day, pH 4.95; fig. 1).
The factors under evaluation were as follows:

- ‘Baseline substrate condition’ at three levels: sound, lowly demineralized or highly demineralized
- ‘Intervention’ at three levels: application of dentifrice containing: (1) sodium fluoride (1,100 ppm F, pH 7.8) and triclosan (0.30%; Colgate Total; Colgate-Palmolive, New York, N.Y., USA; standard treatment, NaF), (2) sodium gluconate-stabilized stannous fluoride (1,100 ppm F, pH 5.6: Procter & Gamble, Weybridge, UK; experimental treatment, SnF₂) or (3) fluoride-free dentifrice (0 ppm F, pH 7.2; Procter & Gamble; negative control)

The participants wore intraoral mandibular appliances for three legs of 4 weeks each, with the in situ exposure only being interrupted during meals and for oral hygiene, resulting in a total wearing time of 22–23 h per day. Each of the three treatment legs were immediately preceded by a 1-week ‘lead-in’ period. For each test cycle the participants received a new toothbrush (Oral-B Indicator; Proctor and Gamble, Schwalbach am Taunus, Germany), a nutrition protocol and the dentifrice in a white packaging containing no commercial names.

Twice daily (in the morning and evening), after removing the intraoral appliance from the mouth, 1 g of the respective dentifrice was applied on the toothbrush. After brushing the teeth for 30 s, the participants spat out the resultant saliva dentifrice mixture (natural slurry) and applied it extraorally on the specimens for 2 min. Subsequently, the participants completed brushing their own teeth (total brushing time: 2 min). Specimens were not brushed at any time to allow plaque to grow. Afterwards the appliances were washed with tap water and re-inserted into the oral cavity, and left for at least 30 min without any intake of drink or food during this period. During additional extraoral times the appliances were stored in plastic boxes in a humid environment. These extra times were noted and added at the end of each phase to ensure similar wearing times for each phase for each volunteer.

The participants were informed not to use high fluoride-containing products (food and oral hygiene products) and received fluoride-free salt for home use. To trigger demineralization, the appliances were placed in 10% sugar solution for 20 min 3 times daily [Meyer-Lueckel et al., 2015a, b].

**Randomization**

After baseline examination a computerized random allocation sequence was generated by the study sponsor who coded the dentifrices with a subject number. The code was provided by a third person not directly involved in this study in a sealed envelope to be broken only in the case of an emergency.

**Specimen Preparation**

Bovine incisors were obtained from freshly slaughtered cattle (negative BSE test) and stored in 0.08% thymol (fig. 1A). Teeth covered with resin for in situ appliance; j = specimens inserted 1 mm below the acrylic under a plastic mesh. B Design of the intraoral mandibular appliances. L = Lowly demineralized lesions; H = highly demineralized lesions. Two specimens with lowly demineralized lesions and 2 specimens with highly demineralized lesions were inserted into the appliances. Each specimen included 1 sound surface.

**Fig. 1.** A Specimen preparation. C = Control surface; S = sound surface; L/H = lowly or highly demineralized lesion; a = frontal view of bovine front tooth; b = separation of crown and root; c, d = cuts perpendicular and parallel to the long axis of the tooth crown; e = obtained specimens (5 × 3.5 × 3 mm); f = specimen covered with resin; g = predemineralized specimen; h = obtain- ment of the 100 μm slices for baseline TMR analysis; i = specimen
were cleaned and approximately 350 enamel blocks (5 × 3.5 × 3 mm) were prepared (Exakt 300; Exakt Apparatebau, Norderstedt, Germany). After sterilization with ethylene dioxide the enamel blocks were embedded in epoxy resin (Technovit 4071; Heraeus Kulzer, Hanau, Germany), ground flat and hand polished (4,000 grit; Mikroschleifsystem Exakt). Baseline mineral loss and lesion depth were determined. For this ΔZBaseline and LDBaseline were assumed to be zero.

Data are presented as means (SD). Negative values for ΔZ, ΔΔZ as well as LD and ΔLD indicate demineralization, and positive values indicate remineralization. Values were only included when at least 1 specimen of a duplet of a participant could be analyzed; therefore n = 13. Values of the mean mineral density profiles were used to calculate SZmax. p values indicate significant differences in mineral loss before and after the in situ period.

### Table 1. Mean mineral loss, SZmax and lesion depth for sound surfaces as well as lowly and highly demineralized lesions before and after the in situ phase

<table>
<thead>
<tr>
<th>Mineral loss</th>
<th>ΔZBaseline</th>
<th>ΔZEffect</th>
<th>ΔΔZ</th>
<th>p</th>
<th>SZmaxBaseline</th>
<th>SZmaxEffect</th>
<th>LDBaseline</th>
<th>LDEffect</th>
<th>ΔLD</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>1,100 ppm F (NaF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sound</td>
<td>0°</td>
<td>-1,679 (909)</td>
<td>-1,679 (909)</td>
<td>-</td>
<td>a</td>
<td>68</td>
<td>0°</td>
<td>-72 (26)</td>
<td>-72 (26)</td>
<td>-</td>
</tr>
<tr>
<td>Lowly demineralized</td>
<td>-3,031 (905)</td>
<td>-4,219 (1,929)</td>
<td>-1,189 (1,835)</td>
<td>0.038</td>
<td>42</td>
<td>59</td>
<td>-99 (19)</td>
<td>-136 (48)</td>
<td>-37 (50)</td>
<td>0.020</td>
</tr>
<tr>
<td>Highly demineralized</td>
<td>-5,270 (1,091)</td>
<td>-5,835 (3,340)</td>
<td>-564 (2,995)</td>
<td>0.510</td>
<td>34</td>
<td>42</td>
<td>-151 (22)</td>
<td>-178 (48)</td>
<td>-27 (68)</td>
<td>0.175</td>
</tr>
<tr>
<td>Lowly demineralized (7-day lesion)</td>
<td>-3,238 (1,340)</td>
<td>-3,466 (2,090)</td>
<td>-228 (2,696)</td>
<td>0.765</td>
<td>41</td>
<td>70</td>
<td>-106 (26)</td>
<td>-124 (45)</td>
<td>-17 (59)</td>
<td>0.312</td>
</tr>
<tr>
<td>Highly demineralized (21-day lesion)</td>
<td>-5,804 (1,301)</td>
<td>-5,431 (2,709)</td>
<td>-373 (2,942)</td>
<td>0.656</td>
<td>37</td>
<td>40</td>
<td>-160 (27)</td>
<td>-169 (57)</td>
<td>-10 (59)</td>
<td>0.552</td>
</tr>
</tbody>
</table>

0 ppm F | | | | | | | | | | |
| Sound | 0° | -1,794 (1,092) | -1,794 (1,092) | - | a | 70 | 0° | -83 (52) | -83 (52) | - |
| Lowly demineralized (7-day lesion) | -3,134 (871) | -3,843 (1,755) | -709 (1,862) | 0.195 | 42 | 65 | -102 (15) | -130 (36) | -27 (43) | 0.003 |
| Highley demineralized (21-day lesion) | -5,537 (882) | -5,633 (2,834) | -96 (2,666) | 0.899 | 35 | 41 | -155 (17) | -174 (57) | -18 (56) | 0.014 |

### Free Fluoride Analysis

Fluoride concentrations of all dentifrices were measured (Orion Autochemistry System 960; Fisher Scientific, Ulm, Germany) using a calibrated ion-specific electrode (type 96-09 BNC; Fisher Scientific). For low-level measurement four fluoride solutions (3.8, 1.9, 0.38 and 0.19 mg/l) were prepared. These concentrations narrowly bracket the expected sample concentrations. The electrode potentials (millivolts) of the four standard solutions were measured and plotted on the linear axis against their concentrations (milligrams/liter) on the log axis. After calibration the fluoride concentrations of the dentifrices were determined. For this, 200 mg of the dentifrices were diluted in 100 ml distilled water at room temperature. Then 4 ml of each solution were centrifuged at 2,500 g and 1 ml of the supernatant was added to 1 ml TISAB II (Fisher Scientific). The percentage of free fluoride in relation to the given total fluoride concentration (manufacturer’s information) was determined. Two solutions for each dentifrice were prepared and analyzed in triplelicate.
Re- and Demineralization Characteristics of Enamel

Electron Microprobe Analysis

The contents of MgO, Na₂O, CaO, Cl, P₂O₅ and SnO₂ being incorporated in the enamel surface of specimens in group SnF₂/NaF were measured using a JEOL JXA 8,900 microprobe (JOEL, Eching, Germany) equipped with five WD spectrometers. An accelerating voltage of 15 kV, a probe current of 15 nA and a probe diameter of about 5 μm were used. Characteristic X-rays were recorded using a TAP crystal for Mg and Na, a PETJ crystal for Cl and Ca and a PETH crystal for P and Sn. As standard materials spinel (Mg), jadeite (Na), tugtupite (Cl), apatie (P, Ca) and stannous oxide (Sn) were used. After the in situ period 3 specimens of intervention SnF₂/NaF were prepared and analyzed in triplicate. The percentage of stannous in relation to given total ions was determined. The calculated 1-sigma detection limit of Sn is about 200 ppm (0.02 wt%).

Statistical Analysis

The data from the participants completing the study (n = 13; per-protocol analysis) were statistically analyzed using the SAS statistical software (SAS 9.2; SAS, Cary, N.C., USA). Variables were tested for normal distribution (Shapiro-Wilk test). For lowly and highly demineralized lesions differences in integrated mineral loss and lesion depth before and after in situ exposure were analyzed using the t test. Analysis of covariance for crossover design (ANCOVA) and the t test were used to detect differences in the changes in mineral loss (ΔΔZ) and lesion depth (ΔLD) between interventions. More technically, the ANCOVA statistical model may be described as a general linear mixed model with baseline, period and treatment as fixed effects and participant as a random effect. Correlations between ΔZ Baseline and ΔΔZ as well as between LD Baseline and ΔLD were assessed using the Spearman’s rank correlation coefficient. All tests were performed at a 5% level of significance.

Results

Regarding the participants’ consumption of dentifrice no significant differences between NaF (100.6 g), SnF₂ (116.0 g) and fluoride-free (116.0 g) toothpastes were observed (p > 0.05, ANCOVA). Calculus formation or staining was also not detected for any participant. Of the participants, 6 did not complete the study and 2 were excluded due to protocol violation. Out of ethical reasons specified by the ethics committee of the Medical Faculty of the RWTH University, participants who decide to quit the study should be able to do it at any time (even after signing the informed consent) and without mentioning the reasons. A total of 13 specimens broke during preparation, resulting in 143 specimens that could be analyzed after the in situ phase.
Mean (SD) baseline mineral loss and lesion depth were 2,930 vol% × μm (1,487) and 100 μm (31) for lowly demineralized (7-day) lesions, respectively, and 5,208 vol% × μm (1,600) and 149 μm (34) for highly demineralized (21-day) lesions (table 1).

In general, independently of the intervention, sound surfaces presented the highest demineralization and highly demineralized lesions the lowest. Both toothpastes with 1,100 ppm F showed similar effects with no significant differences in ΔΔZ and ΔLD for all three baseline substrate conditions (p > 0.05, ANCOVA). Thus, for further analysis of the influence of the baseline or lesion characteristics on further de- or remineralization, the two toothpastes were combined in a category named 1,100 ppm fluoride intervention (table 1). The reactions and the mean mineral profiles for the three baseline substrate conditions for interventions with (1,100 ppm F) and without (0 ppm F) fluoride can be seen in figures 2 and 3.

The lowly demineralized lesions tended to demineralize more than highly demineralized lesions, although no significant differences were observed regardless of the intervention (p > 0.05, adjusted t test; fig. 2). Between sound surfaces and highly demineralized lesions significant differences could only be observed for the intervention 1,100 ppm F (p < 0.05, adjusted t test; fig. 2). Irrespectively of the baseline substrate condition specimens of group 0 ppm F showed a significantly higher change in mineral loss (demineralization) than specimens of group 1,100 ppm F. A similar behavior was observed regarding lesion depth. Regardless of the interventions sound surfaces showed the highest increase in lesion depth, being significantly higher than for lowly and highly demineralized lesions (p < 0.05, adjusted t test). Highly demineralized lesions revealed the lowest increase in lesion depth.

According to Spearman’s rank correlation coefficient a low but significant correlation could be found between baseline mineral loss and change of mineral loss (r_{1,100 ppm F} = 0.436, p_{1,100 ppm F} < 0.001; r_{0 ppm F} = 0.246, p_{0 ppm F} = 0.008) as well as between baseline lesion depth and change of lesion depth (r_{1,100 ppm F} = 0.462, p_{1,100 ppm F} < 0.001; r_{0 ppm F} = 0.436, p_{0 ppm F} < 0.001) for both kinds of intervention.

**TMR: Mineral Density Profiles**

After the in situ period specimens of group 1,100 ppm F revealed a surface layer more mineralized than before the in situ period, whereas specimens of group 0 ppm F revealed a surface layer less mineralized than before (fig. 3; table 1). Before and after the in situ period specimens with
highly demineralized lesions showed the least mineralized surface layer and specimens with sound surfaces showed the most mineralized surface layer (fig. 3; table 1).

Fluoride Analysis and Electron Microprobe Analysis

The percentage of free/soluble fluoride in relation to given total fluoride concentration (SD) was 92.5% (4.6) [1,017.5 (50.6) ppm F] for NaF and 91.7% (7.9) [1,008.7 (86.9) ppm F] for SnF₂. In the fluoride-free dentifrice no free/soluble fluoride was measured. No stannous could be found in the lesions and in the sound surfaces.

Discussion

The present in situ study evaluated the re- and demineralization characteristics of sound enamel as well as lowly and highly demineralized caries-like enamel lesions in an established in situ caries model. The study hypothesis was partially rejected since for the samples treated with fluoride-free toothpastes no significant differences in the changes of mineral loss were observed for the different baseline substrate conditions (sound, lowly and highly demineralized). However, for the samples treated with the fluoride-containing toothpastes, indeed significantly more demineralization was observed for the sound surfaces than for highly demineralized lesions. Independent-ly of the intervention, a trend could be observed for high-ly demineralized lesions to demineralize less than lowly demineralized lesions or sound surfaces.

A low but significant correlation between baseline substrate condition and demineralization could be ob-served for all interventions. In our demineralizing in situ model the change in mineral loss and lesion depth de-creased with increasing baseline values (more mineral loss). This reflects the increasing potential for demineralization with decreasing mineral loss and lesion depth under demineralizing conditions. Since the design of an in situ model and the environment created by the model will have an overriding impact on its response (net demineralization or net remineralization) [Zero, 1995], the effect observed in the present study is presumed to be reversed under remineralizing conditions. Indeed, in-creasing potential for remineralization with increasing baseline mineral loss and lesion depth under remineral-izing conditions has been observed [Strang et al., 1987]. Thus, in demineralizing in situ models lowly demineral-ized baseline lesions are presumably more applicable, whereas in remineralizing in situ models highly demineral-ized baseline lesions seem to be more applicable.

We hypothesized that the effect observed in the present demineralizing in situ study is related to the nature of the subsurface caries lesions. Thus, a more mineralized surface layer could be observed in the presence of fluoride than in its absence. For lowly and highly demineralized lesions this mineralized surfaces layer could presumably be built within a few days, whereas for sound samples this surface layer could only be built after initial deminerali-zation [Arends and Christoffersen, 1986]. Once the mineral-ized surface layer has been established, it is a barrier firstly for the dissolution of mineral and secondly for the diffusion of acids into deeper parts of the lesions. This inhibiting mechanism increases when lesions becomes deeper [Arends and Christoffersen, 1986]. At the end it might even reach an equilibrium at which neither re- or demineralization occurs [ten Cate and Duijsters, 1982]. The previous findings are confirmed through the present results. In the presence of fluoride significantly more de-mineralization was observed for the sound surfaces than for highly demineralized lesions. Secondly, fewer mineral-als could be bound in the surface layer of highly demineralized specimens than in the sound ones, due to the hindered process of dissolution and incorporation of minerals, especially in initially larger lesions [Arends and Christoffersen, 1986]. Hence, a lower S\text{Z}_\text{max} of the lesion surface was observed in highly demineralized specimens. Contrastingly, in the absence of fluoride these effects could not be observed. Here the degree of mineralization of the lesion surface layer for all three types of lesions was in the same order of magnitude, so no ‘protective’ effect of the surface layer could be established (in the absence of fluoride), resulting in only slight differences (in ΔZ, ΔLD and S\text{Z}_\text{max}) between the various baseline substrate conditions.

In addition to the impact of the lesion surface zone on the observed effects, the mineral density may have played a role in the different degrees of de- and remineralization effects observed in the present study. It has recently been shown that lesions with the same Δ\text{Z}_\text{Baseline} but different LD\text{Baseline}, and consequently high or low R values, react differently under net remineralizing conditions. Lesions with lower mineral density (high R values) showed the highest reaction to remineralizing solutions [Lynch et al., 2007]. Since the observed effect is presum-ably reversed under demineralizing conditions, this is in agreement with the results of the present study. The lowly demineralized lesions (low R value: 29, SD = 3) de-mineralized more than the highly demineralized lesions (high R value: 35, SD = 3) under demineralizing condi-tions.
Sound enamel as well as lowly demineralized (7-day demineralization; 100 μm) and highly demineralized (21-day demineralization; 150 μm) caries-like enamel lesions were used to analyze re- and demineralization characteristics. From a clinical point of view the lesions classified here as highly demineralized were ‘only’ 150 μm deep and could be considered ‘subclinical’. Some of them would probably be difficult to detect in vivo [ten Cate et al., 2008]. However, the initiation and progression of enamel caries lesions seems to be extremely slow [Bjarnason and Finnbogason, 1991], and fluoride seems to be the predominant factor influencing remineralization and arresting the process of ‘subclinical’ caries lesions [Silverstone, 1982; ten Cate, 1984]. This anticares effect is assumed to be limited to the outer 150–200 μm of the lesion (at deeper levels no difference was observed compared with placebo) [ten Cate and Rempt, 1986; White and Featherstone, 1987; Bjarnason and Finnbogason, 1991]. However, various types of deeper lesions formed in vitro and in vivo [Al-Khateeb et al., 2002] should be analyzed in further studies, firstly to get more information on the tested agent, secondly to get more information on the model being used and thirdly to choose the perfect setting for the scientific issue under investigation (before analyzing the scientific issue under clinical conditions).

In the present study the participants were instructed not to brush the specimens at any time to allow plaque to grow and to compare the re- and demineralization characteristics of enamel depending on baseline mineral loss and lesion depth of the caries challenge in the worst-case scenario. This is in contrast to other in situ studies analyzing baseline characteristics [Strang et al., 1987; Mellberg, 1991b; Lippert et al., 2011] but might be a realistic sequence for proximal surfaces since many people do not clean these areas on a regular basis. Previous studies have also shown that this model seems preferable to simulating proximal caries [Hara et al., 2003; Itthagarun et al., 2005; Meyer-Lueckel et al., 2007; Cochrane et al., 2012; Meyer-Lueckel et al., 2015a].

The best way to check the validity of an in situ model is to demonstrate a fluoride dose response similar to the anticipated clinical response. The more likely the model reflects the natural caries process, the more likely the model will be responsive to clinically proven cariostatic agents [Wefel, 1992]. Referring to the known dose-dependent clinical effects of dentifrices containing 0, 500 and 1,100 ppm F [Walsh et al., 2010], this effect could recently be shown for the present in situ model [Meyer-Lueckel et al., 2015a]. A fluoride dose-dependent effect was observed for dentifrices containing 0, 500 or 1,450 ppm F. Under the conditions tested here, again an effect of the same order could be observed for the dentifrice containing 1,100 ppm F, and significant differences in the change of mineral loss could be observed for all baseline substrate conditions compared with fluoride-free dentifrice. Regarding the lesion size this does not always seem to be the case, as a former in situ study observed much smaller differences in dentifrices containing between 0 and 1,100 ppm F for highly than for lowly demineralized lesions after 14 days in situ [Mellberg, 1991b].

All former in situ studies evaluating baseline mineral loss and lesion depth in in situ re- or demineralization studies used predemineralized human enamel specimens [Strang et al., 1987; Schafer et al., 1992; Lippert et al., 2011], except one study presumably using a mixture of predemineralized human and bovine specimens [Mellberg, 1991b]. Contrastingly, in the present study we used bovine enamel. Compared with human enamel bovine teeth are easier to obtain and mineralization patterns show lower variations, resulting in a more consistent experimental response [Mellberg, 1992; Kiellbassa et al., 2006]. However, bovine enamel is more porous, resulting in faster mineral changes and diffusion rates and consequently faster lesion formation [Edmunds et al., 1988].

Four bovine enamel specimens were inserted in the buccal flanges in the present in situ appliances and plaque accumulation was triggered by recessed specimens 1 mm below the acrylic under a plastic mesh. In contrast to former studies [Meyer-Lueckel et al., 2007, 2015b], in which 8 bovine enamel specimens were inserted, the size of the buccal flanges of the appliance was reduced in order to increase the wearing comfort. Although the reduced buccal flanges did not result in an increased risk of fracture, no additional comfort was diagnosed (e.g. by fewer pressure sores compared with former studies) or reported by participants. Thus, for further in situ studies buccal flanges with up to 8 specimens seem to be recommendable since more information can be gained simultaneously by using a second (different) substrate (e.g. dentine), a different brushing mode (e.g. brushing) or a different position (e.g. easily cleanseable).

SnF2 combines therapeutic effects against dental caries [Mellberg, 1991a], gingivitis [Beiswanger et al., 1995], sensitivity [Schiff et al., 2006] and dental erosion [Huysmans et al., 2011]. Nonetheless, it is very reactive and has to be prevented from being oxidized (to stannous IV) and consequently becoming insoluble and ineffective [Lippert, 2013]. Therefore, several approaches to stabilize and to increase the effectiveness have recently been made. It has been combined with, for example, sodium fluoride.
be interpreted as ‘enhanced’ remineralization. Nonetheless, the maximum mineral density of the lesion surface zones ($S_{\text{max}}$) indicated that Sn did not influence mineral quantification in the present investigation. This is confirmed by the results of the electron microprobe analysis. The maximum percentage of stannous in relation to total ions present in enamel was under the detection threshold (0.02 wt%).

It can be concluded that baseline mineral loss and lesion depth directly affect in situ lesion response. This relationship was not influenced by SnF$_2$, NaF or fluoride-free dentifrices. Treatment groups should therefore be well balanced with respect to baseline mineral loss and lesion depth. Particularly in demineralizing in situ models, lowly demineralized baseline lesions are presumably more applicable.

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**Author Contributions**

H.M.-L. and R.J.W. designed and planned the study. R.J.W. and J.L. prepared the samples. R.J.W. performed the measurements and statistical analysis. R.J.W. and M.E.-O. wrote the manuscript. All authors revised the manuscript.

**Disclosure Statement**

The authors declare no conflicts of interest.

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