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Demineralization Inhibitory Effects of Highly Concentrated Fluoride Dentifrice and Fluoride Gels/Solutions on Sound Dentin and Artificial Dentin Caries Lesions in vitro

Daniel Erdwey[a, b] Hendrik Meyer-Lueckel[c] Marcella Esteves-Oliveira[b, d] Christian Apel[e] Richard Johannes Wierichs[c, e]

[a]Department of Oral and Maxillofacial and Facial Plastic Surgery, School of Medicine, University Hospital RWTH Aachen, Aachen, Germany; [b]Department of Operative Dentistry, Periodontology and Preventive Dentistry, RWTH Aachen University, Aachen, Germany; [c]Department of Restorative, Preventive and Pediatric Dentistry, zmk Bern, University of Bern, Bern, Switzerland; [d]Department of Cariology, Endodontology and Periodontology, University of Leipzig, Leipzig, Germany; [e]Department of Biohybrid and Medical Textiles, Institute of Applied Medical Engineering, RWTH Aachen University, Aachen, Germany

Keywords
Dentin · Demineralization · Fluoride gel · Fluoride solution · pH-cycling

Abstract
Objectives: The aim of this in vitro study was to compare the demineralization inhibitory effect of gels/solutions used in combination with either standard or highly fluoridated dentifrices on sound dentin as well as on artificial dentin caries-like lesions. ***Methods:*** Bovine dentin specimens (n = 240) with two different surfaces each (sound [ST] and artificial caries lesion [DT]) were prepared and randomly allocated to twelve groups. Weekly interventions during pH-cycling (28 days, 6 × 120 min demineralization/day) were: the application of gels/solutions containing amine fluoride/sodium fluoride (12,500 ppm F [ppm]; pH = 4.4; AmF); NaF (12,500 ppm; pH = 6.6; NaF1); NaF (12,500 ppm; pH = 6.3; NaF2); silver diamine fluoride (14,200 ppm; pH = 8.7; SDF); acidulated phosphate fluoride (12,500 ppm; pH = 3.8; APF), and no intervention (standard control; S). Furthermore, half of the specimens in each group were brushed (10 s; twice per day) with dentifrice slurries containing either 1,450 ppm (e.g., AmF_{1450}) or 5,000 ppm (e.g., AmF_{5000}). Differences in integrated mineral loss ($\Delta\Delta Z$) and lesion depth (ΔLD) were calculated between values before and after pH-cycling using transversal microradiography. ***Results:*** After pH-cycling Ss showed significantly increased ΔZ_{DT} and LD_{DT} values, indicating further demineralization. In contrast, except for one, all groups including fluoride gels/solutions showed significantly decreased ΔZ_{DT} values. Additional use of most fluoride gels/solutions significantly enhanced mineral gain, mainly in the surface area; however, acidic gels/solutions seemed to have negative effects on lesion depths. ***Significance:*** Under the present pH-cycling conditions the highly fluoridated dentifrice significantly reduced caries progression and additional application of nearly all of the fluoride gels/solutions resulted in remineralization. However, there was no difference in the remineralizing capacity of fluoride gels/solutions when used in combination with either standard or highly fluoridated dentifrices.

karger@karger.com
www.karger.com/cre

Karger

Daniel Erdwey
Department of Oral and Maxillofacial and Facial Plastic Surgery
School of Medicine, University Hospital RWTH Aachen
Pauwelsstrasse 30, DE–52074 Aachen (Germany)
derdwey@ukaachen.de

Introduction

Fluoride gels are commonly used as an additional topical fluoride treatment, especially for high-risk patients [Marinho et al., 2015]. The demineralization inhibitory effect of a gel is supposed to result from its local reaction at the tooth-gel interface [Featherstone and Ten Cate, 1988]. After application calcium fluoride precipitates on the tooth surface, providing a reservoir of fluoride ions [Ogard et al., 1994]. Application frequency ranges from weekly, if applied by the patients themselves, up to semi-annually, if applied by a dental profession.

One of the most widely used gels in Germany contains 12,500 ppm F as AmF/NaF. Acidic amine fluoride has been reported to protect enamel against erosion [Lennon et al., 2006; Wegehaupt and Attin, 2010] and demineralization [Esteves-Oliveira et al., 2017]. On dentin, however, the demineralization inhibitory effect has not been analyzed in a cariogenic in vitro model. Nonetheless, a few models mimicking the oral environment of patients suffering from xerostomia indicated that an acidic AmF gel significantly hampers demineralization in dentin, which was not the case when using an acidic NaF gel [Walther et al., 2019].

To increase the demineralization inhibitory effects of gels, fluoride compounds have been modified (e.g., acidulated phosphate fluoride; APF). APF contains sodium fluoride at pH 3.0 and phosphate [Brudevold et al., 1963]. Thus, fluorapatite formation is supposed to be increased and calcium fluoride formation is supposed to be depressed [Ogard et al., 1994]. Several studies on enamel show beneficial demineralization inhibitory effects [Clark et al., 1985; Agrawal and Pushpanjali, 2011]. In situ, it could be shown that the single application of a gel containing APF (12,300 ppm F) plus the daily use of a dentifrice (1,100 ppm F) inhibited enamel demineralization as effectively as a highly fluoridated dentifrice containing NaF (5,000 ppm F) [Fernandez et al., 2017]. However, when this combination was analyzed using dentin the results were inconsistent. For sound dentin surfaces the percentage of surface hardness recovery was significantly higher for the highly fluoridated dentifrice, whereas for initially demineralized dentin specimens the combined use of the gel and the standard fluoridated dentifrice showed a significantly higher reduction of the lesion area [Fernandez et al., 2017]. In a second in situ study using dentin lesions the demineralization inhibitory effect of the combined use of a gel containing APF (12,500 ppm F) plus standard fluoridated dentifrice (1,100 ppm F) was (non-)significantly superior compared with the dentifrice only [Vale et al., 2011]. However, the legs of the in situ studies lasted only 7 [Vale et al., 2011] or 14 days [Fernandez et al., 2017], respectively. Within this relatively short period natural remineralization from saliva is not exhausted [Neuhaus and Lussi, 2009], since natural remineralization by saliva seems only to be enhanced with a study design which lasts at least 21 days [Leach et al., 1989]. Thus, the effects of APF on sound dentin as well as on dentin lesions remain unclear.

Moreover, other fluoride compounds (e.g., silver diamine fluoride; SDF; for a more intuitive reading SDF is also called gel, although it is a solution) have been proposed instead of NaF or AmF. A considerable antibacterial effect could be observed for SDF [Rosenblatt et al., 2009]. Silver nitrate ($AgNO_3$) nanoparticles are able to penetrate the bacterial cell walls [Mei et al., 2012] disrupting the DNA replication [Targino et al., 2014]. Even under bacteria-free conditions SDF containing 35,400 ppm F prevented further demineralization on enamel [Delbem et al., 2006] and dentin [Wierichs et al., 2018a] caries lesions, indicating a considerable demineralization inhibitory effect based solely on the fluoride compound. However, the demineralization inhibitory effect of SDF containing lower fluoride concentrations (14,200 ppm) has not been analyzed in a chemical model so far.

The demineralization inhibitory effects of the above-mentioned gels have mainly been studied using initially demineralized as well as sound enamel surfaces, but only once for various dentin conditions [Fernandez et al., 2017]. The cited study is also the only study comparing the use of a highly fluoridated dentifrice with the use of a standard fluoridated dentifrice and additional application of fluoride gels on dentin. Moreover, previous systematic reviews on the caries-preventive efficacy of root caries highlighted that there is a lack of data on the optimal use of professional applied fluoride agents combined with the daily use of (standard) fluoride dentifrices [Gluzman et al., 2013; Twetman and Keller, 2016]. Thus, until know there is a gap in the knowledge. The aim of the present study was, therefore, to compare the demineralization inhibitory effect of different fluoride gels/solutions in addition to the use of either standard or highly fluoridated dentifrices on sound dentin surfaces as well as artificial dentin caries-like lesions under net-demineralizing conditions using a bacteria-free pH-cycling model. We hypothesized that no significant differences in mineral loss and lesion depth would be observed between the groups with additional use of fluoride gels/solutions, but for all compared with the dentifrice-only groups.

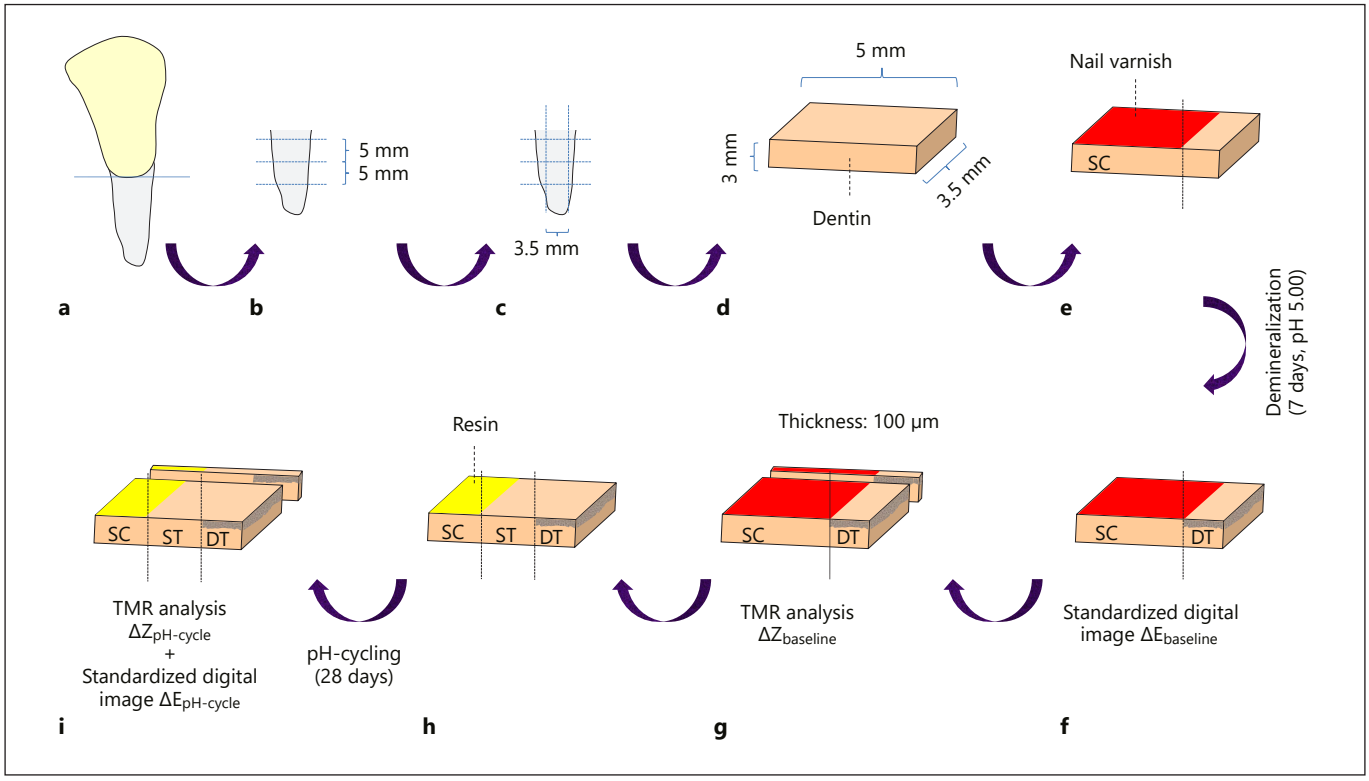


Fig. 1. Specimen preparation. **a** Frontal view of a bovine incisor. **b**, **c** Lines for cutting perpendicular and parallel to the root axis. **d** Prepared specimens (5 × 3 × 3.5 mm). **e** Specimen covered with acid-resistant nail varnish (SC, red). **f** Initially demineralized specimen (DT; gray). **g** Thin section of 100 µm for baseline TMR analysis. **h** Specimen covered with resin (ST; brown). **i** Thin section of specimen after pH-cycling for 28 days and application of the agents.

Materials and Methods

Specimen Preparation

Bovine incisors were obtained from freshly slaughtered cattle (negative BSE test) and stored in 0.08% thymol. Teeth were cleaned and 460 dentin blocks (5 × 3.5 × 3 mm) were prepared (Exakt 300; Exakt Apparatebau, Norderstedt, Germany) [Wierichs et al., 2016, 2020] (Fig. 1). The dentin blocks were embedded in acrylic resin (Technovit 4071; Heraeus Kulzer, Hanau, Germany), ground flat, and hand-polished (waterproof silicon carbide papers, FEPA grit sizes: 800, 1,200, 2,400, 4,000, Phoenix Alpha; Wirtz-Buehler, Düsseldorf, Germany).

Lesion Formation

Two thirds of the surface of each specimen was covered with nail varnish (sound control, SC, and sound treatment area, ST) in order to ensure enough mechanical and acid resistance. SC was used as a control to register possible damage (e.g., destruction, erosion, abrasive surface loss) of the specimen during the in vitro study. The other third remained uncovered (demineralized treatment area, DT). To create subsurface artificial dentin caries lesions in uncovered areas, specimens were stored in a demineralization solution for 7 days (2.0 mL solution/mm^2 dentin surface). The solution contained 50 mM acetic acid, 2.2 mM $CaCl_2 \cdot H_2O$, 2.2 mM KH_2PO_4, 10 mM KOH, 47.6 µM NaF, and traces of thymol (pH 5.00; 37 °C) [Wierichs et al., 2018a]. During that period, the pH was monitored daily and, if necessary, adjusted with small amounts of either 10% HCl or 10 M KOH to maintain a constant pH value.

After demineralization of the specimens, artificial caries lesions with intact surface layers were observed in all samples. In order to calculate baseline mineral loss and lesion depth, thin slices of 100 (±10) µm of each partially demineralized dentin specimen were cut perpendicularly to the surface, as described below. These slices were prepared for transversal microradiographic (TMR) analysis in order to select specimens with comparable and homogeneous demineralization. This way, 240 specimens with a mean (95% confidence interval; CI) baseline mineral loss (ΔZ_B) of 932 (841; 1,024) vol% × µm and a mean lesion depth (LD_B) of 138 (135; 141) µm were chosen from the 460 specimens originally prepared [Esteves-Oliveira et al., 2017]. Specimens were randomly allocated to twelve experimental groups.

pH-Cycling Conditions

A computer-controlled pH-cycling and brushing machine [Wierichs et al., 2017b] was used to simulate oral pH fluctuation patterns and daily oral care. Recently, it was shown that the model

Table 1. The pH-cycling regimen (schedule for 24 h)

Cycle		Phase	Total time, h:min:s
1	Demineralization	Demineralizing phase Rinsing phase (30 s)	02:00:00
	Brushing	Brushing (10 s) Slurry exposure (additional 110 s) Rinsing (30 s)	00:02:30
	Remineralization	Remineralizing phase Rinsing phase (30 s)	01:00:00
2	Demineralization	Demineralizing phase Rinsing phase (30 s)	02:00:00
	Remineralization	Remineralizing phase Rinsing phase (30 s)	01:00:00
3	Demineralization	Demineralizing phase Rinsing phase (30 s)	02:00:00
	Remineralization	Remineralizing phase Rinsing phase (30 s)	01:00:00
4	Demineralization	Demineralizing phase Rinsing phase (30 s)	02:00:00
	Remineralization	Remineralizing phase Rinsing phase (30 s)	01:00:00
5	Demineralization	Demineralizing phase Rinsing phase (30 s)	02:00:00
	Remineralization	Remineralizing phase Rinsing phase (30 s)	01:00:00
6	Demineralization	Demineralizing phase Rinsing phase (30 s)	02:00:00
	Remineralization	Remineralizing phase Rinsing phase (30 s)	01:00:00
	Brushing	Brushing (10 s) Slurry exposure (additional 110 s) Rinsing (30 s)	00:02:30
"Night" period	Remineralization	Demineralizing phase Rinsing phase (30 s)	05:55:00
Once weekly the respective gels were applied directly after the brushing (before the "night period").			

is able to reveal a dose-response characteristic on bovine enamel [Wierichs et al., 2018b] and dentin [Wierichs et al., 2020] similar to the anticipated clinical efficacy. pH-cycling lasted 28 days and conditions were chosen with a daily schedule of 6 cycles, where specimens were consecutively subjected to a demineralizing (120 min), a rinsing (30 s), a remineralizing (60 min), and again a rinsing (30 s) phase. During a 6-h "night" period the specimens were subjected to a remineralizing solution [Wierichs et al., 2018a] (Table 1). The remineralizing solutions contained 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, and 20 mM HEPES, pH 7.0. The demineralization solutions contained 50 mM acetic acid, 2.2 mM $CaCl_2 \cdot H_2O$, 2.2 mM KH_2PO_4, 10 mM KOH, and 23.8 μM NaF, pH 5.0 [Wierichs et al., 2018a]. For both solutions the pH was adjusted with small amounts of 10% HCl or 10 M KOH. New pH-cycling solutions were used for every cycle. The amounts of each solution were large enough to prevent the solutions from becoming saturated with or depleted of mineral ions (0.7 mL solution/mm^2 dentin surface).

Twice a day (before the first and last remineralizing phase) the specimens were brushed (Oral-B Indicator; Procter & Gamble, Schwalbach am Taunus, Germany) for 10 s with either a standard fluoridated dentifrice (group name, e.g., AmF_{1450}; 1,450 ppm F as NaF) or a highly fluoridated dentifrice (e.g., AmF_{5000}; 5,000 ppm F

Table 2. Description of dentifrices and gels, fluoride content and active ingredients

Group	Dentifrice	Fluoride content, ppm F[1]	Free fluoride content (SD), ppm F[2]	pH[1]	pH[2]	Active ingredients[1]	Inactive ingredients[1]
Standard fluoridated dentifrice	Blend-a-med Frisch (Procter & Gamble, Schwalbach am Taunus, Germany)	1,450	1,527 (17) [105 (1) %]	nm	7.3	NaF	Sorbitol, aqua, hydrated silica, sodium lauryl sulfate, trisodium phosphate, flavor, sodium phosphate, cellulose gum, carbomer, sodium saccharin, titanium dioxide, blue 1
Highly fluoridated dentifrice	Colgate® Duraphat® 5,000 ppm fluoride toothpaste (Colgate-Palmolive Ltd, Guildford, UK)	5,000	5,434 (35) [109 (1) %]	nm	8.4	NaF	Liquid sorbitol (non-crystallizing), dental type silica, dental type silica (precipitated), macrogol 600, tetrapotassium pyrophosphate, xanthan gum, sodium benzoate (E211), sodium lauryl sulfate, spearmint flavoring (containing peppermint oil, carvone, spearmint oil, menthol, anethol, and lemon oil), saccharin sodium, brilliant blue FCF (E133), and purified water.
Group	Varnish	Fluoride content (ppm F)[1]	Free fluoride content (SD) (ppm F)[2]	pH[1]	pH[2]	Active ingredients[1]	Inactive ingredients[1]
AmF	Elmex gelee (CP GABA GmbH, Hamburg, Germany)	12,500	13,907 (184) [111 (2) %]	4.5–5.0	4.4	AmF NaF	Purified water, propylene glycol, hyetellose, saccharin, apple aroma, peppermint aroma, crisped mint oil, menthol aroma, banana aroma.
NaF1	Sensodyne PROSCHMELZ Fluorid Gelee (GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Munich, Germany)	12,500	14,430 (349) [115 (3) %]	7.0	6.6	NaF	Purified water, disodium hydrogen phosphate, carbomer, sodium dodecyl sulfate, peppermint flavor, sodium saccharin, grapefruit aroma type A
NaF2	Paro Fluor Gelée (Esro AG, Kilchberg, Switzerland)	12,500	15,081 (230) [121 (2) %]	6.7–7.3	6.3	NaF	Flavor, sodium saccharin, E131, E217, E219, sodium lauryl
SDF	Cariestop 12% (Biodinâmica Química E Farmacêutica LTDA, Ibiporã, Brazil)	14,200	11,023 (1,947) [78 (14) %]	8.5	8.7	SDF	Fluoridric acid, silver nitrate, ammonia hydroxide, deionized water
APF	NUPRO® acidulated phoshpate fluoride topical gel (Dentsply Sirona, York, PA, USA)	12,500	13,741 (144) [110 (1) %]	3.0–4.0	3.8	NaF, APF	Hydrofluoric acid, methylparaben, carbomer homopolymer type B, phosphoric acid, benzoic acid, FD&C red No. 40, water, xanthan gum, sodium saccharin
S (negative control)	–	–	–	–	–	–	

S, standard control; SD, standard deviation; nm, not measured.
[1] According to the manufacturer.
[2] According to the present measurements.

as NaF; Table 2). The dentifrice slurries remained on each specimen for another 110 s. Subsequently, the specimens were perfused with distilled water to remove the slurry. In total, the brushing procedure for each specimen lasted 120 s, hence simulating the recommended brushing time of 2 min [Ganss et al., 2009]. The machine was adjusted to a constant brushing frequency of 60 strokes/min and a constant brushing load of 0.5 N [Wierichs et al., 2020]. These settings ensured the prevention of brushing abrasion in the present study. For brushing, dentifrice slurries were prepared with deionized water at a ratio of 2:1 parts by weight and renewed every second day [Wierichs et al., 2020].

Treatment

Once weekly interventions during pH-cycling were as followed: application of a gel containing amine fluoride and sodium fluoride (AmF); sodium fluoride (NaF1); sodium fluoride (NaF2);

silver diamine fluoride (SDF); acidulated phosphate fluoride (APF), and no additional gel intervention (standard control, S; Table 2). The respective gels were applied directly after toothbrushing (before the last remineralizing phase) according to the manufacturer's instructions, with the exception of a prolonged application time (5 min instead of 1–3 min as instructed) and a modified application frequency (SDF is recommended to be applied once or twice a year). Nonetheless, SDF was chosen as the fluoride concentration and the application methods are similar to fluoride gels. Subsequently, the specimens were perfused with distilled water to remove the gels. Thus, any carry over effect could be avoided. Furthermore, by using prolonged application times a "best-case scenario" under net-demineralizing conditions was simulated.

Determination of Free Fluoride and pH in the Dentifrice and the Fluoride Gels

For the gels and dentifrices total soluble fluoride concentrations were measured using an ion-specific electrode (type 96–09 BNC; Fisher Scientific) [Wierichs et al., 2020]. For calibration, 4 fluoride solutions (3.8, 1.9, 0.38, and 0.19 mg/L) were prepared. After calibration the fluoride concentrations of the gels and dentifrices were determined. For this 200 mg of the freshly prepared gels and dentifrices were diluted in 100 mL of distilled water at room temperature. Four milliliters of the diluted gels and dentifrices were centrifuged at 3,200 *g* for 10 min and 1 mL of the supernatant was added to 1 mL of TISAB II (Thermo Fisher Scientific, Beverly, MA, USA) and analyzed in triplicates. The pH values of the gels were measured with a pH-sensitive electrode (pH-Meter GMH 3,510, pH electrode GE 100, Greisinger, Regenstauf, Germany) according to the manufacturer's instructions [Esteves-Oliveira et al., 2017].

TMR Analysis

After the pH-cycling period from each specimen slices of approximately 100 µm thickness (Exakt GmbH, Norderstedt, Germany) were obtained and TMR images were taken as described previously [Wierichs et al., 2020]. TMR software (version 5.25, Joop de Vries, Groningen, The Netherlands) was used by 1 blinded examiner to calculate the mineral loss ($\Delta Z_{baseline}$ and $\Delta Z_{pH\text{-}cycle}$) and lesion depth ($LD_{baseline}$ and $LD_{pH\text{-}cycle}$) [Wierichs et al., 2017b].

Changes in mineral losses ($\Delta\Delta Z_{DT} = \Delta Z_{baseline\ DT} - \Delta Z_{pH\text{-}cycle\ DT}$) and lesion depths ($\Delta LD_{DT} = \Delta LD_{baseline\ DT} - \Delta LD_{pH\text{-}cycle\ DT}$) of initially demineralized surfaces were calculated by subtracting values after pH-cycling from the respective values before pH-cycling. Furthermore, graphics of mean mineral density profiles were prepared for all groups with the TMR/WIM Calculation Program.

Colorimetric Analysis

Discoloration of the dentinal surface was evaluated as described previously [Wierichs et al., 2017a]. Images were taken at the following points: 1 day after initial demineralization ($\Delta E_{baseline,DT}$) and 1 day after pH-cycling ($\Delta E_{pH\text{-}cycle,DT}$ and $\Delta E_{pH\text{-}cycle,ST}$). Since discoloration was only expected for SDF, pictures were taken for SDF and two "controls" (AmF and APF) only.

Energy-Dispersive X-Ray Spectroscopy: SEM-EDX

The contents of Ag being incorporated in the dentinal surface of specimens in group SDF were measured using an environmental scanning electron microscope (ESEM XL 30 FEG, FEI, Eindhoven, The Netherlands) in a high-vacuum environment (0.8 Torr, acceleration voltage: 10 kV, probe diameter: approx. 1 µm) [Wierichs et al., 2018a]. After pH-cycling, 3 specimens of intervention SDF and 2 of intervention AmF were prepared and examined. Analyses of sound (ST) and demineralized (DT) treatment areas were carried out across the flat dentin slice in areas of 200 µm. Assessments of elements were made in triplicates. The detection limit of Ag was about 1 wt%.

Power Calculation

The number of specimens per group was calculated based on previous studies [unpubl. data]. The α-error was set at 5%. Considering the differences (SD) between the S_{1450} and $NaF1_{1450}$ ($\Delta\Delta Z_{DT}$: mean difference of 922 (940) vol% × µm; $\Delta\Delta Z_{ST}$: mean difference of 4,037 (1,200) vol% × µm) the statistical power calculated for $\Delta\Delta Z_{DT}$ was 82% and for $\Delta\Delta Z_{ST}$ was 100%. The dropout rate was assumed not to exceed 20%. Approximately 20 specimens should have been enrolled into the study for analyses of at least 16 specimens per group. Since the retro-perspective power analysis for the smallest difference (difference between S_{1450} and S_{5000}) with 15 specimens still provided a power of at least 100% for $\Delta\Delta Z_{DT}$ (mean difference: 1,162 (490) vol% × µm), 100% for $\Delta\Delta Z_{ST}$ (mean difference: 1,731 (555) vol% × µm), 75% for ΔLD_{DT} (mean difference: 24 (24) µm) and 96% for ΔLD_{ST} (mean difference: 44 (31) µm), no additional specimens were involved in the study.

Statistical Analysis

Data were analyzed using SPSS statistical software (SPSS 25.0; SPSS, Munich, Germany). Variables were tested for normal distribution (Shapiro-Wilk test). Changes in mineral loss, lesion depth, and colorimetric value before and after pH-cycling (e.g., $\Delta Z_{Baseline}$, $\Delta Z_{pH\text{-}cycle}$) were analyzed using two-tailed paired *t* tests [Wierichs et al., 2018b].

Analysis of covariance (ANCOVA) for the standard and highly fluoridated dentifrices was used to detect differences in changes of mineral losses ($\Delta\Delta Z_{DT}$, $\Delta\Delta Z_{ST}$), lesion depths (ΔLD_{DT}, ΔLD_{ST}), and colorimetric values ($\Delta\Delta E_{DT}$, $\Delta\Delta E_{ST}$) [Wierichs et al., 2018b]. More technically the ANCOVA statistical model may be described as a general linear mixed model with TMR/colorimetric data and treatment as fixed effects. All tests were performed at a 5% level of significance. Thus, for both baseline substrate conditions (ST and DT) the factors under evaluation were:

- application of a gel at six levels: AmF, NaF1, NaF2, SDF, APF, or S
- brushing at two levels: 1,450 ppm (e.g., AmF_{1450}) or 5,000 ppm (e.g., AmF_{5000}).

Results

After initial demineralization the treatment groups did not differ significantly in mineral loss ($p = 1.000$; ANCOVA) and lesion depth ($p = 1.000$; ANCOVA). The mean $\Delta Z_{baseline,DT}$ was 932 vol% × µm (95% CI 841–1,024) and $LD_{baseline,DT}$ was 138 µm (95% CI 135–141). Due to losses during preparation, the final TMR analysis was performed with 15–20 specimens per group.

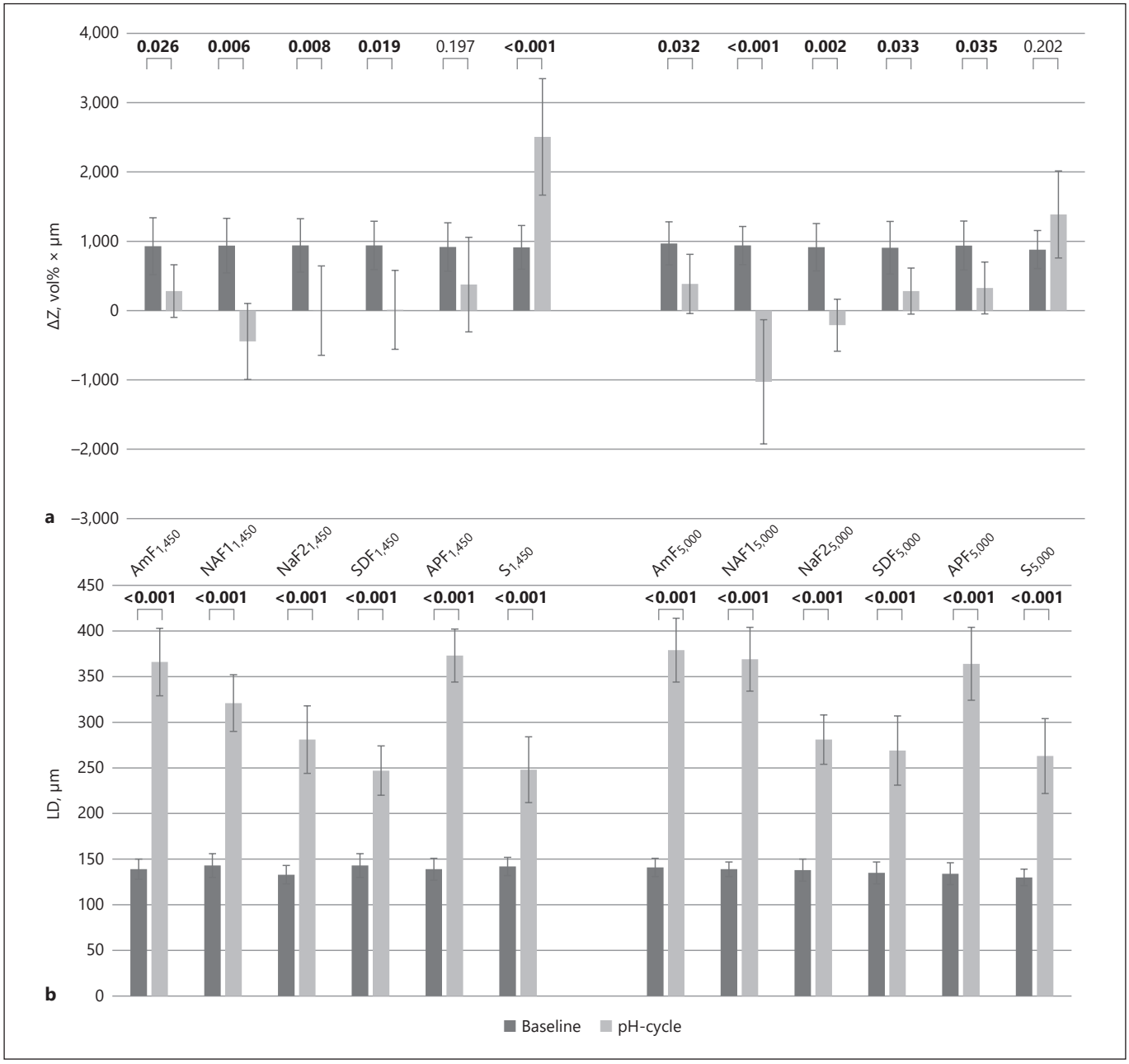


Fig. 2. Means with 95% CIs of mineral losses (**a**) and lesion depths (**b**) after initial demineralization ($\Delta Z_{baseline}$ and $LD_{baseline}$) and after pH-cycling ($\Delta Z_{pH\text{-}cycle}$ and $LD_{pH\text{-}cycle}$). Bold *p* values indicate significant differences in mineral losses and lesion depths before and after pH-cycling (two-tailed paired *t* test). AmF, sodium fluoride and amine fluoride; NaF1, sodium fluoride; NaF2, sodium fluoride; SDF, silver diamine fluoride; APF, acidulated phosphate fluoride; S, standard control.

TMR: Mineral Content

For mineral content (significantly) increased values between before and after pH-cycling were observed in S_{1450} ($p < 0.001$; two-tailed paired *t* test; Fig. 2) and S_{5000} ($p = 0.202$; two-tailed paired *t* test), indicating further demineralization. In contrast, all groups with additional fluoride gel application except for one (APF_{1450}) showed significantly decreased ΔZ_{DT} values, indicating remineralization ($p \leq 0.035$; two-tailed paired *t* test; Fig. 2).

For comparisons between the groups initially demineralized specimens of all interventions showed significantly higher changes in mineral content ($\Delta\Delta Z_{DT}$) than S_{1450} and S_{5000}, respectively ($p \leq 0.023$; ANCOVA; Fig. 3). Furthermore, specimens of $NaF1_{5000}$ showed significantly higher changes in mineral content ($\Delta\Delta Z_{DT}$) than SDF_{5000}, AmF_{5000}, and APF_{5000} ($p \leq 0.014$; ANCOVA; Fig. 3).

For $\Delta\Delta Z_{ST}$ the highest values could be observed for $NaF1_{1450}$ and $NaF1_{5000}$. Both groups showed significantly higher $\Delta\Delta Z_{ST}$ values than all other groups ($p \leq 0.026$; ANCOVA; Fig. 3). Furthermore, specimens of S_{1450} and S_{5000}

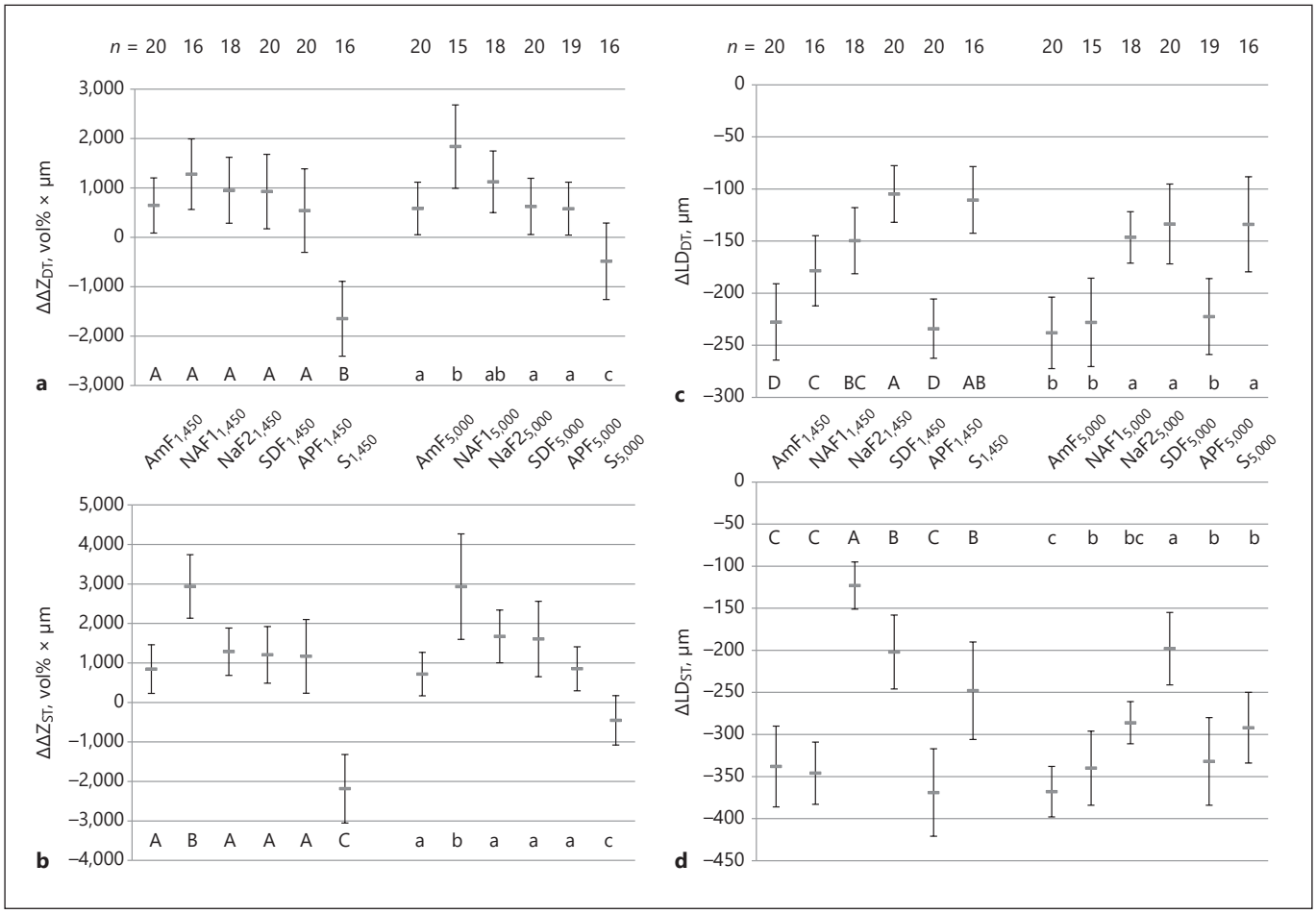
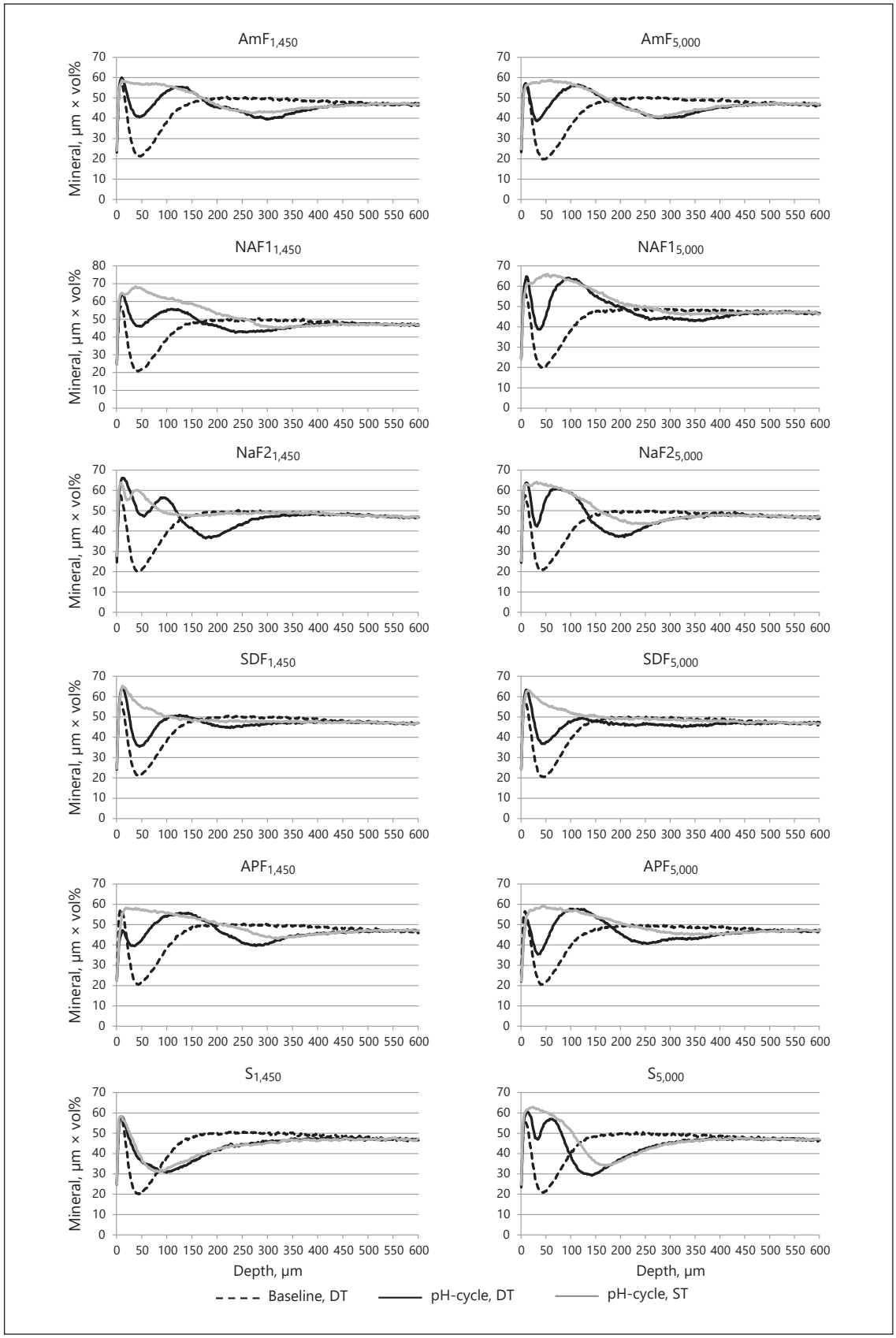


Fig. 3. Means with 95% CIs of the changes in mineral loss ($\Delta\Delta Z$; **a**, **b**) and lesion depths (ΔLD; **c**, **d**) of initially demineralized surfaces ($\Delta\Delta Z_{DT}$ and ΔLD_{DT}) and sound surfaces ($\Delta\Delta Z_{ST}$ and ΔLD_{ST}). Different letters indicate significant differences between treatments among specimens being brushed with 1,450 ppm F (e.g., AmF_{1450}; uppercase) and with 5,000 ppm F (e.g.: AmF_{5000}; lowercase; $p < 0.05$; ANCOVA). Negative $\Delta\Delta Z$ values indicate demineralization, positive $\Delta\Delta Z$ values indicate remineralization. For a more intuitive reading, $\Delta\Delta Z_{ST}$ and ΔLD_{ST} values were calculated as well, although the values were measured only after pH cycling. For this, the baseline values were assumed to be zero. AmF, sodium fluoride and amine fluoride; NaF1, sodium fluoride; NaF2, sodium fluoride; SDF, silver diamine fluoride; APF, acidulated phosphate fluoride; S, standard control.

Fig. 4. Mean mineral density profiles of the initially demineralized dentin surfaces before (baseline, DT) and after pH cycling (pH-cycle, DT) as well as the profiles of sound surfaces (pH-cycle, ST). Lesions were assessed using the TMR/WIM calculation program. All initially demineralized specimens (except specimens in groups S_{1450} and SDF_{5000}) showed the formation of a second lesion body. For sound surfaces a second lesion body could only be observed in NaF1 and NaF2. *(For figure see next page.)*


AmF1,450
AmF5,000
NAF11,450
NAF15,000
NaF21,450
NaF25,000
SDF1,450
SDF5,000
APF1,450
APF5,000
S1,450
S5,000
Mineral, µm × vol%
Depth, µm
Baseline, DT
pH-cycle, DT
pH-cycle, ST

4

showed significantly lower $\Delta\Delta Z_{ST}$ values than all other groups ($p \leq 0.027$; ANCOVA; Fig. 3).

For comparison between standard and highly fluoridated dentifrices a significantly lower change in mineral content ($\Delta\Delta Z_{DT}$ and $\Delta\Delta Z_{ST}$) for the standard fluoridated dentifrice could only be observed in the dentifrice-only group ($p \leq 0.019$; ANCOVA; Fig. 3).

TMR: Lesion Depth

For lesion depth, significantly increased values between before and after pH-cycling were observed in all groups ($p < 0.001$; two-tailed paired *t* test; Fig. 2). For comparisons between the groups, specimens of S_{1450} showed a significantly lower change in lesion depths (ΔLD_{DT} and ΔLD_{ST}) compared to AmF_{1450}, $NaF1_{1450}$, and APF_{1450} ($p \leq 0.006$; ANCOVA; Fig. 3). Furthermore, for specimens of S_{5000} a significantly lower change in lesion depth compared to AmF_{5000}, $NaF1_{5000}$, and APF_{5000} could only be observed for ΔLD_{DT} ($p < 0.001$; ANCOVA). Interestingly, specimens of SDF showed no significant difference compared to S regardless of the respective dentifrice and baseline substrate condition ($p \geq 0.805$; ANCOVA; Fig. 3).

For comparison between standard and highly fluoridated dentifrices no significant difference in the change of lesion depths (ΔLD_{DT} and ΔLD_{ST}) could be observed, except for the sound specimen of NaF2 ($p \leq 0.01$; ANCOVA; Fig. 3).

TMR: Mineral Density Profiles

All specimens revealed subsurface lesions without abrasive surface losses (Fig. 4). After pH-cycling a second layer of demineralized tissue could be observed in all initially demineralized specimens being treated with highly fluoridated dentifrice and in specimens being treated additionally with a fluoride gel (except SDF_{5000}). For sound surfaces a second lesion body could only be observed for NaF1 and NaF2. Furthermore, the lesion front moved deeper into the dentin in all groups while the surface layer became broader.

Colorimetric Analysis

After initial demineralization the treatment groups did not show significantly different colorimetric values ($p = 1.000$; ANCOVA; online suppl. Fig. 1; see www.karger.com/doi/10.1159/000509931 for all online suppl. material). The mean $\Delta E_{baseline,DT}$ was 6.4 (95% CI 6.0–6.8). Significantly increased values between before and after pH-cycling were only observed for SDF_{1450} and SDF_{5000}, indicating discoloration ($p < 0.001$; two-tailed paired *t* test). Furthermore, a significantly higher increase in colorimetric values ($\Delta\Delta E_{DT}$ and $\Delta\Delta E_{ST}$) could be observed for SDF compared to AmF and APF ($p < 0.001$, ANCOVA).

Energy-Dispersive X-Ray Spectroscopy: SEM-EDX

After pH-cycling, silver could not be found in ST or in DT areas. Furthermore, no silver was deposited on the surface.

Fluoride Analysis

The free fluoride content and the percentage of free fluoride in relation to given fluoride content are given in Table 2. The biggest differences between the measured and given fluoride contents was observed for NaF2 and SDF.

Discussion

The present in vitro study compared the demineralization inhibitory effect of fluoride gels being used in combination with either standard or highly fluoridated dentifrices on sound dentin and on artificial dentin lesions under net-demineralizing conditions. Significant differences in the change of mineral loss could be observed between the dentifrice-only groups and all fluoride gel groups. Furthermore, a significantly higher mineral gain could be observed for NaF1 compared to the other fluoride gels. For the change in lesion depth a significantly lower increase could be observed for SDF (not for all comparisons) compared to the acidic fluoride gels, therefore rejecting the hypothesis that no differences in mineral loss and lesion depth would be observed between the groups with additional use of fluoride gels but for all compared with the dentifrice-only groups.

In the present study the use of a standard or highly fluoridated dentifrice and additional application of fluoride gels could significantly hamper further mineral loss compared with the dentifrice-only groups (S_{1450} and S_{5000}). It even induced significant mineral gain under net-demineralizing conditions. Until now there are no data on the demineralization-inhibiting effects of the use of highly fluoridated dentifrices and additional application of fluoride gels on root dentin. However, the present results of the use of standard fluoridated dentifrices and additional application of fluoride gels seem to be in agreement with previous in vitro studies on sound dentin and initially demineralized dentin lesions [Tschoppe and Meyer-Lueckel, 2011; Walther et al., 2019]. Furthermore, the results are consistent with the current understanding

that fluoride inhibits demineralization and enhances remineralization not only in enamel but also in dentin [Buzalaf et al., 2011].

Additionally, a significantly higher increase in lesion depth could be observed in all groups with acidic fluoride gels (except $NaF2_{1450}$) compared with the dentifrice-only groups. This seems somehow to be in contrast to the observed effect that in the presence of fluoride, minerals and fluorides are forming a more mineralized surface layer than in the absence of fluoride. In enamel the mineralized surface layer is, indeed, a barrier for, firstly, the dissolution of mineral and, secondly, for the diffusion of acids into deeper parts of the lesions [Arends and Christoffersen, 1986]. However, in dentin the interface with cariogenic acids is larger than in enamel [Buzalaf et al., 2011]. Acids may, therefore, bypass the mineralized surface layer while minerals and fluorides will be deposited in the (more mineralized) surface area [Fejerskov et al., 2008; Buzalaf et al., 2011]. This finding was also observed in previous pH-cycling studies [Ten Cate et al., 1995; Wierichs et al., 2018a]. Mineral uptake in dentin lesions predominated at the surface, whereas mineral loss was observed in deeper parts – at the lesion front.

Subsurface artificial dentin caries lesions were used to analyze the caries-preventive effect of different fluoride gels under net-demineralizing conditions. This type of dentin lesion was used before in several studies [Tschoppe and Meyer-Lueckel, 2011; Wierichs et al., 2018a; Walther et al., 2019]. However, when creating these lesions a small amount of sodium fluoride (47.6 μM) had to be added to the demineralization solution to preserve the dentin surface integrity. Thus, it has to be assumed that the mineral density of the lesion surface zones in the present specimens were slightly higher than in natural root dentin caries lesion [Fejerskov et al., 2008]. Nonetheless, previous studies indicated that – although not being identical – the artificial dentin caries lesions most closely mimic the clinical conditions [Ten Cate et al., 2008].

Another issue to be considered is that the present baseline mineral losses seem to be significantly lower when compared to previous studies using the same pre-demineralization protocol [Tschoppe and Meyer-Lueckel, 2011; Wierichs et al., 2018a; Walther et al., 2019]. Due to the large increase in lesion depth during pH-cycling in the present study, the analysis of the radiographic images after pH-cycling had to be slightly altered. In order to be able to compare values before and after pH-cycling, the TMR analysis before pH-cycling had to be altered as well. By using the slightly altered protocol the calculated values for mineral losses were lower than reported previously. If the "original" measurement protocol had been used values for baseline mineral loss would have been in the same range as in the previous studies [Tschoppe and Meyer-Lueckel, 2011; Wierichs et al., 2018a; Walther et al., 2019]. Interestingly, values of the lesion depths were not affected by the altered protocol. Nonetheless, indirect comparison of the previous [Wierichs et al., 2018a] and present results have to be interpreted with caution, although one similar treatment regime (S_{1450}) was used in both studies.

In initially demineralized specimens being treated with highly fluoridated dentifrice and in specimens being treated additionally with most of the fluoride gels a second layer of demineralization (lamination) could be observed. This is in agreement with previous studies on enamel [Lippert and Juthani, 2015; Wierichs et al., 2018b] and dentin [Wierichs et al., 2020]. When fluoride concentrations between 2,800 and 12,500 ppm F were applied a second layer of demineralization was observed. However, inverse correlations between fluoride concentration and the severity of the second layer of demineralization was observed as well [Walther et al., 2019]. Recently, it was speculated that the contradictory lamination characteristics might depend on different pre-demineralization protocols [Wierichs et al., 2018b]. However, until now it is still unclear which pre-demineralization protocol most closely mimics the clinical conditions. Thus, further in vitro studies analyzing the re- and demineralization characteristics of dentin are needed, firstly to get more information on the tested agents, secondly to get more information on the lamination characteristics, and thirdly to choose the perfect setting for the scientific issue under investigation (before analyzing the scientific issue under clinical conditions).

In previous studies acidic fluoride agents induced a significantly higher remineralizing effect than neutral agents [Ogard et al., 1994; Yamazaki and Margolis, 2008; Vale et al., 2011; Fernandez et al., 2017]. In contrast, in the present study a significantly higher mineral gain was observed for the neutral NaF1 gel compared to the slightly (AmF and NaF2) and the highly (APF) acidic gels. However, the present results are in agreement with one previous study [Walther et al., 2019]. Here, it was speculated that the beneficial effect of the rather low pH of the fluoride agent was superimposed by the low pH of the used saliva substitute. Consequently, the results of the present study might be explained by the low pH of the demineralization solution and the net-demineralizing conditions of the pH-cycling model. Furthermore, it might be speculated that under net-demineralizing conditions acidic products (being supposed to increase min-

eral diffusion) might not be required to increase mineral gain. Nonetheless, gels and dentifrices not only differed in pH values and fluoride compounds, but also in other inactive ingredients. It might, thus, also be speculated that the observed effects were influenced by other ingredients (e.g., antimicrobial, abrasive, or remineralizing inhibiting ingredients) [Walther et al., 2019]. However, in a chemical caries model no antimicrobial interferences are expected, and all 218 specimens revealed subsurface lesions without abrasive surface losses. Nonetheless, the standard fluoridated dentifrices and 2 fluoride gels (APF and NaF1) contained Carbopol polymer (carbomer 956). Carbomer 956 is used as a thickening agent. Although the formation of calcium fluoride (CaF_2) on the dentin surface has not been analyzed in the present study, it might be speculated that Carbomer 956 also impaired the formation of unbound fluoride (CaF_2) and deposition over the dentin surfaces after the contact with the dentifrice slurries [Backfolk et al., 2002]. Thus, the bioavailability of CaF_2 was presumably reduced during the following demineralization period. Consequently, this could have resulted in a reduced mineral gain.

The demineralization inhibitory effects of highly fluoridated dentifrices as well as the use of standard fluoridated dentifrices and additional application of fluoride gels have only been compared once when using dentin [Fernandez et al., 2017]. In that study, for sound dentin surfaces the percentage of surface hardness recovery was significantly higher for the highly fluoridated dentifrice. Nonetheless, for initially demineralized dentin specimens the combined use of the gel and the dentifrice showed a significantly higher reduction of the lesion area [Fernandez et al., 2017]. In contrast, in the present study for sound surfaces as well as initially demineralized specimens a significant difference between the highly fluoridated dentifrice and the use of standard fluoridated dentifrices and additional application of fluoride gels could be observed between S_{5000} and $NaF1_{1450}$, but not for the other gels (data not shown). Nonetheless, in the previous and the present study the demineralization inhibitory effects of the two treatment regimens (highly fluoridated dentifrice only and the use of standard fluoridated dentifrices and additional application of fluoride gels) were significantly higher than the demineralization inhibitory effect of a fluoride-free [Fernandez et al., 2017] or a standard fluoridated dentifrice (present study). Thus, it seems plausible that patient preferences (i.e., additional efforts and costs to acquire the agents, taste of the available products, etc.) should be included when deciding for one of the treatment regimes.

However, although the present model was able to demonstrate significant differences between the three different treatment regimens (S_{1450}, S_{5000}, and $NaF1_{1450}$) further in vitro and in vivo studies are needed to evaluate which regime is clinically more applicable and more effective and to evaluate the risks of long-term use of the various regimes.

In a recent study using the same pH-cycling protocol no discoloration or staining after the application of SDF on dentin surfaces was observed [Wierichs et al., 2018a]. This is in contrast with several clinical studies [Yee et al., 2009] and the present study, where specimens of SDF_{1450} and SDF_{5000} showed black discolorations after pH-cycling. One reason for the contradictory results might be a slightly altered methodical procedure. In a recent study specimens were pH-cycled for 14 days immediately after the application of the SDF [Wierichs et al., 2018a]. Subsequently, photographic images were taken. Since specimens were kept in darkness the whole time, the influence of light on SDF was not analyzed. In contrast, in the present study, specimens were stored in distilled water for 24 h after pH-cycling and before photographic images were taken. In this time laboratory lighting (T5 Eco Saver H0 36W/840, Aura Light, Hamburg, Germany) was switched on and silver phosphate – presumably being deposited on the dentin surface during pH-cycling [Lou et al., 2011; Willershausen et al., 2015] – could be reduced to black metallic silver [Lou et al., 2011]. Thus, a significant discoloration after the application of SDF could be observed in the present study.

Conclusion

Under the present pH-cycling conditions, the highly fluoridated dentifrice significantly reduced caries progression and the additional application of nearly all fluoride gels resulted in mineral gain. However, there was no difference in the remineralizing capacity of fluoride gels or solutions when used in combination with either standard or highly fluoridated dentifrices.

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Statement of Ethics

This study is reported according to the COPE guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest with respect to the authorship and/or publication of this article.

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Author Contributions

R.J.W., D.E., M.E.-O. and H.M.-L. designed and planned the study. D.E. prepared the samples. R.J.W. and D.E. performed the measurements and statistical analysis. D.E. wrote the manuscript. R.J.W. and C.A. commented on, and all authors revised the manuscript.

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