Original Paper

Caries Research

Caries Res 2016;50:170–179
DOI: 10.1159/000445169

Received: November 4, 2015
Accepted after revision: March 1, 2016
Published online: April 27, 2016

Effects of Erosion Protocol Design on Erosion/Abrasion Study Outcome and on Active Agent (NaF and SnF_2) Efficacy

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Key Words
Abrasion · Citric acid · Enamel · Erosion · Fluoride · Study design · Tin

Abstract
There is no standard for testing anti-erosive/anti-abrasive agents, making the assessment and comparison of study results difficult. Factors which are varied in study designs are amongst others the erosive medium regarding concentration and pH or movement type of acid. The present study therefore investigated the impact of these factors on dimension of tissue loss and on efficacy of active agents used as anti-erosive/anti-abrasive therapeutics. In 8 experiments, consisting of 8 groups each (n = 20 each), resulting in a total of 64 groups, enamel specimens were demineralised (10 days, 6 × 2 min/day) using different acids (1, 0.5 and 0.3% citric acid at native pH 2.3, 2.5 and 2.8, respectively, and 0.3% citric acid adjusted to pH 3.6) with two different movement types (jerky or smooth). Specimens were immersed (2 × 2 min/day) in slurries of 1,450 ppm F^- toothpaste (NaF), 1,450 ppm F^- and 3,436 ppm Sn^{2+} toothpaste (NaF/SnF_2), 970 ppm F^- and 3,000 ppm Sn^{2+} gel (SnF_2) or placebo, or were additionally brushed during immersion (15 s, 200 g). All groups were in between stored in a mineral salt solution. Tissue loss was determined profilometrically. Movement type, pH and concentration of acid had a substantial impact on study outcome. The combination of jerky movement and concentrated acid masked, to some extent, differences between erosive and erosive-abrasive tissue loss. The acid at low concentration (0.3%), independent of pH, was too mild to produce any tissue loss. The model with the best ability to demonstrate effects of abrasive impacts and active agents used the 1% acid concentration combined with smooth acid movements.

Options for the prevention and treatment of dental erosion or of combined erosive-abrasive tissue loss have been increasingly investigated within the last few decades. A PubMed search performed in January 2015 including the years between 1960 and 2015 with the terms 'dental', 'erosion', 'abrasion' and 'enamel' retrieved a total of 238 articles. Amongst these, 68 in vitro, in situ and combined in vitro/in situ studies were found investigating the effect of various types of acid on erosive or erosive-abrasive tissue loss, or the preventive or therapeutic effect of different active agents. Retrospectives, reviews and clinical studies were excluded. The included studies varied tremendously in the parameters of total erosion time (range

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between 30 s and 60 h), acid type (acidic beverages, citric acid, phosphoric acid and hydrochloric acid), acid concentration (range between 0.3 and 6.0%), acid pH (range between 1.4 and 4.0), movement of acid (not named, static, shaking bath, magnetic stirrer and continuous flow), brushing force (range between 20 and 400 g), brushing duration (range between 30 s and 56 min or between 20 and 144,000 brushing strokes) and, in case of using toothpastes as a vehicle for the application of an active agent, method for preparation of slurry (solution used for dilution of toothpaste: none, distilled water, tap water, mineral salt solution; range of dilution ratio between toothpaste and solution from no dilution up to 1:10). Other than these factors, several other parameters such as the temperature of the demineralisation solution [Barbour et al., 2006], the saturation of the demineralisation solution with respect to hydroxyapatite [Barbour et al., 2003, 2005] and the abrasivity of the toothpaste used [Hara et al., 2009] have been found to potentially impact the study outcome.

Even if recommendations for the design of in vitro and in situ studies have been suggested [Shellis et al., 2011; Wiegand and Attin, 2011; West et al., 2011; Young and Tenuta, 2011], there is currently no established standard for testing the effect of anti-erosive/anti-abrasive agents. It is clear that the factors mentioned above impact the dimension of erosive/abrasive loss [Shellis et al., 2013], but it is not known to what extent, in particular if various factors are combined; furthermore, little is known about their impact on the relative effects of active agents.

The aim of the present study was therefore to investigate the impact of varying the erosive challenge in terms of concentration and pH of one acid (citric acid), which is the most often used acid type in erosion/abrasion studies, and the dynamic conditions at the enamel-acid interface on the relative effects of two active agents (NaF, SnF_2). The experiment was designed to cover the different ranges of experimental conditions identified in the PubMed search. A series of cyclic erosive or erosive/abrasive in vitro experiments were performed in parallel, using citric acid at different concentrations (0.3–1.0%) and pH (2.3–3.6) and varying the dynamic conditions in terms of the type of acid movement. The impact of these parameters on dimension of tissue loss, effect of brushing abrasion and relative efficacy of active agents was investigated. The null hypotheses were that there is no difference between the various experimental conditions and that the relative effects of the active agents to each other and to placebo are constant, irrespective of the experimental conditions.

Materials and Methods

Specimen Preparation

In the present study, a total of 1,280 longitudinal human enamel specimens were prepared from previously impacted, freshly extracted third molars, which were disinfected with saturated thymol solution (Exakt Abrasive Cutting System, Exakt-Apparatebau, Norderstedt, Germany), as described previously [Schlueter et al., 2013]. The natural surface was removed. A standardised grinding (Exakt Mikrogrinder, Exakt-Apparatebau; P1200 silicon carbide abrasive paper, Leco, St. Joseph, Mich., USA) and polishing (Apex DGD diamond grinding disc, 3 μm, Buehler GmbH, Düsseldorf, Germany) procedure was performed under a constant water flow of 50 ml/min, resulting in a specimen surface of at least 3 × 3 mm. All specimens were checked for cracks and damage under a stereomicroscope (10-fold magnification; Nikon SMZ-2T, Tokyo, Japan). The flatness of specimens was measured profilometrically (MicroProf, Fries Research and Technology GmbH, Bergisch-Gladbach, Germany; maximum deviation ±0.5 μm). Specimens were mounted on holders for the brushing machine with a light-curing acrylic (Technovit 7230 VLC, Kulzer-Exakt, Wehrheim, Germany) as described previously [Ganss et al., 2014]. One half of each specimen was covered with the acrylic and served as the reference area for profilometric tissue loss measurement. All specimens were again checked for damage or acrylic remnants on the uncovered (experimental) area (10-fold magnification) and then stored in 100% humidity at 4°C until further use.

Treatment

The study was conducted using a variety of treatments. The specimens were cyclically demineralised with various types of citric acid, which were used with two types of movement, and immersed in or abraded with different toothpaste slurries.

Demineralisation procedures were performed either in a water bath (Model 1083, GFL mbH, Burgwedel, Germany) or on a shaking plate (GFL Shaker 3006, GFL mbH). Both devices performed reciprocating movements, were adjusted to a constant movement frequency (35/min) and had the same travel path (20 mm). The motion in the water bath was constant within the travel path, with nearly no additional acceleration or deceleration, inducing a smooth movement of the demineralisation solution. The movement of the shaking plate, however, included a clearly visible acceleration within the travel path and a distinct deceleration at the end point. This led to clearly visible, jerky movements of the acid with a small wave built up in the liquid.

For demineralisation, acids with 4 different combinations of citric acid concentration and pH values were used (1% native pH 2.3; 0.5% native pH 2.5; 0.3% native pH 2.8 or 0.3% pH adjusted to 3.6 with NaOH). The respective amount of citric acid monohydrate (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) was dissolved in 1 litre of distilled water. The combination of movement types and acids resulted in 8 experiments, which were performed in parallel. Demineralisation was performed for a total of 10 days (2 × 5 workdays) for 6 × 2 min per day. All procedures were performed at 22°C (room temperature).

The active agents used were NaF and SnF_2, either alone or in combination; in addition, a placebo toothpaste was included. A total of 3 toothpastes and 1 gel were used (for details and composition, see table 1). Slurries were mixed with a mineral salt solution (1 part toothpaste/gel and 3 parts mineral salt solution by weight). The mineral salt solution was supersaturated with respect to hydroxyapatite

Table 1. Composition, relative dentine abrasivity (RDA) and active agents of the toothpastes and pH of the toothpaste slurries used (abbreviation for groups is given in parentheses)

Product	Ingredients	pH	Active agents
Fluoride-free toothpaste (placebo), RDA 77	Aqua, dicalcium phosphate dihydrate, dicalcium phosphate, propylene glycol, glycerine, hydroxyethylcellulose, sodium lauryl sulphate, titanium dioxide, aroma, silica, methylparaben, sodium saccharin, retinyl palmitate, propylparaben	7.2	None
NaF toothpaste (NaF), RDA 80	Aqua, sorbitol, hydrated silica, glycerine, PEG-12, sodium lauryl sulphate, cellulose gum, aroma, sodium fluoride, sodium saccharin, saliva officinalis oil, *Mentha piperita* oil, *Chamomilla recutita* extract, *Commiphora myrrha* extract	7.0	1,450 ppm F^- as NaF
SnF_2 gel (SnF_2), RDA 60	Glycerine, stannous fluoride, hydroxymethylcellulose, aroma	4.3	970 ppm F^- as SnF_2 3,000 ppm Sn^{2+} as SnF_2
NaF/SnF_2 toothpaste (NaF/SnF_2), RDA 119	Glycerine, hydrated silica, sodium hexametaphosphate, propylene glycol, PEG-6, aqua, zinc lactate, CI 77891, sodium lauryl sulphate, sodium gluconate, aroma, *Chondrus crispus,* trisodium phosphate, stannous fluoride, sodium saccharin, xanthan gum, silica, sodium fluoride	6.0	350 ppm F^- as NaF 1,100 ppm F^- and 3,436 ppm Sn^{2+} as SnF_2

The RDA was determined by Missouri Analytical Laboratories Inc., St. Louis, Mo., USA [Ganss et al., 2011]. Fluoride-free toothpaste: Aronal fluoride free, GABA International AG, Daishing Trading Co., Japan; NaF toothpaste: Dentagard, Colgate-Palmolive GmbH, Hamburg, Germany; SnF_2 gel: Colgate GelKam, Colgate Oral Pharmaceuticals, N.Y., USA; NaF/SnF_2 toothpaste: blend-a-med ProExpert Zahnfleischschutz, P and G Manufacturing GmbH, Gross-Gerau, Germany.

Table 2. Profilometrically determined step height values (μm, means ± standard deviation) between the reference and the experimental area on specimens of all groups

Acid treatment conc.		Placebo		NaF		SnF_2		NaF/SnF_2	
		slurry	brushing	slurry	brushing	slurry	brushing	slurry	brushing
1%	S	23.3±9.1[a]	25.8±5.7	21.8±6.7[a, b]	29.1±5.7[a, b]	11.9±5.6[a, b]	17.0±8.2[a, b]	22.0±7.8[a, b]	37.7±12.2[a, b]
	W	9.8±2.9[a, b]	19.2±3.7[b]	7.1±3.1[a, b]	12.6±2.3[a, b]	3.7±1.1[a, b]	8.2±1.4[a, b]	5.3±1.2[a, b]	23.1±5.8[a, b]
0.5%	S	9.1±4.6[a, b]	12.1±3.5[b]	3.0±4.7	5.7±4.3	2.1±2.2[b]	4.1±2.3[b]	6.9±3.2[a]	9.3±5.9[a]
	W	3.1±0.8[a, b]	10.2±2.0[b]	*–0.1±1.7*	5.3±2.4	*–1.4±2.0*	*–0.3±2.9*	2.9±0.7[a, b]	4.3±2.0[a, b]
0.3%	S	*–0.1±3.0*	2.1±2.0	*–23.1±6.2*	*–10.9±6.1*	*–13.0±6.7*	2.1±1.2	2.0±1.1[a, b]	8.4±4.4[a, b]
	W	*–2.9±4.1*	*–1.2±4.4*	*–22.4±14.4*	*–23.6±11.8*	*–15.0±10.7*	*–6.7±8.2*	1.2±0.3[a, b]	4.3±1.7[a, b]
0.3%	S	*–14.5±10.7*	*–14.0±10.7*	*–25.5±21.7*	*–27.9±15.5*	*–2.8±5.1*	*–0.1±2.6*	0.7±0.6	0.6±0.3
pH 3.6	W	*–23.1±7.9*	*–10.7±5.7*	*–11.9±19.9*	*–23.8±6.5*	*–20.6±15.5*	*–4.4±7.1*	0.6±0.3	0.5±0.3

Negative values indicate precipitation on the surface (data shown in italics); positive values indicate substance loss. Statistics are only given for the groups that showed tissue loss. The table shows the comparisons of different movement types and the comparison of erosive and erosive/abrasive conditions. The comparison of the effects of active agents can be found in figure 2. S = Demineralisation performed on a shaking plate; W = demineralisation performed in a water bath; conc. = acid concentration. [a] $p \leq 0.05$: statistical significance between groups with different acid movement (water bath or shaking plate) but treated with the same acid and the same preparation (immersion only or brushing); [b] $p \leq 0.05$: statistical significance between slurry and brushing groups, which were treated with the same acid, the same acid movement and the same preparation.

and contained 4.08 mmol H_3PO_4, 11.90 mmol $NaHCO_3$, 20.10 mmol KCl and 1.98 mmol $CaCl_2$ [Gerrard and Winter, 1986].

Specimens were immersed after the first and the last demineralisation procedure for 2 min in the toothpaste/gel slurries, and in half of the groups, specimens were additionally brushed within the slurry immersion time for 15 s with a 200-gram load in an automatic brushing machine (SD Mechatronik GmbH, Feldkirchen-Westerham, Germany; ADA reference brush soft; 'zig-zag' pattern with 150 oscillations/min, linear travel path 6 mm, travel velocity 60 mm/s). Between the demineralisation procedures or after treatment with toothpaste slurries, specimens were stored for 1 h in the mineral salt solution. Specimens were rinsed with tap water for 30 s prior to a change into the next solution/slurry. All slurries and solutions were renewed daily.

Combinations of shaking types, acids, toothpastes and treatment (slurry immersion or slurry immersion plus brushing) re-

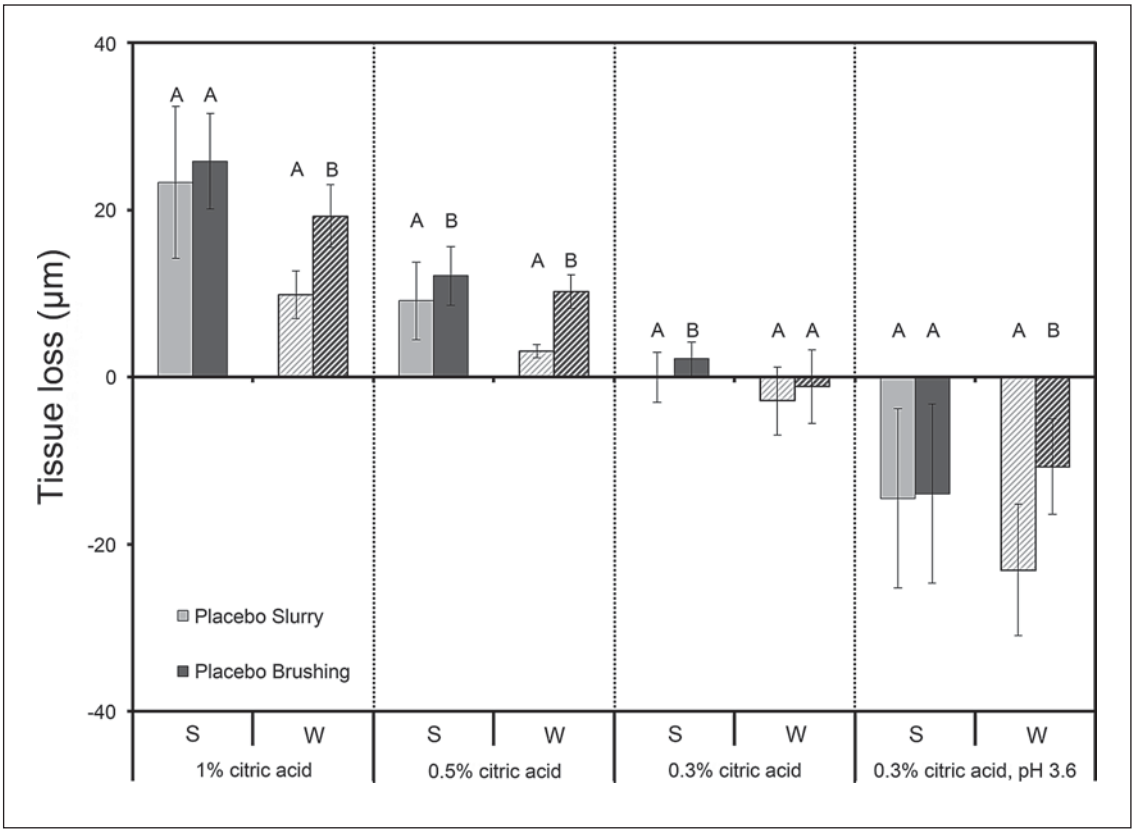

Fig. 1. Tissue loss (µm, means ± standard deviation) in the placebo groups (light grey: slurry control group, immersion in fluoride-free toothpaste slurry; dark grey: brushing control group, immersion in and brushing with fluoride-free toothpaste slurry; negative values indicate precipitation on the specimens' surfaces). Statistically significant differences between control groups (slurry and brushing) under the same demineralisation conditions are indicated by different capital letters. S = Demineralisation performed on a shaking plate (solid bars); W = demineralisation performed in a water bath (hatched bars).

sulted in a total number of 64 groups (n = 20 specimens each); for group details, see table 2. Sample size calculation was based on the results of two studies with a similar study design as in the present study using 0.5% [Ganss et al., 2012] and 1% [Ganss et al., 2011] citric acid for demineralisation. A relevant difference of 2.3 µm and an SD of 2 µm were estimated, resulting in a sample size of 13. As a smaller difference was expected in the groups using 0.3% citric acid, 20 specimens were assigned to each group.

Tissue Loss Measurement

Tissue loss was measured profilometrically at the end of the study as described previously [Schlueter et al., 2014]. Prior to measurement, the acrylic coverage on the reference area was carefully removed, and all specimens' surfaces were controlled with respect to damage or coverage remnants. Measurement was performed with an optical, contactless measuring device (MicroProf; Fries Research and Technology GmbH; sensor H0). On each specimen, 3 traces were made with a total length of 2 mm (200 pixels, 1 mm on the reference and 1 mm on the experimental area) at 200-µm intervals. Traces were analysed with special software (Mark III; Fries Research and Technology GmbH). At the end of both the reference and the experimental area, parallel regression lines were constructed with a length of 0.5 mm each. The vertical distance between the regression lines was defined as the amount of tissue loss. A specimen's tissue loss was expressed as the mean of 3 traces.

Statistical Analysis

Statistical analysis was performed with SPSS 20 for Windows (Armonk, N.Y., USA). Data were checked with regard to whether they deviated significantly from a normal distribution (Kolmogorov-Smirnov test). Since no significant deviation was found, comparisons of groups within one experiment and after use of the same acid, of the corresponding groups treated with different acids, and of the corresponding groups of the various experiments were performed with an ANOVA and Tamhane's post hoc test (significant deviation from the homogeneity of variances, Levene's test). A comparison of results after slurry immersion and after slurry immersion plus brushing was performed with an ANOVA. Except for placebo (control) groups, the comparison of groups was only performed for groups showing substance loss. Interaction between the different factors was analysed for the 1 and 0.5% citric acid groups by a factorial ANOVA (GLM 3) and a test of between-subject effects. The level of significance was set at 0.05.

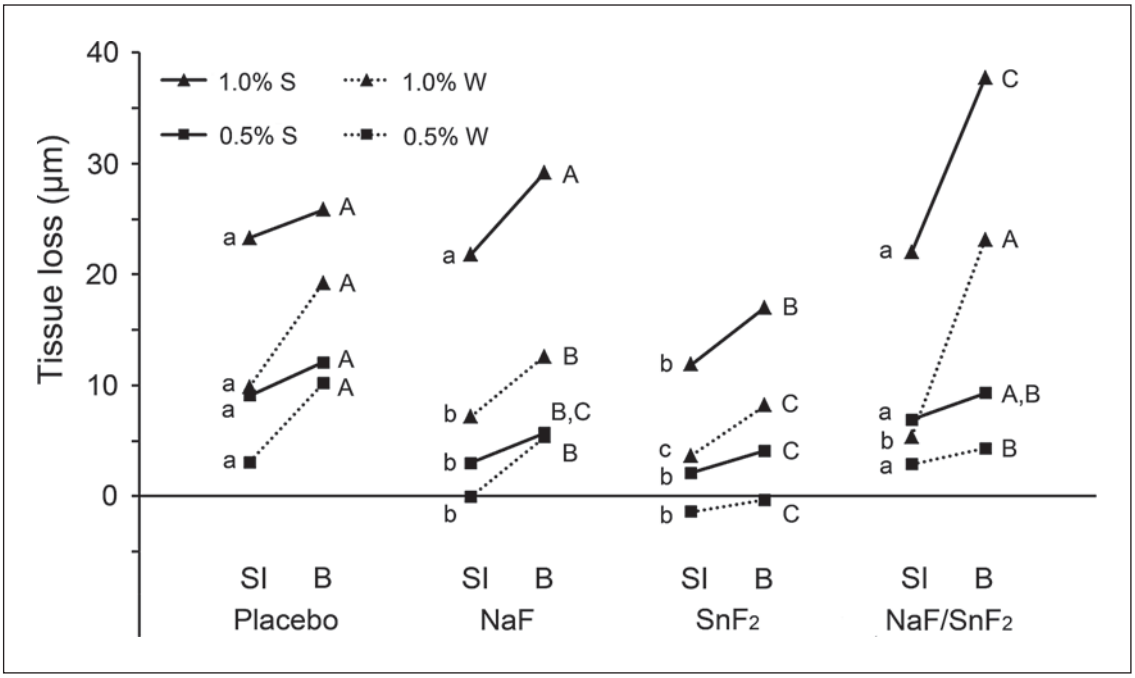

Fig. 2. Impact of brushing on tissue loss (µm) in the 1% (triangles) and 0.5% (squares) citric acid models moved on the shaking plate (S; solid lines) or in the water bath (W; dotted lines). Significant differences ($p \leq 0.05$) between toothpaste efficacies within one demineralisation model are labelled with different lower case letters for slurry immersion (SI) and with different upper case letters for slurry immersion and brushing (B). For the sake of clarity, no standard deviations are given. All data are provided in table 2.

Results

Except for 1 specimen (loss of coverage on the reference area), all specimens were analysed. A comparison of the placebo (control) groups is given in figure 1; a comparison of the groups demineralised with 1 and 0.5% citric acid is displayed in figure 2; measured values (means ± standard deviation) can be found in table 2.

In the placebo groups (fig. 1), tissue loss was observed after demineralisation with 1 and 0.5% citric acid. Amongst the 0.3% citric acid groups, tissue loss was only observed in the 0.3% citric acid group with native pH moved on the shaking plate; in the other groups, mineral precipitates with different thicknesses were found on the specimens' surfaces. Tissue loss was significantly increased by brushing in the groups with 1 and 0.5% citric acid, moved in a water bath (both $p \leq 0.001$), as well as 0.5% ($p \leq 0.05$) and 0.3% ($p \leq 0.01$) citric acid when using a shaking plate.

After the use of SnF_2 and NaF slurries, tissue loss was observed after demineralisation with 1% (shaking plate and water bath) and with 0.5% citric acid (shaking plate). After the use of 0.3% citric acid, independently of pH and type of acid movement, no tissue loss was induced, and mineral precipitates were found in each case. Tissue loss was significantly increased by brushing with SnF_2 and NaF after demineralisation with 1 and 0.5% citric acid, except for NaF 0.5% citric acid (shaking plate) and SnF_2 0.5% citric acid (water bath). In the 0.3% citric acid groups, no tissue loss was induced by brushing except for 0.3% citric acid at native pH (SnF_2, shaking plate).

In all groups treated with the NaF/SnF_2 toothpaste, tissue loss was observed independently of the acid type or shaking device. Tissue loss (means ± SD) ranged from 0.6 ± 0.3 to 22.0 ± 7.8 µm in the slurry immersion groups and from 0.5 ± 0.3 to 37.7 ± 12.2 µm in the brushing groups. Brushing with the toothpaste significantly ($p \leq 0.05$) increased tissue loss compared to slurry immersion in all groups except for 0.5% citric acid (shaking plate) and 0.3% citric acid at pH 3.6 (shaking plate and water bath).

Since tissue loss was mainly found in the groups demineralised with 1 and 0.5% citric acid, the comparison of active agents' efficacy among each other and in relation to the placebo group is only described for these groups (fig. 2). After demineralisation with 1% citric acid on a shaking plate, only immersion in, as well as brushing

Table 3. Significance values of the factorial ANOVA (GLM 3) to analyse the impact of the interaction between the various parameters and the active agents on study outcome for data from the 1 and 0.5% citric acid groups (n = 319)

Source	Significance values
Toothpaste (active agent)	0.000
Acid type (concentration)	0.000
Treatment type (slurry immersion or brushing)	0.000
Shaking type (water bath or shaking plate)	0.000
Toothpaste × acid type	0.000
Toothpaste × treatment type	0.000
Toothpaste × shaking type	0.002
Acid type × treatment type	0.000
Acid type × shaking type	0.000
Treatment type × shaking type	0.064
Toothpaste × acid type × treatment type	0.000
Toothpaste × acid type × shaking type	0.000
Toothpaste × treatment type × shaking type	0.019
Acid type × treatment type × shaking type	0.806
Toothpaste × acid type × treatment type × shaking type	0.262

with, the SnF_2 slurry significantly ($p \leq 0.01$) reduced tissue loss. In the 1% citric acid water bath model, immersion in all slurries containing active agents significantly reduced tissue loss ($p \leq 0.05$); SnF_2 performed better than both other preparations ($p \leq 0.01$). In the case of brushing, SnF_2 and NaF significantly reduced tissue loss ($p \leq 0.001$), but not NaF/SnF_2; SnF_2 reduced tissue loss to a greater extent than NaF ($p \leq 0.001$). In both 0.5% models, immersion in NaF and SnF_2 but not NaF/SnF_2 slurries reduced tissue loss ($p \leq 0.001$); NaF and SnF_2 showed comparable effects. In the case of additional brushing in the 0.5% citric acid shaking plate model, NaF and SnF_2 reduced tissue loss ($p \leq 0.001$) and were again comparable in efficacy; NaF/SnF_2 showed no reduction in tissue loss compared to the placebo group. In the 0.5% citric acid water bath model, brushing with all active agents reduced tissue loss ($p \leq 0.001$); however, SnF_2 was more effective ($p \leq 0.001$) than NaF and NaF/SnF_2.

The comparison of the results of both movement types showed in the 1 or 0.5% citric acid groups that the jerky motions by the shaking plate induced, except when brushing with NaF (0.5% citric acid), higher tissue losses ($p \leq 0.05$) than the smooth motion in the water bath.

For details of factorial ANOVA results of the values from the 1.0 and 0.5% citric acid groups, see table 3. A significant impact of a single factor or of the combination of factors on outcome was found, except for the interaction (1) of the 2 factors treatment type and acid movement type, (2) of the 3 factors acid type, treatment type and movement type, and (3) of all 4 factors.

Discussion

The demineralisation protocol is a key parameter in erosion and erosion/abrasion experiments, but little is known about how variations in the protocol impact study outcomes such as dimension of tissue loss, increase in tissue loss due to brushing or efficacy of active agents. Thus, the present study included a series of experiments that tested the effects of 3 different formulations and a placebo under changing erosive conditions using 1 type of acid. Citric acid was used because it is often the demineralisation agent in erosion or erosion/abrasion studies; ranges of concentrations and pH values were chosen according to the literature (for example Lagerweij et al. [2006]; Attin et al. [2007]; Hara et al. [2009]; Ganss et al. [2012]; Hara et al. [2013]). The other parameters (erosion time, brushing time, brushing force and remineralisation procedure) were kept constant and followed our previous experiments [Ganss et al., 2011, 2012] as well as recommendations for erosion and erosion/abrasion studies [Wiegand and Attin, 2011; Shellis et al., 2011]. The formulations with active agents were chosen in order to produce a wide enough range of effects. There is evidence that a combination of stannous and fluoride ions provides good protection against erosion [Ganss et al., 2010; Huysmans et al., 2011; Carvalho and Lussi, 2014] and erosion/abrasion [Schlueter et al., 2013]. In particular, the SnF_2 gel used here has been shown to be effective in earlier studies when applied as a slurry as well as in combination with brushing [Ganss et al., 2012; Schlueter et al., 2014]. This product is a water-free formulation without abrasives. The other stannous and fluoride formulation contained 2 fluoride compounds: NaF and SnF_2. This preparation has shown promising effects in the literature when applied as a slurry which, however, was counteracted when brushing was added [Ganss et al., 2011]. NaF alone seems to be even less effective, although there are so far unexplained differences between the different marketed NaF toothpastes. The product used here was of interest because it showed good effects when applied as a slurry [Ganss et al., 2011].

Distinct differences in tissue loss were found in the placebo slurry groups depending on the severity of the erosive challenge. The concentration and the pH of the acid are some of the key components in designing an erosion or erosion/abrasion study. However, regarding the

demineralising effect, not only the hydrogen concentration, but also the titratable acidity is of importance. The effects of citric acid on enamel dissolution have been intensively studied [Hughes et al., 2000; West et al., 2001; Barbour et al., 2005; Shellis et al., 2013]. It dissociates in 3 steps, can therefore act as a buffer and can resist pH changes. This behaviour results in a non-linear relationship between mineral loss and pH or concentration, which was found in the present study and corroborated data from the literature [Hughes et al., 2000; Shellis et al., 2013]. In the present study, demineralisation with 1 and 0.5% citric acid produced distinct tissue loss, but not at a concentration of 0.3%, regardless of the pH. In principle, enamel dissolution can occur from such solutions [Hughes et al., 2000; West et al., 2000], but the cited studies used citric acid not at room temperature but at 37°C. It is well known that the erosivity of an acid increases approximately linearly with increasing temperature [Barbour et al., 2006]. Furthermore, these studies were performed without remineralisation phases. The mineral salt solution used in the present study was supersaturated with respect to hydroxyapatite and produced distinct mineral precipitation under neutral conditions. Obviously, these precipitates survived 0.3% citric acid at both pH values to an extent that even no enamel loss occurred. These macroscopically visible precipitates detached in toto or over a wide area from the sample surface when a certain thickness was reached. This might explain the wide range of step height values and the relatively high standard deviations in the groups showing precipitates and was the reason for deciding to perform only statistics on groups showing no precipitates at least in the groups treated with active agents. Furthermore, precipitates indicate that no erosive or erosive/abrasive tissue loss occurred, making a statistical analysis not meaningful.

The dissolution rate of a solid depends on two conditions at the enamel-acid interface. One aspect is the provision of new hydrogen ions at this interface. Free dissolved minerals consume hydrogen ions with a concomitant local increase in pH, if no new hydrogen ions are provided. The saturation stage of the liquid is another aspect that depends on the dissolution and enrichment of mineral at the interface. Considering this information, it is clear that the dynamic conditions at the enamel-acid interface are a determinant for the dimension of tissue loss [Eisenburger and Addy, 2003; Attin et al., 2013]. Under dynamic conditions, more new hydrogen ions are provided, and dissolved Ca^{2+} is more quickly removed than under slow motion or static conditions, where local saturation phenomena can occur [Gray, 1962; Shellis et al., 2014]. It has been shown that citric acid at pH 3.2 produced tissue loss of only 8.6 μm even after 2 h under static conditions, while stirring at 270 and 540 rpm increased values by 258% (22.2 μm) and 465% (40.9 μm), respectively [Eisenburger and Addy, 2003]. The present study, however, revealed that it is not only the frequency of shaking or stirring that makes a difference but also, at a given frequency, the type of movement. Changing the acid immersion movement from a water bath (smooth movement of the acid) to a shaking plate (jerky movement, showing clear motion of the acid in the demineralisation container), both at a frequency of 35/min with a travel path of 20 mm, resulted in a 169 and 294% increase in enamel loss for 0.5 and 1% citric acid, respectively (placebo slurry groups). Obviously, the jerky movement led to a better exchange of the solution near the enamel surface, providing new hydrogen ions that dissolved enamel. As a practical consequence, this means that even relatively small dynamic changes may be significant with respect to the amount of tissue loss and therefore important for designing a study. The provision of new H^+ ions depends also on the viscosity of the acid, which was kept constant in the present study. However, an increase in viscosity would decrease the H^+ ion exchange and following the erosivity [Aykut-Yetkiner et al., 2013], making a more jerky movement of the solution necessary to achieve the same demineralisation power of the acid. The results cannot be directly transferred from citric acid to other acids; however, trends might be deduced from these findings obtained.

Considering the effects of abrasion with the placebo toothpaste, it could be expected that brushing would produce greater tissue loss in the groups with more concentrated citric acid and more dynamic movement conditions because a severer acid challenge is associated with greater loss of surface microhardness [Attin et al., 1997]. The present study, however, revealed contradicting results. An explanation for this finding is that mineral precipitates may play a role. It is conceivable that less dynamic conditions at the enamel surface resulted in survival and/or reprecipitation of mineral regardless of the pH of the acid because of a local supersaturation of the acid. These structures may be of greater thickness than the partly demineralised surface layer of enamel eroded under dynamic conditions and might act as a 'protective' layer. Under dynamic conditions, however, dissolved mineral is readily moved away and, as a consequence, the partially demineralised surface layer might be directly removed by abrasion, resulting in a greater increase in step height values in the profilometric readings. In the case of the 0.3% citric acid model, the acidic challenge was too mild to remove or even reduce the precipitates on the sur-

face, with the effect that brushing on these surfaces induced no profilometrically detectable substance loss.

After the use of the SnF_2 or the NaF formulation, tissue loss was found in the groups treated with 1 and 0.5% citric acid, but not after demineralisation with the 0.3% solutions. Thus, only the former will be discussed in the following. The effects after treatment with the NaF/SnF_2 formulation differ distinctly from those of NaF or SnF_2 alone; therefore the effects will be addressed separately.

The SnF_2 gel showed the best efficacy and confirmed earlier results [Ganss et al., 2012; Schlueter et al., 2014], irrespective of the experimental conditions. Overall, the effect size increased with decreasing aggressiveness of the demineralisation process, which corroborates the results of another study in which the aggressiveness of the model was controlled by increasing the duration of demineralisation [Schlueter et al., 2009a]. The mode of action of Sn^{2+}-containing formulations is quite well understood and depends on the precipitation of various Sn/Ca/PO_4 salts on and the incorporation of the stannous ion into enamel [Schlueter et al., 2009b], thereby improving its acid resistance. In contrast, the NaF toothpaste was sufficiently effective only under milder conditions. In principle, topical sodium fluoride applications led to the formation of CaF_2-like deposits on the surface, the amount of which depends on the fluoride concentration, the application time and the pH value [Saxegaard and Rolla, 1988]. These deposits are readily soluble in acids [Ganss et al., 2008] and are therefore less stable under more erosive conditions, which explains the present findings. In the absence of sources of Ca^{2+} other than from the enamel itself and from neutral solutions, however, CaF_2 precipitation occurs only after prolonged application times [Petzold, 2001]. It is therefore difficult to explain how the neutral low-concentrated NaF toothpaste slurry, used in the present study, could have any effect at all. Thus, the provision of Ca^{2+} seems of major importance for efficacy. The relevant calcium ions in the present study could arise from two sources. It could be speculated that, on the one hand, the more pronounced mineral precipitations in the case of less dynamic movement conditions may have enabled fluoride effects by providing calcium ions and by forming more CaF_2-like material. On the other hand, the mineral salt solution used for the preparation of slurries should also be considered as a potential source of calcium ions. The role of mineral salt solutions in experimental designs used either as a storage solution or for preparation of active agents, warrants further investigation. Especially, since there are indications that not only the calcium ion plays an important role in the formation of CaF_2-like deposits on the surface and for the resulting efficacy of a fluoride-containing toothpaste. Other ions in the environment also seem to be relevant for modulating the efficacy [Koeser et al., 2014].

The most relevant finding of the present study was, however, that the effects of NaF, in relation to SnF_2, depend on the erosion protocol. After demineralisation with 1% citric acid and the use of dynamic movements, only SnF_2 showed significant effects, but when the same acid was used under less dynamic movement conditions, both NaF and SnF_2 were effective (NaF less than SnF_2). This seems plausible since these two erosion protocols represent different acid challenges as shown by the amount of tissue loss in the placebo groups. However, an interesting finding is that demineralisation in 0.5% citric acid under dynamic movement conditions revealed the same tissue loss as demineralisation with 1% citric acid under less dynamic movement conditions in the placebo groups but resulted in a different relative effect when using NaF and SnF_2. Whilst it was possible to discriminate effects of the two formulations under the former conditions, the latter revealed that both formulations provided protecting effects but that there was no significant difference between them. It may be that the Sn^{2+}-containing gel is more active when an acidic surrounding is maintained at the surface layer, e.g. under the more dynamic movement conditions, which provokes a more pronounced H^+ ion exchange.

Brushing with formulations containing active agents increased tissue loss in most groups, but the relative effects of the NaF and the SnF_2 formulation remained, with a reduction between 35 and 100% (the same as after slurry application alone). The effects of toothpastes on eroded enamel surfaces are complex and not very well understood [Ganss et al., 2013]. On the one hand, active agents may impact the loss of hardness, thus reducing abrasive loss, but these respective effects seem to be of a minor order [Ganss et al., 2013]. On the other hand, the type and amount of abrasive particles may play a role. To date, there is only one published study that systematically investigated the impact of relative enamel/dentine abrasion in the presence or absence of fluoride, demonstrating that the abrasive may play a greater role in the presence than in the absence of fluoride [Hara et al., 2009]. In the NaF groups, a pattern similar to that after placebo brushing was observed, with more pronounced effects in the groups with less dynamic movement conditions, perhaps for the same reasons as discussed above. In the SnF_2 groups, this effect was less clear, which might be due to the different surface properties that arise in the presence of Sn ions. Overall, however, the present results indicate that abrasives may play a minor role as long as the respective tooth-

pastes are of low or medium abrasivity, irrespective of the severity of the erosion protocol.

The results obtained from the NaF/SnF_2-treated groups differed distinctly from the other groups since tissue loss occurred even under the mildest erosive conditions. The reason for this is not completely clear, but it seems that the toothpaste hampers the formation of mineral precipitates. The NaF/SnF_2 preparation contains sodium hexametaphosphate in order to stabilise the stannous ion in the formulation. However, this compound can also complex with calcium ions [Changgen and Yongxin, 1983] and can, for example, be used as a water softener [Larson, 1957] or as an anticalculus agent in humans [van Loveren and Duckworth, 2013] and in animals [Stookey et al., 1995; Willis et al., 1999; Hennet et al., 2007]. Even in calcium-saturated solutions, such as soy milk, the compound is able to decrease the free calcium concentration significantly [Pathomrungsiyounggul et al., 2007]. Sodium hexametaphosphate is negatively charged and is therefore able to bind to surfaces containing polyvalent charges [Choi et al., 1993] such as eroded enamel and can potentially be retained on it for a longer period. The compound showed a notable degree of substantivity since it was capable of preventing mineral precipitation from the mineral salt solution. This could explain the finding that this slightly acidic formulation had almost no protective effect when applied as a slurry. Regarding the results of the brushing groups, a distinct increase in tissue loss, larger than that shown for the other groups, was only found in the 1% citric acid models. One could argue that the abrasivity (relative dentine abrasion) of the NaF/SnF_2 preparation is higher than that of the other preparations. However, in the groups treated with lower concentrated acids, the increase in tissue loss due to brushing was comparable to the other preparations. This led to the suggestion that the concentration of the acid and the dynamic conditions were more important for the dimension of abrasive loss than the abrasivity of the preparation used. Regarding the effect of hexametaphosphate, one has to bear in mind that under intra-oral conditions, the mode of action might be different. One in situ study, for example, has shown an erosion/abrasion-protecting effect of a 1% hexametaphosphate additive to a NaF-containing toothpaste [Conceição et al., 2015]. Probably, the difference in effect depends on the proneness of calcium ions to precipitate. The saliva contains proteins, such as statherin, which is able to prevent calcium ions to precipitate from the saliva on the dental hard tissue.

In conclusion, the results clearly demonstrate that designing a study is complex and challenging. As distinct differences in the dimension of tissue loss, the effect of brushing abrasion and the relative effects of the active agents to each other and to the placebo depended on the demineralisation conditions, the null hypotheses were rejected.

The basic finding is that, in principle, erosive conditions that are too severe or too mild may lack discriminating power, resulting in either a masking of effects or in the production of experimental artefacts pretending an effect, which cannot be upheld under clinical conditions. The erosion protocol with the best discriminating power in the present comparison was demineralisation with 1% citric acid under less dynamic movement conditions (water bath). The pH of acid seems to play a minor role, the concentration and the titratable acidity depending on the acid concentration seem to be of higher relevance. The dynamic conditions at the sample surface play an important role not only in terms of the dimension of tissue loss, but also with respect to complex demineralisation and reprecipitation phenomena that are obviously decisive for the study outcome. The factorial ANOVA revealed that no interaction between treatment type (brushing or slurry immersion) and shaking type, between treatment type, shaking type and acid type or between all factors can be found, reflecting well the visual effects shown in figure 2.

The present design primarily addressed extrinsic erosive challenges since citric acid, the most common acid in foodstuff, was used. The behaviour of other acids, for example hydrochloric acid to simulate intrinsic erosion, would be of particular interest for further studies. The findings also indicate that the storage medium in cyclic erosion/abrasion studies is of relevance, which justifies further investigation of this issue. All in all, the results clearly emphasise the need for thorough standardisation of experimental protocols, since results obtained by one study design may not be comparable with that of another design.

Acknowledgement

We gratefully thank Birgit Meier for her invaluable help with all technical procedures.

Author Contributions

Contribution to the paper of each author (in alphabetical order): conceived and designed the experiments: A.T., C.G., N.S.; performed the experiments, all practical work and PubMed search: A.T.; analysed the data: A.L., C.G., N.S.; wrote the paper: A.L, A.T., C.G., N.S.

Disclosure Statement

There are no conflicts of interest for any of the authors.

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