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Artificial Saliva Formulations versus Human Saliva Pretreatment in Dental Erosion Experiments

Graziela Ribeiro Batista[a, b] Carlos Rocha Gomes Torres[b] Beatrice Sener[a]
Thomas Attin[a] Annette Wiegand[c]

[a]Department of Preventive Dentistry, Periodontology and Cariology, University of Zurich, Zurich, Switzerland; [b]Department of Restorative Dentistry, Institute of Science and Technology, UNESP – São Paulo State University, São José dos Campos, Brazil; [c]Department of Preventive Dentistry, Periodontology and Cariology, University of Göttingen, Göttingen, Germany

Key Words
Artificial saliva · Dental erosion · Human saliva · In vitro · In vivo

Abstract
The aim of this study was to evaluate the erosion-preventive effect of different artificial saliva formulations and human saliva in vitro compared to human saliva in situ. In the in vitro experiment, bovine enamel and dentin specimens were stored in artificial saliva (4 different formulations, each n = 20), deionized water (n = 20) or human saliva (n = 6 enamel and dentin specimens/volunteer) for 120 min. In the in situ experiment, each of the 6 enamel and dentin specimens was worn intraorally by 10 volunteers for 120 min. The specimens were then eroded (HCl, pH 2.6, 60 s). Half of the specimens were subjected to microhardness analysis (enamel) and the determination of calcium release into the acid (enamel and dentin), while the other half were again placed in the respective medium or worn intraorally, respectively, for 120 min before a second erosion was performed. Knoop microhardness of enamel and the calcium release of enamel and dentin into the acid were again determined. Statistical analysis was conducted by two-way repeated-measures ANOVA or two-way ANOVA (α = 0.05). Enamel microhardness was not significantly different between all test groups after the first and the second erosive challenge, respectively. Enamel calcium loss was significantly lower in situ compared to the in vitro experiment, where there was no significant difference between all test groups. Dentin calcium loss was significantly lower than deionized water only after the first and than all except one artificial saliva after the second erosion. Under the conditions of this experiment, the use of artificial saliva formulations and human saliva in vitro does not reflect the intraoral situation in dental erosion experiments adequately.

Dental erosion is caused by the direct contact of teeth with acids from extrinsic or intrinsic origin, but the development and progression of erosive lesions is modified by various behavioral or biological factors. As erosive tooth wear is a growing problem affecting adults and children [Lussi and Carvalho, 2014], research in dental erosion and erosive tooth wear is steadily increasing. Most research in dental erosion is still done in in vitro setups, as in vitro experiments allow the analyzing of principal mechanisms by controlling and standardizing several variables while one variable is systematically varied. Compared to in situ and clinical studies, in vitro experiments

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Graziela Ribeiro Batista, Department of Restorative Dentistry
Institute of Science and Technology, UNESP – São Paulo State University
Av. Engenheiro Francisco José Longo, 777, Jd. São Dimas
São José dos Campos, SP 12.245-000 (Brazil)
E-Mail graziribat@gmail.com

on dental erosion are relatively inexpensive and enable a fast assessment of products or treatments without the need to consider ethical aspects. On the other hand, in vitro experiments should simulate clinical conditions as closely as possible to generate results relevant for the clinical situation.

One important cofactor in the development and progression of erosive lesions is saliva, which forms an acid-protective pellicle on tooth surfaces and minimizes the acid effects by dilution and buffering properties [Hannig and Hannig, 2014; Hara and Zero, 2014]. Ideally, these effects should be also achieved in in vitro experiments when using saliva substitutes.

A literature search revealed that different formulations of artificial saliva were used in in vitro experiments on dental erosion. The following search terms were used for searching a literature database (PubMed, March 2013): dental erosion AND saliva AND in vitro. One hundred and eighteen studies were retrieved, but only full papers in English were taken into consideration. In 76 papers, in vitro experiments were performed by using artificial saliva formulations, in which the following formulas were used most often – artificial saliva according to: (1) Klimek et al. [1982], which was the first artificial formula introduced for in vitro studies (differences in mucin content) – 17 studies [Attin et al., 1998, 2000; Lennon et al., 2006; Wiegand et al., 2006, 2007; Magalhaes et al., 2008a; Wiegand et al., 2008a, 2009a, b; Souza et al., 2010; Wegehaupt and Attin, 2010; Yu et al., 2010a; Levy et al., 2011; Rochel et al., 2011; Magalhaes et al., 2011; Comar et al., 2012; Magalhaes et al., 2012]; (2) Vieira et al. [2005] – 17 studies [Kato et al., 2007; Magalhaes et al., 2007; Francisconi et al., 2008; Magalhaes et al., 2008b; Kato et al., 2009; Magalhaes et al., 2009; Rios et al., 2009; Bueno et al., 2010; Kato et al., 2010; Magalhaes et al., 2010a, b; Moretto et al., 2010; Barbosa et al., 2011; De Carvalho Filho et al., 2011; Manarelli et al., 2011; Barbosa et al., 2012; Cruz et al., 2012]; (3) Amaechi et al. [1998b] – 13 studies [Amaechi et al., 1998a, 1999a–c; Amaechi and Higham, 2001; Yamaguchi et al., 2006; Messias et al., 2008; Panich and Poolthong, 2009; Jitpukdeebodintra et al., 2010; Meyer-Lueckel and Tschoppe, 2010; Poggio et al., 2010; Messias et al., 2011; Turssi et al., 2012], and (4) Eisenburger et al. [2001a] – 10 studies [Eisenburger et al., 2001b; Vanuspong et al., 2002; Lippert et al., 2004; Fowler et al., 2006, 2009; Austin et al., 2010; Gracia et al., 2010; Rodriguez and Bartlett, 2010; Venasakulchai et al., 2010; Austin et al., 2011]. The compositions of these formulations are given in table 1. Other formulas were found, but not frequently used [Featherstone et al., 1986; Zero et al., 1990; Newby et al., 2006; Wongkhantee et al., 2006; Piekarz et al., 2008; Engle et al., 2010; Min et al., 2011; Scaramucci et al., 2011; Wang et al., 2011]. Due to the different compositions of the artificial saliva formulations, the erosion process might have been differently affected. In order to establish a valid protocol for in vitro erosion studies, it was necessary to determine whether the artificial solutions were comparable to the effects of human saliva in situ and in vitro.

Therefore, this study aimed to analyze the effects of different artificial saliva formulations and human saliva before dental erosion in an in vitro model and to compare the results with the effects of human saliva in an in situ model. Calcium release (enamel and dentin erosion) and microhardness (enamel erosion only) were analyzed as response variables.

The hypotheses were: (1) all artificial saliva formulations and human saliva are less effective in reducing the calcium release of enamel and dentin and enamel microhardness loss in vitro than human saliva under clinical conditions (in situ, positive control), and (2) all artificial saliva formulations and human saliva are more effective in reducing the calcium release of enamel and dentin and enamel microhardness loss compared to deionized water (negative control).

Materials and Methods

Sample Preparation and Allocation to the Groups

Each of the 220 enamel and 220 cylindrical dentin specimens were prepared from freshly extracted, undamaged bovine incisors which were stored in 0.5% thymol solution until use [Attin et al., 2003]. The enamel and dentin specimens (diameter: 3 mm) were gained from the buccal surface of crowns or roots, respectively, by use of a water-cooled diamond trephine mill. They were embedded in acrylic resin blocks (diameter: 6 mm, height: 3 mm; Paladur; Heraeus Kulzer, Germany). The labial surfaces of the specimens were ground flat and polished with water-cooled carborundum discs (1,200, 2,500 and 4,000 grit, waterproof silicon carbide paper; Stuers, Erkrath, Germany). The polished specimens were cleaned in distilled water in an ultrasonic cleaner (M. Scherrer, Wil, Switzerland) for 1 min to remove any debris. The specimens used in situ were sterilized by gamma radiation (12 kGy, 4 h; Paul Scherrer Institute, Villigen, Switzerland) [Wiegand et al., 2008b; Yu et al., 2010b]. Before use, all specimens were kept in deionized water.

Each of the 20 enamel and 20 dentin specimens were subjected to the storage media listed in table 1 or to the negative control group (deionized water). Each of the 60 enamel and 60 dentin specimens were subjected to the groups where human saliva was used in vitro or in the in situ experiment. The study design is shown in figure 1.

Table 1. Composition of tested artificial saliva formulations and degree of saturation with respect to hydroxyapatite, octacalcium phosphate and dicalcium phosphate dehydrate according to Shellis [1988]

Compound	Artificial saliva formulations			
	according to Klimek et al. [1982]	according to Vieira et al. [2005]	according to Amaechi et al. [1998b]	according to Eisenburger et al. [2001a]
$C_6H_8O_6$	2 mg/l	–	–	–
$C_6H_{12}O_6$	30 mg/l	–	–	–
NaCl	580 mg/l	–	–	–
$CaCl_2$	170 mg/l	–	–	–
KCl	1,270 mg/l	11,182.50 mg/l	624.73 mg/l	2,236.50 mg/l
NaSCN	160 mg/l	–	–	–
KH_2PO_4	330 mg/l	–	326.620 mg/l	544.360 mg/l
CH_4N_2O	200 mg/l	–	–	–
Na_2HPO_4	340 mg/l	–	–	–
Mucin	2,700 mg/l	–	–	–
$Ca(NO_3)_2{\cdot}4H_2O$	–	60.12 mg/l	–	–
NaF	–	0.066 mg/l	–	–
$NaH_2PO_4{\cdot}2H_2O$	–	160.19 mg/l	–	–
$C_4H_{11}NO_3$ Tris buffer	–	12,114.00 mg/l	–	–
K_2HPO_4	–	–	804.712 mg/l	–
$CaCl_2{\cdot}2H_2O$	–	–	166.130 mg/l	77.690 mg/l
$C_8H_8O_3$	–	–	2,000 mg/l	–
CMC-Na	–	–	10,000 mg/l	–
$MgCl_2{\cdot}6H_2O$	–	–	58.96 mg/l	–
$MgCl_2$	–	–	–	19.04 mg/l
$C_8H_{18}N_2O_4S$ HEPES	–	–	–	4,766.20 mg/l
Deionized water	1,000 ml	1,000 ml	1,000 ml	1,000 ml
pH	6.4	7.0	6.75	7.0
Hydroxyapatite	6.51	6.69	11.26	9.50
Octacalcium phosphate	1.57	1.20	2.46	1.89
Dicalcium phosphate dehydrate	1.15	0.54	1.66	1.06

Preparation of Artificial Saliva

Artificial saliva formulations were prepared according to the descriptions in previous studies: Klimek et al. [1982], Vieira et al. [2005], Amaechi et al. [1998b] and Eisenburger et al. [2001a]. The degrees of saturation with respect to hydroxyapatite, dicalcium phosphate dehydrate and octacalcium phosphate were calculated according to Shellis [1988] and are presented in table 1.

Volunteers and Saliva Collection

Ethical approval of the study was granted by the local ethics committee (StV 07/11). Ten healthy subjects (3 male, 7 female) aged between 28 and 43 years took part in the study. The inclusion criteria were as follows: ≥18 years old, healthy and mean stimulated saliva flow rate ≥1 ml/min. The exclusion criteria were the following: use of fixed or removable orthodontic appliances, general/systemic illness, smoking, hyposalivation, pregnancy or breastfeeding.

The participants were instructed to refrain from the consumption of any dietary products and oral hygiene treatment 1 h before saliva collection or the insertion of the intraoral appliances, during the interval before the second saliva collection (in vitro experiment) and while the appliances were in place (in situ experiment) [Attin et al., 2004]. The saliva collection or the insertion of appliances in the oral cavity, respectively, started between 7.30 and 8.30 a.m.

The same volunteers were used for the in situ experiment and for the collection of saliva for the in vitro experiment. For the in vitro experiment, saliva was stimulated by the chewing of Parafilm® M (Brand GmbH + Co. KG, Wertheim, Germany). Whole-mouth saliva was freshly collected for both parts of the in vitro experiment; each volunteer donated at least 12 ml of saliva each time.

In vitro Experiment

Enamel and dentin specimens (each n = 20) were stored individually in 1 ml of each medium at 37°C for 120 min prior to erosion. Each specimen was eroded by hydrochloric acid (1 ml, pH 2.6, 2.5 mmol/l, 60 s), which was kept for calcium analysis. Erosion was done in an Eppendorf tube, which was gently shaken (180° rotation, 60×/min). After erosion, the specimens were washed with deionized water (pH 5.5) for 10 s, and half of them (each n = 10) were placed again in the respective medium for an additional 120 min. The other half (each n = 10) were submitted to microhardness evaluation after the first erosion (only for enamel specimens); the dentin specimens were discarded.

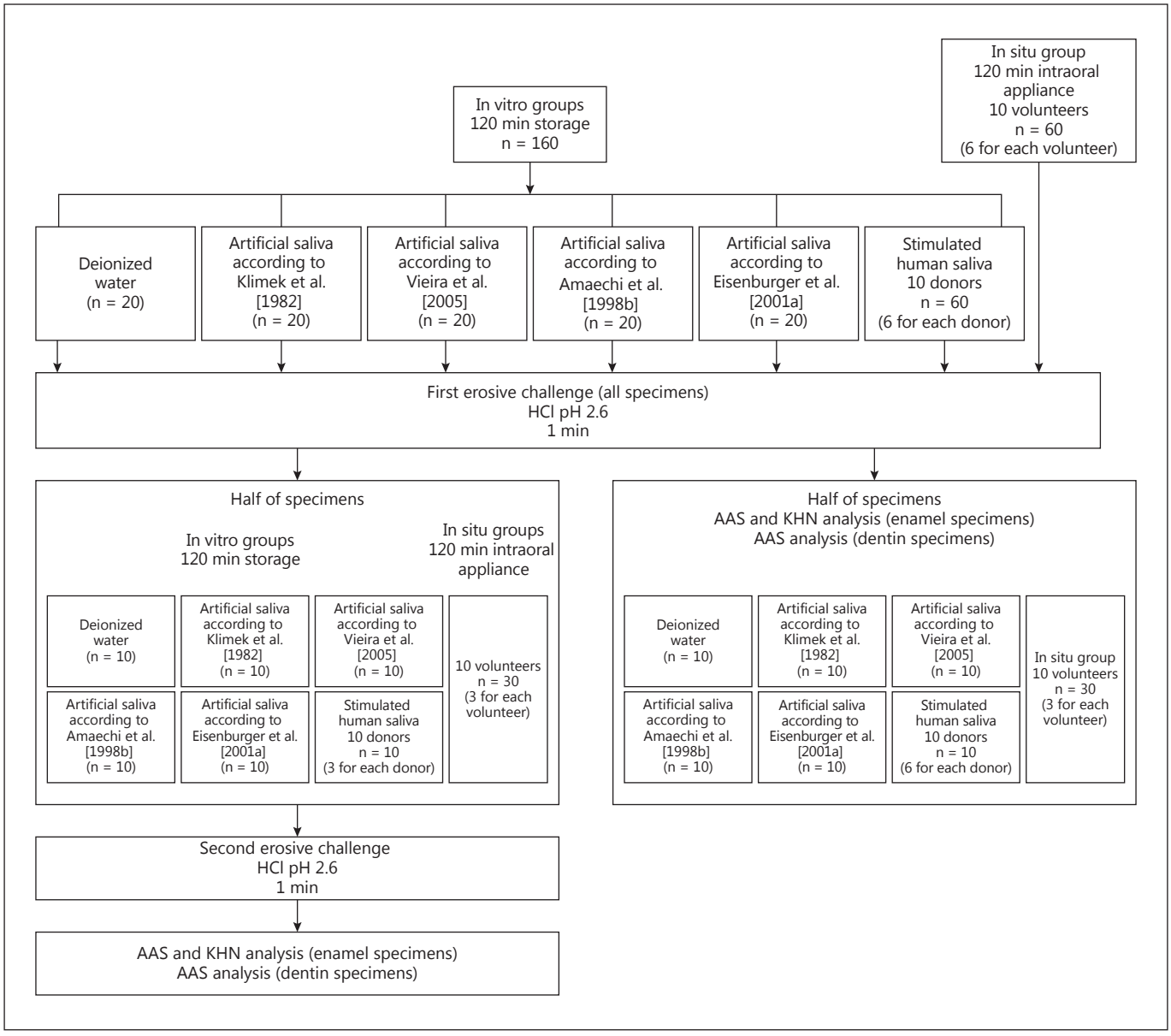

Fig. 1. Flowchart of the experimental setup. AAS = Atomic absorption spectroscopy. KHN = Knoop hardness number.

After additional storage in the respective medium for 120 min, the remaining specimens were eroded a second time (1 ml HCl, pH 2.6, 60 s), and the acid was again kept for calcium analysis. The second erosive challenge was followed by microhardness testing of the enamel specimens.

In situ Experiment

The subjects used custom-made acrylic devices of the upper jaw, provided with buccal recesses in the areas of the left and right second premolars and first and second molars for fixing of the specimens [Hellwig et al., 1987]. Each volunteer received 6 enamel or 6 dentin specimens on 2 consecutive days. The sequence of experiments and the allocation of the specimens to the appliances were randomly assigned.

The appliances were inserted in the oral cavity and used for 120 min. The specimens were extraorally submitted to erosion (1 ml HCl, pH 2.6, 2.5 mmol/l, 60 s), and the acid was kept for calcium analysis. The specimens were then washed with deionized water for 10 s, and half of them were placed intraorally for an additional 120 min prior to the second erosion (1 ml HCl, pH 2.6, 60 s). The other half were submitted to microhardness evaluation after the first erosion (only for enamel specimens); the dentin specimens

Table 2. Enamel microhardness (KHN) in the respective tested groups initially and after the first and second erosive challenge

Groups/saliva composition	Initial microhardness	Microhardness after 1st erosion	Microhardness after 2nd erosion
In vitro			
Deionized water	297.3±22.9[a, A]	247.3±20.7[a, B]	254.8±14.5[a, B]
Klimek et al.	290.2±23.6[a, A]	254.5±31.6[a, B]	251.0±39.2[a, B]
Vieira et al.	292.3±30.9[a, A]	241.2±19.2[a, B]	246.9±41.0[a, B]
Amaechi et al.	282.9±34.6[a, A]	256.7±24.1[a, A, B]	240.1±49.6[a, B]
Eisenburger et al.	291.4±27.8[a, A]	259.0±15.8[a, B]	270.6±30.7[a, A, B]
Human saliva	282.5±28.8[a, A]	231.6±34.1[a, B]	245.9±45.1[a, B]
In situ	275.6±38.3[a, A]	232.2±41.4[a, B]	229.1±33.8[a, B]

Values are means ± SD. In each column, the groups followed by the same lower case letters were not significantly different. In each row, the groups marked by the same upper case letters were not significantly different. KHN = Knoop hardness number.

were discarded. The second erosive challenge was again followed by analysis of calcium release and microhardness testing of enamel specimens..

Measurement Methods

Surface microhardness of the enamel specimens was determined at baseline, and after the first and second erosive experiment using the average values of three indentations at a distance of 50 µm from each specimen (Knoop diamond, 100-gram load per 20 s, high-quality hardness tester; Buehler, Düsseldorf, Germany).

To evaluate the amount of calcium dissolved from the enamel and dentin specimens into the acid, 0.3 ml from the acid sample was mixed with 2 ml of strontium chloride (0.75%) and 3.7 ml of bidistilled water prior to atomic absorption spectroscopy (ConfrAA300; Analytic Jena, Germany; detection limit: 0.025 µg calcium/ml).

Statistical Analysis

Mean enamel microhardness (± standard deviation, SD) was calculated and analyzed by two-way repeated-measures ANOVA, considering the time points of measurement and the type of saliva as variables. The two-way repeated-measures ANOVA was followed by Tukey's or Sidak's multiple comparison tests ($p < 0.05$).

Mean calcium loss (± SD) of the enamel and dentin specimens was calculated and statistically analyzed by two-way repeated-measures ANOVA, separately for enamel and dentin specimens, followed by Tukey's or Sidak's multiple comparison tests ($p < 0.05$).

To compare the protective effect of the different artificial saliva formulations and human saliva on enamel and dentin, the percentage reduction of calcium loss (compared to the negative control) was calculated for each group and statistically analyzed by two-way ANOVA separately for the first and second time point of measurement. Two-way ANOVA was followed by Sidak's multiple comparison tests ($p < 0.05$). All the statistical analyses were performed by GraphPad Prism 6 software (San Diego, Calif., USA).

Results

The enamel microhardness loss is presented in table 2. Two-way repeated-measures ANOVA revealed a significant reduction of Knoop hardness after the first ($p < 0.0001$, compared to baseline) and second ($p < 0.0001$, compared to baseline except for the artificial saliva according to Eisenburger et al. [2001a]) erosive challenge, while the microhardness of specimens after the first and second challenge was not significantly different from each other ($p = 0.65$). However, there was no statistically significant difference among all test groups in the respective time point of measurement ($p > 0.05$).

Two-way repeated-measures ANOVA revealed that both the type of saliva ($p < 0.0001$) and the time point of measurement ($p = 0.0009$), as well as the interaction between both variables ($p = 0.039$), were significant with respect to enamel calcium loss. The calcium release of the enamel specimens was significantly lower in the in situ experiment compared to the in vitro experiment at both time points. No differences between the artificial saliva formulations, deionized water (negative control) and human saliva in vitro were detected. Between-time point comparisons revealed no significant differences except for the artificial saliva according to Amaechi et al. [1998b].

Two-way repeated-measures ANOVA revealed that both the type of saliva ($p = 0.0005$) and the time point of measurement ($p < 0.0001$), but not the interaction between both variables ($p = 0.22$), were significant with respect to dentin calcium loss. The calcium release of the dentin specimens after the first erosive challenge was low-

Table 3. Enamel and dentin calcium release (μg) in the respective tested groups after the first and second erosive challenge

Groups/saliva composition	Enamel		Dentin	
	after 1st erosion	after 2nd erosion	after 1st erosion	after 2nd erosion
In vitro experiment				
Deionized water	$2.90 \pm 0.06^{b, A}$	$3.21 \pm 0.56^{b, A}$	$3.08 \pm 0.76^{b, A}$	$2.40 \pm 0.53^{b, B}$
Klimek et al.	$2.85 \pm 0.50^{b, A}$	$3.09 \pm 0.56^{b, A}$	$2.88 \pm 0.60^{a, b, A}$	$2.43 \pm 0.41^{b, A}$
Vieira et al.	$2.74 \pm 0.54^{b, A}$	$2.96 \pm 0.62^{b, A}$	$2.84 \pm 0.36^{a, b, A}$	$2.52 \pm 0.33^{b, A}$
Amaechi et al.	$2.78 \pm 0.43^{b, A}$	$3.29 \pm 0.47^{b, B}$	$2.71 \pm 0.39^{a, b, A}$	$2.49 \pm 0.43^{b, A}$
Eisenburger et al.	$2.69 \pm 0.52^{b, A}$	$3.08 \pm 0.68^{b, A}$	$2.84 \pm 0.57^{a, b, A}$	$2.14 \pm 0.82^{a, b, B}$
Human saliva	$2.55 \pm 0.53^{b, A}$	$2.76 \pm 0.44^{b, A}$	$2.72 \pm 0.50^{a, b, A}$	$2.42 \pm 0.48^{b, A}$
In situ experiment	$1.83 \pm 0.87^{a, A}$	$1.49 \pm 0.83^{a, A}$	$2.28 \pm 0.44^{a, A}$	$1.52 \pm 0.40^{a, B}$

Values are means ± SD. In each column, the groups followed by the same lower case letters were not significantly different. Separately for enamel and dentin, significant differences in calcium release between the first and second challenge are marked by different upper case letters.

er in the in situ experiment compared to deionized water. There was no statistically significant difference among all other groups. After the second erosive challenge, calcium release in the in situ experiment was significantly lower than in all other groups except for the artificial saliva according to Eisenburger et al. [2001a]. The artificial saliva and human saliva in vitro did not differ significantly from each other. Between-time point comparisons revealed significant differences for the groups 'human saliva in situ', 'deionized water' and 'artificial saliva' according to Eisenburger et al. [2001a] (table 3).

A comparison between the relative calcium release of the enamel and dentin specimens revealed no significant effect of the type of substrate after the first erosive challenge ($p = 0.77$), but there was an effect after the second erosive challenge ($p < 0.0001$). However, Sidak's post hoc tests revealed no significant differences among all groups.

Discussion

In this study the erosion-preventive effect of different artificial saliva formulations and human saliva in vitro was compared to human saliva in situ. While enamel microhardness loss did not show differences among the experimental groups, calcium release in the in situ experiment was significantly lower compared to all (enamel) or most of the groups (dentin, second erosive challenge) of the in vitro experiment.

The specimens were short-time eroded using hydrochloric acid to simulate clinical conditions in patients suffering from gastric reflux or bulimia [Wiegand et al., 2008a; Wegehaupt et al., 2012; Passos et al., 2015]. To address the erosion-protective effect of the salivary pellicle, the specimens were stored in the artificial saliva solutions or placed intraorally for 2 h before the erosive attacks. In former studies, short-time pellicle formation up to 2 h was shown to have a significant protective effect on enamel and dentin erosion [Amaechi et al., 1999c; Hannig et al., 2003, 2004; Wetton et al., 2006]. The pellicle might act as a diffusion barrier, inhibiting the contact of acids to the dental surface and thus decreasing the diffusion of calcium and phosphate ions into the surrounding fluid exposure [Siqueira et al., 2010]. A previous study found that the protective effect of the pellicle was higher on enamel compared to dentin [Wiegand et al., 2008a], but this was not observed in the present study, probably as the artificial saliva formulations are generally unable to form a protective surface layer independently of the type of substrate. In the in situ experiment, the specimens were placed in the buccal region of the upper jaw to minimize abrasion (as seen in specimens localized palatally due to tongue abrasion) and allow for continuous contact with saliva. However, in contrast to the earlier study of Wiegand et al. [2008a], the protective effect of the salivary pellicle was only slightly, but not significantly, different between the enamel and dentin specimens.

However, the results of the present study are conflicting as microhardness loss did not differ between the in situ experiment and the artificial saliva formulations, while calcium loss was significantly reduced in the in situ experiment. Chemical analysis of calcium allows for the

detection of very small mineral losses [Attin and Wegehaupt, 2014], which might not be detected by hardness measurement. Although microhardness measurement allows for the discrimination of erosive softening even after short-term demineralization, it can be assumed that the differences between the various test groups in the present study were too small to be detected by Knoop hardness measurement.

After the first erosion, half of the specimens were again stored in the respective media or in the oral cavity to address potential rehardening effects of saliva [Gedalia et al., 1991]. It has also to be considered that a new surface pellicle is formed. Only half of the specimens were used for the further experiment as the microhardness measurement was very time-consuming and did not allow for an immediate replacement in the artificial saliva formulations or in the oral cavity, respectively. The enamel specimens used for the microhardness measurement after the first erosion were discarded and not used for the further experiment. To ensure the same number of enamel and dentin specimens in the further experiment, half of the dentin specimens were randomly chosen and also discarded.

As shown in an early study by Hall et al. [1999], the protective effect of saliva in vitro is significantly reduced compared to the in situ environment. Saliva collected in vitro might be altered or degraded due to protein breakdown and pH changes, thus resulting in a reduced capacity to prevent erosion. In an in vitro experiment cycling model over 14 days, enamel and dentin mineral loss was highest when specimens were stored in water between the erosive cycles. Storage in human saliva samples resulted in significantly less mineral loss, but was less effective compared to the in situ experiment, where the specimens were worn in the oral cavity. These differences were explained by the depletion of inorganic components of human saliva and by the degradation of saliva proteins. However, in the present study human saliva in vitro was not even different from artificial saliva formulations and water. This might be explained by two reasons. Firstly, the extraoral storage time was too long, resulting in the complete degradation of human saliva. Secondly, the present study design did not allow the revealing of possible differences between human saliva in vitro and the artificial saliva formulations as no cycling treatment of specimens was performed.

In contrast to the results of the present study, a recent study by Ionta et al. [2014] found differences in the rehardening potential of various artificial saliva formulations and water. This study did not use a de- and remineralization protocol, but focused on the remineralization of erosively softened enamel (citric acid, pH 2.5, 15 s) after 2 h of storage time. All tested artificial saliva solutions resulted in higher rehardening of erosively demineralized enamel than water, but remineralization varied distinctly between the artificial saliva test groups. These differences were explained by different degrees of saturation with respect to calcium phosphates, as well as by different concentrations of carboxymethyl cellulose and mucins.

The different compositions of the artificial saliva formulations might also affect the results of the present study. For instance, the artificial saliva containing carboxymethyl cellulose showed a lower protective effect after the second erosion compared to the first erosion, probably due to the fact that carboxymethyl cellulose might form complexes with calcium and/or phosphate ions, which are then no longer available for rehardening of previously eroded enamel.

However, from the results of the present study it can be speculated that the degree of remineralization is generally too low to be relevant when an additional (second) erosive challenge is performed on dental hard tissues pretreated with different saliva formulations, although the degree of saturation between the artificial saliva formulations varied distinctly.

Under the conditions of the present study, artificial saliva formulations and the use of human saliva in vitro were unable to adequately reflect in situ conditions of enamel and dentin erosion. This aspect has to be taken into consideration when performing in vitro studies using artificial saliva formulations or human saliva.

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Disclosure Statement

No potential conflicts of interest exist in this study for any author.

References

Amaechi BT, Higham SM: In vitro remineralisation of eroded enamel lesions by saliva. J Dent 2001;29:371–376.

Amaechi BT, Higham SM, Edgar WM: The influence of xylitol and fluoride on dental erosion in vitro. Arch Oral Biol 1998a;43:157–161.

Amaechi BT, Higham SM, Edgar WM: Use of transverse microradiography to quantify mineral loss by erosion in bovine enamel. Caries Res 1998b;32:351–356.

Amaechi BT, Higham SM, Edgar WM: Techniques for the production of dental eroded lesions in vitro. J Oral Rehabil 1999a;26:97–102.
Amaechi BT, Higham SM, Edgar WM: Factors influencing the development of dental erosion in vitro: enamel type, temperature and exposure time. J Oral Rehabil 1999b;26:624–630.
Amaechi BT, Higham SM, Edgar WM, Milosevic A: Thickness of acquired salivary pellicle as a determinant of the sites of dental erosion. J Dent Res 1999c;78:1821–1828.
Attin T, Buchalla W, Gollner M, Hellwig E: Use of variable remineralization periods to improve the abrasion resistance of previously eroded enamel. Caries Res 2000;34:48–52.
Attin T, Meyer K, Hellwig E, Buchalla W, Lennon AM: Effect of mineral supplements to citric acid on enamel erosion. Arch Oral Biol 2003; 48:753–759.
Attin T, Siegel S, Buchalla W, Lennon AM, Hannig C, Becker K: Brushing abrasion of softened and remineralised dentin: an in situ study. Caries Res 2004;38:62–66.
Attin T, Wegehaupt FJ: Methods for assessment of dental erosion. Monogr Oral Sci 2014;25: 123–142.
Attin T, Zirkel C, Hellwig E: Brushing abrasion of eroded dentin after application of sodium fluoride solutions. Caries Res 1998;32:344–350.
Austin RS, Rodriguez JM, Dunne S, Moazzez R, Bartlett DW: The effect of increasing sodium fluoride concentrations on erosion and attrition of enamel and dentine in vitro. J Dent 2010;38:782–787.
Austin RS, Stenhagen KS, Hove LH, Dunne S, Moazzez R, Bartlett DW, Tveit AB: A qualitative and quantitative investigation into the effect of fluoride formulations on enamel erosion and erosion-abrasion in vitro. J Dent 2011;39:648–655.
Barbosa CS, Kato MT, Buzalaf MA: Effect of supplementation of soft drinks with green tea extract on their erosive potential against dentine. Aust Dent J 2011;56:317–321.
Barbosa CS, Montagnolli LG, Kato MT, Sampaio FC, Buzalaf MA: Calcium glycerophosphate supplemented to soft drinks reduces bovine enamel erosion. J Appl Oral Sci 2012;20:410–413.
Bueno MG, Marsicano JA, Sales-Peres SH: Preventive effect of iron gel with or without fluoride on bovine enamel erosion in vitro. Aust Dent J 2010;55:177–180.
Comar LP, Gomes MF, Ito N, Salomao PA, Grizzo LT, Magalhaes AC: Effect of NaF, SnF_2, and TiF_4 toothpastes on bovine enamel and dentin erosion-abrasion in vitro. Int J Dent 2012; 2012:134350.
Cruz JB, Lenzi TL, Tedesco TK, Guglielmi Cde A, Raggio DP: Eroded dentin does not jeopardize the bond strength of adhesive restorative materials. Braz Oral Res 2012;26:306–312.
De Carvalho Filho AC, Sanches RP, Martin AA, Do Espirito Santo AM, Soares LE: Energy dispersive X-ray spectrometry study of the protective effects of fluoride varnish and gel on enamel erosion. Microsc Res Tech 2011;74: 839–844.
Eisenburger M, Addy M, Hughes JA, Shellis RP: Effect of time on the remineralisation of enamel by synthetic saliva after citric acid erosion. Caries Res 2001a;35:211–215.
Eisenburger M, Hughes J, West NX, Shellis RP, Addy M: The use of ultrasonication to study remineralisation of eroded enamel. Caries Res 2001b;35:61–66.
Engle K, Hara AT, Matis B, Eckert GJ, Zero DT: Erosion and abrasion of enamel and dentin associated with at-home bleaching: an in vitro study. J Am Dent Assoc 2010;141:546–551.
Featherstone JDB, O'Reily MM, Shariati M, Brugler S: Enhancement of remineralization in vitro and in vivo; in Leach SA (ed): Factors Relating to Demineralisation and Remineralisation of the Teeth. Oxford, IRL Press, 1986.
Fowler CE, Gracia L, Edwards MI, Willson R, Brown A, Rees GD: Inhibition of enamel erosion and promotion of lesion rehardening by fluoride: a white light interferometry and microindentation study. J Clin Dent 2009;20: 178–185.
Fowler CE, Willson R, Rees GD: In vitro microhardness studies on a new anti-erosion desensitizing toothpaste. J Clin Dent 2006;17:100–105.
Francisconi LF, Honorio HM, Rios D, Magalhaes AC, Machado MA, Buzalaf MA: Effect of erosive pH cycling on different restorative materials and on enamel restored with these materials. Oper Dent 2008;33:203–208.
Gedalia I, Dakuar A, Shapira L, Lewinstein I, Goultschin J, Rahamim E: Enamel softening with Coca-Cola and rehardening with milk or saliva. Am J Dent 1991;4:120–122.
Gracia LH, Rees GD, Brown A, Fowler CE: An in vitro evaluation of a novel high fluoride daily mouthrinse using a combination of microindentation, 3D profilometry and DSIMS. J Dent 2010;38(suppl 3):S12–S20.
Hall AF, Buchanan CA, Millett DT, Creanor SL, Strang R, Foye RH: The effect of saliva on enamel and dentine erosion. J Dent 1999;27: 333–339.
Hannig M, Fiebiger M, Guntzer M, Dobert A, Zimehl R, Nekrashevych Y: Protective effect of the in situ formed short-term salivary pellicle. Arch Oral Biol 2004;49:903–910.
Hannig M, Hannig C: The pellicle and erosion. Monogr Oral Sci 2014;25:206–214.
Hannig M, Hess NJ, Hoth-Hannig W, De Vrese M: Influence of salivary pellicle formation time on enamel demineralization – an in situ pilot study. Clin Oral Investig 2003;7:158–161.
Hara AT, Zero DT: The potential of saliva in protecting against dental erosion. Monogr Oral Sci 2014;25:197–205.
Hellwig E, Klimek J, Wagner H: The influence of plaque on reaction mechanism of MFP and NaF in vivo. J Dent Res 1987;66:46–49.
Ionta FQ, Mendonca FL, de Oliveira GC, de Alencar CR, Honorio HM, Magalhaes AC, Rios D: In vitro assessment of artificial saliva formulations on initial enamel erosion remineralization. J Dent 2014;42:175–179.
Jitpukdeebodintra S, Chuenarom C, Muttarak C, Khonsuphap P, Prasattakarn S: Effects of 1.23% acidulated phosphate fluoride gel and drinkable yogurt on human enamel erosion, in vitro. Quintessence Int 2010;41:595–604.
Kato MT, de Moraes Italiani F, de Araujo JJ, Garcia MD, de Carvalho Sales-Peres SH, Buzalaf MA: Preventive effect of an iron varnish on bovine enamel erosion in vitro. J Dent 2009; 37:233–236.
Kato MT, Lancia M, Sales-Peres SH, Buzalaf MA: Preventive effect of commercial desensitizing toothpastes on bovine enamel erosion in vitro. Caries Res 2010;44:85–89.
Kato MT, Sales-Peres SH, Buzalaf MA: Effect of iron on acid demineralisation of bovine enamel blocks by a soft drink. Arch Oral Biol 2007;52:1109–1111.
Klimek J, Hellwig E, Ahrens G: Fluoride taken up by plaque, by the underlying enamel and by clean enamel from three fluoride compounds in vitro. Caries Res 1982;16:156–161.
Lennon AM, Pfeffer M, Buchalla W, Becker K, Lennon S, Attin T: Effect of a casein/calcium phosphate-containing tooth cream and fluoride on enamel erosion in vitro. Caries Res 2006;40:154–157.
Levy FM, Magalhaes AC, Gomes MF, Comar LP, Rios D, Buzalaf MA: The erosion and abrasion-inhibiting effect of TiF_4 and NaF varnishes and solutions on enamel in vitro. Int J Paediatr Dent 2011;22:11–16.
Lippert F, Parker DM, Jandt KD: In vitro demineralization/remineralization cycles at human tooth enamel surfaces investigated by AFM and nanoindentation. J Colloid Interface Sci 2004;280:442–448.
Lussi A, Carvalho TS: Erosive tooth wear: a multifactorial condition of growing concern and increasing knowledge. Monogr Oral Sci 2014; 25:1–15.
Magalhaes AC, Kato MT, Rios D, Wiegand A, Attin T, Buzalaf MA: The effect of an experimental 4% TiF_4 varnish compared to NaF varnishes and 4% TiF_4 solution on dental erosion in vitro. Caries Res 2008a;42:269–274.
Magalhaes AC, Levy FM, Rios D, Buzalaf MA: Effect of a single application of TiF_4 and NaF varnishes and solutions on dentin erosion in vitro. J Dent 2010a;38:153–157.
Magalhaes AC, Levy FM, Rizzante FA, Rios D, Buzalaf MA: Effect of NaF and TiF_4 varnish and solution on bovine dentin erosion plus abrasion in vitro. Acta Odontol Scand 2012; 70:160–164.

Magalhaes AC, Moraes SM, Rios D, Buzalaf MA: Effect of ion supplementation of a commercial soft drink on tooth enamel erosion. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2009;26:152–156.

Magalhaes AC, Moraes SM, Rios D, Wiegand A, Buzalaf MA: The erosive potential of 1% citric acid supplemented by different minerals: an in vitro study. Oral Health Prev Dent 2010b;8: 41–45.

Magalhaes AC, Rios D, Machado MA, Da Silva SM, Lizarelli Rde F, Bagnato VS, Buzalaf MA: Effect of Nd:YAG irradiation and fluoride application on dentine resistance to erosion in vitro. Photomed Laser Surg 2008b;26:559–563.

Magalhaes AC, Romanelli AC, Rios D, Comar LP, Navarro RS, Grizzo LT, Aranha AC, Buzalaf MA: Effect of a single application of TiF_4 and NaF varnishes and solutions combined with Nd:YAG laser irradiation on enamel erosion in vitro. Photomed Laser Surg 2011;29:537–544.

Magalhaes AC, Stancari FH, Rios D, Buzalaf MA: Effect of an experimental 4% titanium tetrafluoride varnish on dental erosion by a soft drink. J Dent 2007;35:858–861.

Manarelli MM, Vieira AE, Matheus AA, Sassaki KT, Delbem AC: Effect of mouth rinses with fluoride and trimetaphosphate on enamel erosion: an in vitro study. Caries Res 2011;45: 506–509.

Messias DC, Maeda FA, Turssi CP, Serra MC: Effect of dentifrices against hydrochloric acid-induced erosion. Oral Health Prev Dent 2011; 9:269–273.

Messias DC, Martins ME, Serra MC, Turssi CP: Feasibility of using sodium bicarbonate solution as a damage-limiting strategy for erosion lesions. Oral Health Prev Dent 2008;6:155–158.

Meyer-Lueckel H, Tschoppe P: Effect of fluoride gels and mouthrinses in combination with saliva substitutes on demineralised bovine enamel in vitro. J Dent 2010;38:641–647.

Min JH, Kwon HK, Kim BI: The addition of nano-sized hydroxyapatite to a sports drink to inhibit dental erosion: in vitro study using bovine enamel. J Dent 2011;39:629–635.

Moretto MJ, Magalhaes AC, Sassaki KT, Delbem AC, Martinhon CC: Effect of different fluoride concentrations of experimental dentifrices on enamel erosion and abrasion. Caries Res 2010;44:135–140.

Newby CS, Creeth JE, Rees GD, Schemehorn BR: Surface microhardness changes, enamel fluoride uptake, and fluoride availability from commercial toothpastes. J Clin Dent 2006;17: 94–99.

Panich M, Poolthong S: The effect of casein phosphopeptide-amorphous calcium phosphate and a cola soft drink on in vitro enamel hardness. J Am Dent Assoc 2009;140:455–460.

Passos VF, de Vasconcellos AA, Pequeno JH, Rodrigues LK, Santiago SL: Effect of commercial fluoride dentifrices against hydrochloric acid in an erosion-abrasion model. Clin Oral Investig 2015;19:71–76.

Piekarz C, Ranjitkar S, Hunt D, McIntyre J: An in vitro assessment of the role of tooth mousse in preventing wine erosion. Aust Dent J 2008; 53:22–25.

Poggio C, Lombardini M, Colombo M, Bianchi S: Impact of two toothpastes on repairing enamel erosion produced by a soft drink: an AFM in vitro study. J Dent 2010;38:868–874.

Rios D, Magalhaes AC, Machado MA, da Silva SM, Lizarelli Rde F, Bagnato VS, Buzalaf MA: In vitro evaluation of enamel erosion after Nd:YAG laser irradiation and fluoride application. Photomed Laser Surg 2009;27:743–747.

Rochel ID, Souza JG, Silva TC, Pereira AF, Rios D, Buzalaf MA, Magalhaes AC: Effect of experimental xylitol and fluoride-containing dentifrices on enamel erosion with or without abrasion in vitro. J Oral Sci 2011;53:163–168.

Rodriguez JM, Bartlett DW: A comparison of two-dimensional and three-dimensional measurements of wear in a laboratory investigation. Dent Mater 2010;26:e221–e225.

Scaramucci T, Hara AT, Zero DT, Ferreira SS, Aoki IV, Sobral MA: In vitro evaluation of the erosive potential of orange juice modified by food additives in enamel and dentine. J Dent 2011;39:841–848.

Shellis RP: A microcomputer program to evaluate the saturation of complex solutions with respect to biominerals. Comput Appl Biosci 1988;4:373–379.

Siqueira WL, Margolis HC, Helmerhorst EJ, Mendes FM, Oppenheim FG: Evidence of intact histatins in the in vivo acquired enamel pellicle. J Dent Res 2010;89:626–630.

Souza JG, Rochel ID, Pereira AF, Silva TC, Rios D, Machado MA, Buzalaf MA, Magalhaes AC: Effects of experimental xylitol varnishes and solutions on bovine enamel erosion in vitro. J Oral Sci 2010;52:553–559.

Turssi CP, Vianna LM, Hara AT, do Amaral FL, Franca FM, Basting RT: Counteractive effect of antacid suspensions on intrinsic dental erosion. Eur J Oral Sci 2012;120:349–352.

Vanuspong W, Eisenburger M, Addy M: Cervical tooth wear and sensitivity: erosion, softening and rehardening of dentine; effects of pH, time and ultrasonication. J Clin Periodontol 2002;29:351–357.

Venasakulchai A, Williams NA, Gracia LH, Rees GD: A comparative evaluation of fluoridated and non-fluoridated mouthrinses using a 5-day cycling enamel erosion model. J Dent 2010;38(suppl 3):S21–S29.

Vieira AE, Delbem AC, Sassaki KT, Rodrigues E, Cury JA, Cunha RF: Fluoride dose response in pH-cycling models using bovine enamel. Caries Res 2005;39:514–520.

Wang X, Megert B, Hellwig E, Neuhaus KW, Lussi A: Preventing erosion with novel agents. J Dent 2011;39:163–170.

Wegehaupt FJ, Attin T: The role of fluoride and casein phosphopeptide/amorphous calcium phosphate in the prevention of erosive/abrasive wear in an in vitro model using hydrochloric acid. Caries Res 2010;44:358–363.

Wegehaupt FJ, Taubock TT, Stillhard A, Schmidlin PR, Attin T: Influence of extra- and intraoral application of CPP-ACP and fluoride on re-hardening of eroded enamel. Acta Odontol Scand 2012;70:177–183.

Wetton S, Hughes J, West N, Addy M: Exposure time of enamel and dentine to saliva for protection against erosion: a study in vitro. Caries Res 2006;40:213–217.

Wiegand A, Bichsel D, Magalhaes AC, Becker K, Attin T: Effect of sodium, amine and stannous fluoride at the same concentration and different pH on in vitro erosion. J Dent 2009a;37: 591–595.

Wiegand A, Bliggenstorfer S, Magalhaes AC, Sener B, Attin T: Impact of the in situ formed salivary pellicle on enamel and dentine erosion induced by different acids. Acta Odontol Scand 2008a;66:225–230.

Wiegand A, Gutsche M, Attin T: Effect of olive oil and an olive-oil-containing fluoridated mouthrinse on enamel and dentin erosion in vitro. Acta Odontol Scand 2007;65:357–361.

Wiegand A, Kuhn M, Sener B, Roos M, Attin T: Abrasion of eroded dentin caused by toothpaste slurries of different abrasivity and toothbrushes of different filament diameter. J Dent 2009b;37:480–484.

Wiegand A, Lemmrich F, Attin T: Influence of rotating-oscillating, sonic and ultrasonic action of power toothbrushes on abrasion of sound and eroded dentine. J Periodontal Res 2006;41:221–227.

Wiegand A, Meier W, Sutter E, Magalhaes AC, Becker K, Roos M, Attin T: Protective effect of different tetrafluorides on erosion of pellicle-free and pellicle-covered enamel and dentine. Caries Res 2008b;42:247–254.

Wongkhantee S, Patanapiradej V, Maneenut C, Tantbirojn D: Effect of acidic food and drinks on surface hardness of enamel, dentine, and tooth-coloured filling materials. J Dent 2006; 34:214–220.

Yamaguchi K, Miyazaki M, Takamizawa T, Inage H, Moore BK: Effect of CPP-ACP paste on mechanical properties of bovine enamel as determined by an ultrasonic device. J Dent 2006;34:230–236.

Yu H, Attin T, Wiegand A, Buchalla W: Effects of various fluoride solutions on enamel erosion in vitro. Caries Res 2010a;44:390–401.

Yu H, Wegehaupt FJ, Zaruba M, Becker K, Roos M, Attin T, Wiegand A: Erosion-inhibiting potential of a stannous chloride-containing fluoride solution under acid flow conditions in vitro. Arch Oral Biol 2010b;55:702–705.

Zero DT, Rahbek I, Fu J, Proskin HM, Featherstone JD: Comparison of the iodide permeability test, the surface microhardness test, and mineral dissolution of bovine enamel following acid challenge. Caries Res 1990;24: 181–188.