Artificial Saliva Formulations versus Human Saliva Pretreatment in Dental Erosion Experiments

Batista, Graziela Ribeiro; Rocha Gomes Torres, Carlos; Sener, Beatrice; Attin, Thomas; Wiegand, Annette

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.

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Artificial saliva formulations versus human saliva pre-treatment in dental erosion experiments

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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 in human saliva (10 volunteers, each n=6 enamel and dentin specimens/volunteer) for 120min. In the in-situ-experiment, each n=6 enamel and dentin specimens were worn intraorally by 10 volunteers for 120min. Then, specimens were eroded (HCl, pH 2.6, 60s). Half of the specimens were subjected to microhardness analysis (enamel) and determination of calcium into the acid (enamel and dentin), while the other half of the specimens were again placed in the respective medium or worn intra-orally, respectively, for 120min before a second erosion was performed. Knoop microhardness of enamel and 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 after the first and the second erosive challenge, respectively, was not significantly different between all test groups. Enamel calcium loss was significantly lower in-situ compared to the in-vitro-experiment, where all test groups performed not significantly different. 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 do not reflect the intraoral situation in dental erosion experiments adequately.
Introduction

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, research in dental erosion and erosive tooth wear is steadily increasing. Most research in dental erosion is still done in in-vitro-set ups, as in-vitro-experiments allow analyzing principal mechanisms by controlling and standardizing several variables while one variable is systematically varied. Compared to in-situ and clinical studies, in-vitro-experiments 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 co-factor 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. 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 in 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 Klimek et al. (17 studies, differences in mucin content), which was the first artificial formula introduced for in-vitro-studies; Vieira et al. (17 studies, differences in mucin content), which was the first artificial formula introduced for in-vitro-studies; Amaechi et al. (13 studies) and Eisenburger et al. (10 studies). The compositions of these formulations are given in Table 1. Other formulas were found, but not frequently used. Due to the different compositions of the artificial saliva formulations, the erosion process might be differently affected. In order to establish a valid protocol for in-vitro erosion studies it is necessary to determine if the artificial solutions are comparable to the effects of human saliva in-situ and in-vitro.

Therefore, this study aimed to analyse 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 to reduce calcium release of enamel and dentin and enamel microhardness loss in-vitro than human saliva does under clinical conditions (in-situ, positive control). 2) all artificial saliva formulations and human saliva are more effective to reduce 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 220 enamel and 220 cylindric dentin specimens were prepared from freshly extracted, undamaged bovine incisors which were stored in 0.5% thymol solution until use. 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 (1200, 2500 and 4000 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). Before use, all specimens were kept in deionized water.

Each 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 60 enamel and 60 dentin specimens were subjected to the groups, where human saliva was used in-vitro or to the in-situ-experiment. The study design is shown in Figure 1.

**Preparation of artificial salivas**

Artificial saliva formulations were prepared according the descriptions in previous studies: Klimek et al., Vieira et al., Amaechi et al., and Eisenburger et al.
The degrees of saturation with respect to hydroxyapatite (HA), dicalcium phosphate dehydrate (DCPD) and octacalcium phosphate (OCP) were calculated according to Shellis and are presented in Table 2.

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: ≥18 years old; healthy; mean stimulated saliva flow rate ≥1 ml/min. The exclusion criteria were: use of fixed or removable orthodontic appliances, general/systemic illness, smoking, hyposalivation, pregnancy or breastfeeding.

The participants were instructed to refrain from consumption of any dietary products and oral hygiene treatment 1h before saliva collection or insertion of the intra-oral appliances, during the interval before the second saliva collection (in-vitro-experiment) and while the appliances were in place (in-situ-experiment). The saliva collection or the insertion of appliances in the oral cavity, respectively, started between 07.30 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 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 12ml of saliva each time.

In-vitro-experiment

Enamel and dentin specimens (each n = 20) were stored individually in 1 ml of each medium at 37ºC 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, 60x/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 additional 120 min. The other half (each n = 10) was submitted to microhardness evaluation after 1st erosion (only for enamel specimens); dentin specimens were discarded.
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 2nd erosive challenge was followed by microhardness testing of enamel specimens.

**In-situ-experiment**

The subjects used custom-made acrylic devices of the upper jaw, provided with buccal recesses in the areas of left and right 2nd premolars and 1st and 2nd molars for fixing of the specimens. Each volunteer received six enamel or six dentin specimens on two consecutive days. The sequence of experiments and the allocation of the specimens in the appliance were randomly assigned.

The appliances were inserted in the oral cavity and used for 120 min. The specimens were extra-orally submitted to erosion (1 ml HCl, pH 2.6, 2.5 mmol/l, 60 s), and the acid was kept for calcium analysis. Then, the specimens were washed with deionized water for 10 s, and half of them were placed intraorally for additional 120 min prior to the second erosion (1 ml HCl, pH 2.6, 60 s). The other half was submitted to microhardness evaluation after 1st erosion (only for enamel specimens); dentin specimens were discarded. The 2nd erosive challenge was again followed by microhardness testing and analysis of calcium release of enamel specimens.

**Measurement methods**

Surface microhardness of enamel specimens was determined at baseline, after the first and after the second erosive experiment using the average values of three indentations at a distance of 50 µm (Knoop diamond, 100g load per 20s, High Quality Hardness Tester, Buehler, Düsseldorf, Germany) of each specimen.

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 to 2 ml of strontium chloride (0.75%) and 3.7 ml of bi-distilled water prior to Atomic Absorption Spectroscopy (ConfrAA300, Analytic Jena, Germany, detection limit: 0.025 µg calcium/ml).
**Statistical analysis**

Mean enamel microhardness (± standard deviation) was calculated and analysed by two-way repeated measures ANOVA, considering the time points of measurement and the kind of saliva as variables. Two-way repeated measures ANOVA was followed by Tukey’s or Sidak’s multiple comparisons tests (p < 0.05).

Mean calcium loss (± standard deviation) was calculated and statistically analysed by two-way repeated measures ANOVA, separately for enamel and dentin specimens, followed by Tukey’s or Sidak’s multiple comparisons 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 analysed 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 analysis were performed by Graph Pad Prism 6 software (San Diego, California, USA).

**Results**

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.\textsuperscript{54}) erosive challenge, while microhardness of specimens after the first and second challenge was not significantly different from each other (p = 0.65). However, all test groups did not differ statistically significantly from each other within the respective time point of measurement (p > 0.05).

Two-way repeated measures ANOVA revealed that both the kind 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. Calcium release of 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.\textsuperscript{40}

Two-way repeated measures ANOVA revealed that both the kind 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.
Calcium release of dentin specimens after the first erosive challenge was lower in the
in-situ-experiment compared to deionized water. All other groups were not statistically
significant from each other. 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.\textsuperscript{54} The artificial salivas and human saliva in-vitro
did not differ significantly from each other. Between time-point comparisons revealed
significant differences for groups “human saliva in-situ”, “deionized water” and “artificial
saliva according to Eisenburger et al.\textsuperscript{54} (Table 3).

Comparison between relative calcium release of enamel and dentin
specimens revealed no significant effect of the kind of substrate after the first erosive
challenge \((p = 0.77)\) but 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.

Specimens were short-time eroded using hydrochloric acid to simulate
clinical conditions in patients suffering from gastric reflux or bulimia.\textsuperscript{13, 80, 81} To address
the erosion-protective effect of the salivary pellicle, specimens were stored in the
artificial saliva solutions or placed intra-orally 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.\textsuperscript{46, 82-84} 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. A previous study found that the protective effect of the pellicle is higher on enamel compared to dentin, 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 kind of substrate. In the in situ experiment, 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., the protective effect of the salivary pellicle was only slightly, but not significantly different between 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 for the in-situ-experiment. Chemical analysis of calcium allow for the detection of very small mineral losses, which might not be detected by hardness measurement. Although microhardness measurement allows for discrimination of erosive softening even after short-term demineralisation, it can be assumed that the differences between the various test groups in the present study are 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. 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 microhardness measurement was very time consuming and did not allow for immediate replacement in the artificial saliva formulations or in the oral cavity, respectively. 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., 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 extra-oral storage time was too long resulting in complete degradation of human saliva. Secondly, the present study design does not allow to reveal 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. found differences in the rehardening potential of various artificial saliva formulations and water. This study did not use a de- and remineralisation protocol, but focused on the remineralisation of erosively softened enamel (citric acid, pH 2.5, 15 s) after 2 h storage time. All tested artificial saliva solutions resulted in higher rehardening of erosively demineralised enamel than water, but remineralisation 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 (CMC) and mucins.

The different compositions of the artificial salivas might also affect the results of the present study. For instance, the artificial saliva containing CMC showed a lower protective effect after the second compared to the first erosion, probably due to the fact that CMC might form complexes with calcium and/or phosphate ions, which are then not longer available for rehardening of previously eroded enamel.

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

This project was developed as part of PhD Program of first author, with a scholarship by CAPES Foundation, Ministry of Education of Brazil, process number BEX 10470/12-3.
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Table 1 – Composition of tested artificial salivas and degree of saturation with respect to HAP (Hydroxyapatite), OCP (Octacalcium phosphate), DCPD (Dicalcium phosphate dehydrate) according to Shellis.  

<table>
<thead>
<tr>
<th>Compound</th>
<th>Artificial saliva formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>According to Klimek et al. 4</td>
</tr>
<tr>
<td>CaH₂O₆</td>
<td>2 mg/l</td>
</tr>
<tr>
<td>CaH₁₂O₅</td>
<td>30 mg/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>580 mg/l</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>170 mg/l</td>
</tr>
<tr>
<td>KCl</td>
<td>1270 mg/l</td>
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<tr>
<td>NaSCN</td>
<td>160 mg/l</td>
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<tr>
<td>KH₂PO₄</td>
<td>330 mg/l</td>
</tr>
<tr>
<td>CH₃N₂O</td>
<td>200 mg/l</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>340 mg/l</td>
</tr>
<tr>
<td>Mucin</td>
<td>2700 mg/l</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>--</td>
</tr>
<tr>
<td>NaF</td>
<td>--</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>--</td>
</tr>
<tr>
<td>CaH₁₁NO₃</td>
<td>--</td>
</tr>
<tr>
<td>Tris Buffer</td>
<td>--</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>--</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>--</td>
</tr>
<tr>
<td>CaH₂O₃</td>
<td>--</td>
</tr>
<tr>
<td>CMC-Na</td>
<td>--</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>--</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>--</td>
</tr>
<tr>
<td>CaH₁₈N₂O₄S</td>
<td>HEPES</td>
</tr>
<tr>
<td>Deionized water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.4</td>
</tr>
<tr>
<td>HAP</td>
<td>6.51</td>
</tr>
<tr>
<td>OCP</td>
<td>1.57</td>
</tr>
<tr>
<td>DCPD</td>
<td>1.15</td>
</tr>
</tbody>
</table>
Table 2 – Mean (± Standard Deviation) enamel microhardness (KHN) in the respective tested groups initially and after the first and second erosive challenge.

<table>
<thead>
<tr>
<th>Groups / saliva composition</th>
<th>Initial Microhardness</th>
<th>Microhardness after 1st erosion</th>
<th>Microhardness after 2nd erosion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>297.3 ± 22.9&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>247.3 ± 20.7&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>254.8 ± 14.5&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Klimek et al. &lt;sup&gt;4&lt;/sup&gt;</td>
<td>290.2 ± 23.6&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>254.5 ± 31.6&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>251.0 ± 39.2&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vieira et al. &lt;sup&gt;22&lt;/sup&gt;</td>
<td>292.3 ± 30.9&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>241.2 ± 19.2&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>246.9 ± 41.0&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amaechi et al. &lt;sup&gt;40&lt;/sup&gt;</td>
<td>282.9 ± 34.6&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>256.7 ± 24.1&lt;sup&gt;aAB&lt;/sup&gt;</td>
<td>240.1 ± 49.6&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Eisenburger et al. &lt;sup&gt;54&lt;/sup&gt;</td>
<td>291.4 ± 27.8&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>259.0 ± 15.8&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>270.6 ± 30.7&lt;sup&gt;aAB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human saliva</td>
<td>282.5 ± 28.8&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>231.6 ± 34.1&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>245.9 ± 45.1&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>In-situ</td>
<td>275.6 ± 38.3&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>232.2 ± 41.4&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>229.1 ± 33.8&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*In each column, groups followed by the same lower case letters were not significantly different. In each row, groups marked by the same upper case letters were not significantly different.
Table 3 – Mean (± Standard Deviation) enamel and dentin calcium release (µg) in the respective tested groups after the first and second erosive challenge

<table>
<thead>
<tr>
<th>Groups / saliva composition</th>
<th>Enamel</th>
<th>Dentin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 1st erosion</td>
<td>After 2nd erosion</td>
</tr>
<tr>
<td>In-vitro-experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deionized water</td>
<td>2.90 ± 0.06 b,A</td>
<td>3.21 ± 0.56 b,A</td>
</tr>
<tr>
<td>Klimek et al. 4</td>
<td>2.85 ± 0.50 b,A</td>
<td>3.09 ± 0.56 b,A</td>
</tr>
<tr>
<td>Vieira et al. 22</td>
<td>2.74 ± 0.54 b,A</td>
<td>2.96 ± 0.62 b,A</td>
</tr>
<tr>
<td>Amaechi et al. 40</td>
<td>2.78 ± 0.43 b,A</td>
<td>3.29 ± 0.47 b,B</td>
</tr>
<tr>
<td>Eisenburger et al. 54</td>
<td>2.69 ± 0.52 b,A</td>
<td>3.08 ± 0.68 b,A</td>
</tr>
<tr>
<td>Human Saliva</td>
<td>2.55 ± 0.53 b,A</td>
<td>2.76 ± 0.44 b,A</td>
</tr>
<tr>
<td>In-situ-experiment</td>
<td>1.83 ± 0.87 a,A</td>
<td>1.49 ± 0.83 a,A</td>
</tr>
</tbody>
</table>

* In each column, 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 were marked by different upper case letters.
Figure 1 – Flowchart of the experimental set-up