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Abstract

This study analyzed the anti-erosive property of a self-assembling peptide fibre gel. One-hundred-and-twelve bovine enamel samples were ground flat and subjected to a three times de- and remineralization cycle: erosion (5 min; HCl, pH 2.6) alternated with storage in artificial saliva under agitation. Then, samples were covered with different anti-erosive compounds (2 min): Duraphat toothpaste (DT), Elmex Erosion Toothpaste (EET) or Elmex Gelée (EG) - all mixed with saliva (1:3) -, Elmex Erosion Mouthwash (EEM), Curodont Protect (CP; self-assembling-peptide gel) or MI Paste Plus (MIP). Untreated, water stored samples served as control. In experiment 1, half of the samples of each group were continuously superfused with HCl (pH 2.6, 60 µl/min, 8 min). In experiment 2, second half of samples were subjected to 8 cycles, each consisting of application of the respective anti-erosive compound followed by an erosion (60 s HCl, pH 2.6), followed by remineralization in artificial saliva (45 min). Enamel loss was profilometrically determined.

In experiment 1, EEM and EET performed significantly better compared to all other compounds. Substance loss of all other compounds did not differ significantly from control. In experiment 2, significantly better performance was achieved by EEM and EET. EG showed significantly lower protection than the control. All other applied compounds yielded no significant difference compared to control.

Under the chosen conditions, the self-assembling peptide containing compound showed no anti-erosive effect.

Introduction

Several approaches for prevention of erosive lesions have been developed and examined in recent years. In this context several regimes and materials have been tested, like the application of surface resin sealants, highly concentrated fluoride compounds, solutions/toothpastes containing stannous fluoride, other metallic fluoride compounds or stannous chloride and pastes containing casein phosphopeptide amorphous calcium phosphate [LAGERWEIJ et al. 2006, LENNON et al. 2006, WIEGAND et al. 2008, WIEGAND et al. 2009, GANSS et al. 2010, WEGEHAUPT et al. 2012a, WEGEHAUPT et al. 2012b, CECI et al. 2015]. Some of these substances have been shown to be effective in reducing dental hard tissue loss due to erosion. However, their application might be time consuming or technique-sensitive (e.g. surface resin sealants), may lead to tooth surface staining (e.g. stannous compounds or metallic fluorides), may need a high frequency of regular use (e.g. highly concentrated fluorides) or are critical in terms of pH conditions (e.g. titanium tetrafluorides).

Recently, application of self-assembling peptides, such as P_{11}-4, have shown to be able to reduce enamel surface roughening, when used prior to a erosive attacks induced by an acidic beverage [CECI et al. 2016]. In this study, a product containing fibres made up of P_{11}-4 was applied four times on either previously eroded or un-eroded enamel surfaces. The multi-layer three-dimensional peptide scaffold was still visible on a scanning electron microscopic image, performed after a cumulative erosive challenge of 8 min (4 x 2 min). Another recent study could also show an erosive protection on enamel after application of the peptide P_{11}-4, when used in monomeric form [TAKAHASHI et al. 2016].

Aggeli and co-workers developed the class of fibril and fiber formatting self assembling-peptides [Aggeli et al., 1997] and made them useful for clinical applications [AGGELI et al. 1997, MAUDE et al. 2013]. The peptide P_{11}-4 (CH3CO-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH2), which show a net 2- charge, has proved to be non-cytotoxic to human and murine cells, and did not elicit an immunogenic response in mice [SCANLON et al. 2009, MAUDE et al. 2013]. These peptides undergo a self-assembling process and adopt a beta-sheet conformation depending on pH, ionic strength or other favourable conditions [AGGELI et al. 2003b, CARRICK et al. 2007, KNAPMAN et al. 2008, MAUDE et al. 2013, RAVICHANDRAM et al. 2014]. The peptide self-assembly depends on the protonation state of the charged amino acid side-chains. The transition point, at which P_{11}-4 self-assembly occurs in a low ionic strength solution, is at pH=7 [AGGELI et al. 2003b, AGGELI et al. 2003a, CARRICK et al. 2007]. Moreover, increasing the ionic strength of the P_{11}-4-containing solution by the addition of 130 mM NaCl, similar to ionic strength in saliva, has shown to shift the self-assembly point to higher pH values of about pH 12 [CARRICK et al. 2007]. It might by assumed that the impact of ionic strength on fibril formation and their stability might be due to less electrostatic repulsion of deprotonated glutamine (Glu^-) due to the shielding of negative charge by salt ions. Due to these considerations it is assumed that the fibrils of the peptide gel will not de-assembly and that they will be stable under the conditions of the oral cavity, providing a protective surface coating on teeth.

A fusion protein, with the peptide P_{11}-4 as constituent, has shown to be able to bind to hydroxyapatite [WILSHAW et al. 2008, MELCHER et al. 2016]. Thereby, four negatively charged glutamate residues (two of two monomers in the fibril) form a calcium-binding site. It might be assumed that the negative charges on the peptide-fibers might impart

buffering properties to the peptide and thereby attract calcium ions, inducing nucleation processes and precipitation of hydroxyapatite [MELCHER et al. 2016]. Self-assembling peptides, applied as monomeric P_{11}-4, were reported to hinder mild enamel demineralization (i.e. artificial caries) induced by lactic acid and to promote enamel remineralization of artificial and natural subsurface carious lesions [KIRKHAM et al. 2007, BRUNTON et al. 2013, TAKAHASHI et al. 2016, SCHMIDLIN et al. 2016]

Up to now, the above-mentioned study by Ceci et al. [2016] is the only one available, which deals with the impact of self-assembling peptide fibers on the protection of dental erosion. Moreover, there is still a lack of knowledge, how these peptide-fibres behave under clinical conditions, such as highly acidic attacks due to gastric acid or when applied once before an erosive attack.

Thus, the aim of the present study was to examine the anti-erosive properties of the self-assembling peptide fibres made up of P_{11}-4 and other anti-erosive compounds, when applied on eroded enamel in respect to the progression of erosive loss induced by hydrochloric acid, simulating gastric acid.

The null hypothesis of the study was that the protective potential of the peptide-containing product is not different to the other tested compounds.

Materials and methods:

Sample preparation:

For the study, extracted bovine incisors, which were preserved in 0.1% thymol solution, were used for enamel specimens' preparation. A total of 112 cylindrical enamel specimens with a diameter of 3 mm were prepared using a diamond trephine mill. They

were embedded in acrylic resin blocks (6 mm in diameter, Paladur, Heraeus Kulzer GmbH, Hanau, Germany).

The enamel surface was ground with abrasive paper (1200, 2500 and 4000 grit; Gekko-Papier, Struers, Birmensdorf, Switzerland). Thereby the outermost 200 µm of enamel was removed, which was controlled with a micrometer (Mitutoyo, Tokyo, Japan). Grinding was performed in an automatic grinding machine (Tegramin 30, Struers) with running tap water as coolant. Before starting the experimental procedure, all samples were subjected to de- and remineralization cycles. Samples were eroded three times for five minutes by immersion in hydrochloric acid (pH 2.6). After each erosion step, the samples were stored in artificial saliva for remineralization. The artificial saliva for remineralization was prepared following the formulation given formerly [KLIMEK et al. 1982]. In the study, all immersions in HCl and artificial saliva were performed with agitation of the solutions (1 Hz). The hydrochloric acid and the saliva were applied at room temperature of about 22°C.

The enamel samples were divided into seven groups (six experimental groups and one untreated control group). The anti-erosive compounds used in the study are listed in table 1. Half the samples of each group were used for the experiment 1 while the second half was used for experiment 2:

Experiment 1 (8 min continuous erosion): Single application of an anti-erosive compound with a subsequent continuous erosion for 8 min (n = 8) and no remineralization time.

Experiment 2 (eight erosive/remineralizing cycles): Eight cycles, each consisting of a single application of an anti-erosive compound followed by an erosion (60 s), resulting in a total erosion time of 8 min (n = 8).

The set-ups of the experiments are depicted in Fig. 1 and 2.

Treatment of the samples:

All samples of the experimental groups were treated for 2 min with a copious amount (at least 2 mg) of the following products. Samples treated with the toothpastes (DT and EET) and with the fluoride gel (EG) were covered with a slurry, which was prepared from the respective compound and artificial saliva (1:3). The samples allocated to the treatment with Elmex Erosion Mouthwash (EEM) were immersed in 2 ml of the liquid without agitation. The other compounds were directly placed on the enamel samples.

The samples of the control group were stored in tap water for the same time and not treated with any compound.

Experiment 1 (8 min permanent erosion), erosive procedure

Samples were covered with the compounds (2 min), rinsed with tap water (20 s) and distilled water (10 s) to remove loosely attached remnants. Then, the samples were fixed in superfusion chambers, described in detail previously [ATTIN et al. 2013]. Hydrochloric acid (pH 2.6) was pumped with an eight channel peristaltic pump (Ismatec, Glattbrugg, Switzerland) into superfusion chambers, each containing one enamel sample. The acid flowed over the whole enamel surface of 7.07 mm^2. The layer height of the acid was 0.3 mm. The pump output was 60 µl/min. Samples were exposed to the acid for a total of 8 min.

Experiment 2 (eight erosion/remineralization cycles), erosive procedure

The enamel samples were fixed at the bottom of special vials of 4 ml volume and subjected to eight de- and remineralization cycles, as follows: The anti-erosive compounds were applied (2 min), followed by rinsing with tap water (20 s) and distilled water (10 s). Then, the vials were filled with artificial saliva (45 min) for remineralization. The hydrochloric acid (pH 2.6) was applied into the vials for 60 s. This cycle was run for 8 times, summing up for an erosion time of 8 min.

Measurement of substance loss:

Measurement of enamel substance loss was conducted as described earlier [ATTIN et al. 2013]. A stylus profilometer (Perthometer S2/GD 25, Mahr, Göttingen, Germany) placed on a pneumatic stone desk was used. The device is equipped with a custom-made jig for repositioning of samples for successive measurements. Substance loss was calculated based on the differences between pre- and post-treatment profiles with a customized software. Pre-treatment profiles were recorded before application of the respective anti-erosive compound, while the post-treatment profiles were recorded after the erosive procedure. Five profiles were performed on each specimen via scanning from the reference (embedding material) surface to the treated surface. An average of these five readings (µm) was obtained and used for data analysis.

The limit for reliable measurements with the here used set-up for profilometrical determination of dental hard tissue substance loss amounted to 0.1 µm [ATTIN et al. 2009].

Statistical analysis

Significant differences between the individual compounds within each experimental model were assessed by the non-parametric Kruskal-Wallis test. Post-hoc pairwise comparisons were conducted using the Conover-test with Holm-adjustment for multiple testing. In a second step, performance of each compound was compared between the two experimental models using the Wilcoxon rank-sum test. All tests were calculated with the software R [R-CORE-TEAM 2015] and the significance level was set to alpha = 0.05.

Results

For both experiments, statistical analysis led to rejection of the null-hypothesis, having assumed that all compounds were not different in terms of their anti-erosive potential.

Average absolute enamel substance loss ranged from 0.11 to 1.07 µm in experiment 1 and from 0.03 to 1.75 µm, in experiment 2.

Experiment 1 (8 min permanent erosion)

For experiment 1, figure 3 shows the substance loss in the individual groups. Kruskal-Wallis-test revealed significant overall differences between the groups ($p < 0.001$).

Further analysis showed significantly better anti-erosive performance for Elmex Erosion Mouthwash (EEM) and Elmex Erosion Toothpaste (EET), as compared to all other groups. All other groups did not show a significant difference between each other and from the control.

Experiment 2 (eight erosion/remineralization cycles)

For experiment 2, figure 4 gives the relative substance loss for the individual groups. As in experiment 1, Kruskal-Wallis-test showed significant overall differences between the groups ($p < 0.001$).

Further analysis revealed that Elmex Erosion Mouthwash (EEM) and Elmex Erosion Toothpaste (EET) revealed significantly lowest substance loss compared to all other groups, including control. Elmex Gelée (EG) showed significant lower anti-erosive capacity than all other compounds and behaved even worst than the control. The other compounds did not differ from each other and from the control.

Discussion

This study consisted of two experiments. In a first experiment, with a single application of the compounds, the long-term effect of the tested products was evaluated. This procedure has been implemented in various former studies [AYKUT-YETKINER et al. 2013, AZADI-SCHOSSIG et al. 2015]. It has to be reflected that in experiment 1 minor protective impacts of the compounds, possibly existing during the first few minutes of erosion, might be superimposed by severe enamel dissolution taking place during the following minutes of erosion. This effect might blur possibly existing short-term differences in the anti-erosive properties of the compounds. To overcome this conflict, experiment 2 was additionally designed and performed. In experiment 2, the short-term effect of a single application of the compounds on a short-term erosion of 60 s was evaluated. For achievement of an amount of enamel substance loss, which is reliably measurable with the profilometer, application of the compounds followed by erosion was repeated 8 times for a total of 8 min.

An anti-erosive compound should be at least effective in reducing erosive loss during short-term challenges. This was not true for some of the compounds tested. Thus, the clinical relevance of the above mentioned finding is that these compounds are presumably useless under clinical conditions with longer or multiple erosive challenges during a day.

In line with other studies of our working group [AYKUT-YETKINER et al. 2014], remineralization with saliva was included in the experimental protocol.

All tested products were applied according to the manufacturers' instructions except of the toothpastes and the gel, which were not applied simulating toothbrushing.

It should be reflected that application of toothpastes by brushing under simulated clinical conditions, might reduce their anti-erosive and surface protective potential due to the abrasivity of the paste.

Toothpastes and gels are usually used with a toothbrush and are therefore usually diluted with saliva in the oral cavity, as done in the present study. The other compounds were applied undiluted. MI-Paste and Curodont protect are advised to be applied directly on the teeth, either by a dental professional or at home, using a finger tip. Thereafter, they should stay on the teeth for 2 min. The peptide is recommended to apply for 2 min on a cleaned tooth surface. Also, the rinse was applied without a dilution. This was done, since a significant dilution with saliva in the oral cavity is not presumable. The application times of all compounds were oriented to the recommendation given for the peptide gel.

For achieving a flat surface of the bovine samples, needed for reliable profilometry readings, the enamel surfaces were ground. As a positive aspect, this procedure has also led to a removal of the cementum layer, usually existing on bovine enamel surfaces, thus exposing pure bovine prismatic enamel to be eroded. It has to be taken into consideration in how far the absence of aprismatic surface areas and the use of bovine enamel with a different prismatic structure to human enamel, exerted an impact on the adherence of the anti-erosive compounds to the enamel, when compared to clinical conditions of sound and un-eroded teeth. This should be taking into account as the structure-activity relationship of the proposed mechanism of action for the self-assembling peptide fibres strongly depends on the presentation of available binding sites on the surface of the enamel.

It should also be considered that the enamel samples were pre-conditioned in erosion/remineralization cycles, but that no salivary pellicle was formed on the surfaces. The absence of the salivary pellicle might have an impact of the adherence an dthe interaction of the compounds with the enamel surface.

In experiment 1 only the use of stannous-chloride / fluoride-containing mouthwash (Elmex Erosion Mouthwash) and the application of the toothpaste with stannous-chloride / fluoride and chitosan (Elmex Erosion Toothpaste) led to better surface protection against the hydrochloric acid compared to the controls. This finding corroborated the results of previous studies, where these compounds were also efficient as anti-erosive substrates [MAIA et al. 2014, PINI et al. 2016]. For the stannous chloride containing mouthwash it has been proved that its use leads to a protective surface coating on and incorporation into an eroded enamel surface stabilizing the surface [SCHLUETER et al. 2009b].

In experiment 2, the differences between the different compounds were more pronounced, ranging from a nearly complete surface protection (Elmex Erosion Mouthwash and Elmex Erosion Toothpaste) up to the other products showing no protective properties. The model used in experiment 2 as compared to experiment 1 might explain the greater differences between the different compounds in experiment 2. In experiment 2, a more frequent application of the compounds alternating with the erosive challenge was conducted, thus resulting in better interactions of the compounds with the enamel substrate. This feature was shown to be of importance for the interaction of the tin of the stannous chloride containing compounds (Elmex Erosion Mouthwash and Elmex Erosion Toothpaste) with the enamel surface [SCHLUETER et al. 2009a]. In contrast to some previous studies, also performed by our working group, an erosive protection due to the highly concentrated fluoride gel could not be found [ATTIN et al. 1999, LAGERWEIJ et al. 2006]. A rationale explanation for this unexpected outcome is not obvious. Usually, the effect of the used highly concentrated fluoride gel could be attributed to the formation of a protective calcium fluoride-like layer on the enamel surface especially formed considering the acidic pH of the gel (pH 4.8). For manifesting the results of the present study, the experimental procedure with this test group was repeated four times in total, always presenting the same outcome, i.e. no anti-erosive capacity of the gel.

With regard to the casein phosphopeptide-amorphous calcium phosphate / fluoride compound, the results of the present study are in line with previous studies, showing no benefit in terms of erosion protection [WANG et al. 2011, WEGEHAUPT et al. 2012b], although a recent study was able to attribute an anti-erosive property to this product in an erosive/abrasive study design [ALEXANDRIA et al. 2017]. Also for the highly

concentrated fluoride toothpaste (Duraphate Toothpaste), the present studies are not totally in line with previous studies, in which anti-erosive properties have been documented in erosion/abrasion set-ups [ALEXANDRIA et al. 2017]. It is possible that those different outcomes in the studies might be related to the presence or absence of additional extensive brushing of samples in the experiments.

In the present study, we abstained from additional brushing of the samples, in order to get an insight into the pure anti-erosive capacities of the compounds.

The self-assembling peptide was not effective in reducing enamel erosive loss in the present set-up. In a recent study, enamel showed increase in roughness due to erosion with an acidic beverage (Coca Cola) when the self-assembling peptide was applied on intact enamel for 3 min before the erosive attack [CECI et al. 2016]. However, when applied on previously eroded enamel (likewise the present study) and eroded afterwards again, then the enamel surfaces did not show a significant difference in roughness as compared to sound and un-eroded controls. This finding was interpreted as the ability of the self-assembling peptide to offer a degree of protection against enamel erosion [CECI et al. 2016].

The results of the present study do not corroborate this interpretation, but made clear that under the chosen conditions the self-assembling peptide containing compound is less effective in protection against an erosive challenge as compared to some other of the tested anti-erosive compounds. It might be assumed that the self-assembling peptide fibers, as already present in the product Curodont Protect, did not attach properly to the demineralized enamel surface, thus forming no effective physical barrier against the erosive challenge. This might also explain the difference to the outcome of a

recent study, in which the peptide P11-4 was applied in monomeric form, and showing an erosion protective effect [TAKAHASHI et al. 2016].

The manufacturer of the product did not disclose the concentrations of fluoride, calcium and phosphate. Thus, any assumption about the interaction of these ingredients with the enamel surface remains speculative. However, it should be considered that the peptide gel was applied on dried enamel surfaces and stayed there for 2 min without any salivary contact, according to manufacturer instructions. It might be assumed that, under these conditions, a release of fluoride or calcium/phosphate from the gel and interaction with the enamel surface is rather unlikely.

In how far the self-assembling peptide might possibly act as an anti-erosive compound under other experimental set-ups than the one chosen in the present study, has to be further evaluated.

Conflict of interest: Study was partly granted by Credentis, Windisch, Switzerland.

References

Table 1

Anti-erosive compounds used in experiment 1 and 2

Group	Brand name (company)	Active ingredients*
CP	Curodont Protect (Credentis, Windisch, Switzerland)	- 900 ppm fluoride as sodium monofluorophosphate - 0.1% di-calcium-phoshate - 0.028% calcium-glycerophosphate - self-assembling peptide P_{11-4}
DT	Duraphat Toothpaste (Colgate–Palmolive, Hamburg, Germany)	- 5000 ppm fluoride as sodium fluoride
EG	Elmex Gelée (Gaba, Therwil, Switzerland)	- 12500 ppm fluoride as amine fluoride / sodium fluoride
EEM	Elmex Erosion Mouthwash (Gaba, Therwil, Switzerland)	- 800 ppm tin as stannous chloride - 500 ppm fluoride as amine fluoride / sodium fluoride
EET	Elmex Erosion Toothpaste (Gaba, Therwil, Switzerland)	- 3500 ppm tin as stannous chloride - 1400 ppm fluoride as amine fluoride / sodium fluoride - 0.5% chitosan
MIP	MI Paste Plus (GC Corp., Leuven, Belgium)	- 10% casein phosphopeptide-amorphous calcium phosphate - 900 ppm fluoride as sodium fluoride

* as given by manufacturers

Legends

Fig. 1: Set-up of experiment 1, with 8 min continuous erosion.

Fig. 2: Set-up of experiment 1, with eight erosive/remineralizing cycles.

Fig. 3: Substance loss [µm] of the individual groups in experiment 1, given as boxplots, with median, and 25- and 75-percentile, whiskers with the highest and the lowest values within 1.5x interquartile range, and outliers.
Groups indicated with same letters are not statistically significantly different.

Fig. 4: Substance loss [µm] of the individual groups in experiment 2, given as boxplots, with median, and 25- and 75-percentile, whiskers with the highest and the lowest values within 1.5x Interquartile range, and outliers.
Groups indicated with same letters are not statistically significantly different.

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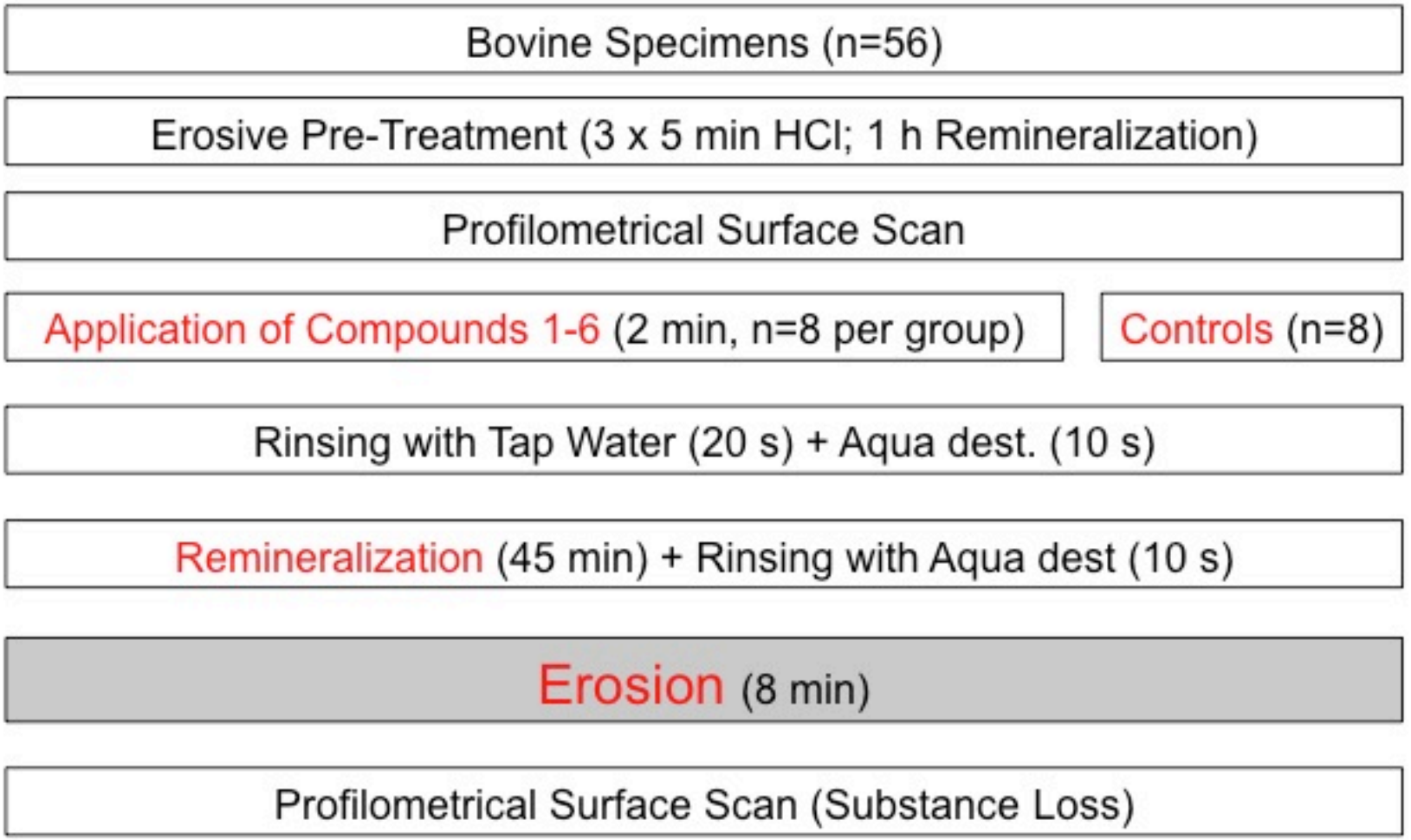
Bovine Specimens (n=56)
Erosive Pre-Treatment (3 x 5 min HCl; 1 h Remineralization)
Profilometrical Surface Scan
Application of Compounds 1-6 (2 min, n=8 per group)
Controls (n=8)
Rinsing with Tap Water (20 s) + Aqua dest. (10 s)
Remineralization (45 min) + Rinsing with Aqua dest (10 s)
Erosion (8 min)
Profilometrical Surface Scan (Substance Loss)

Figure 1

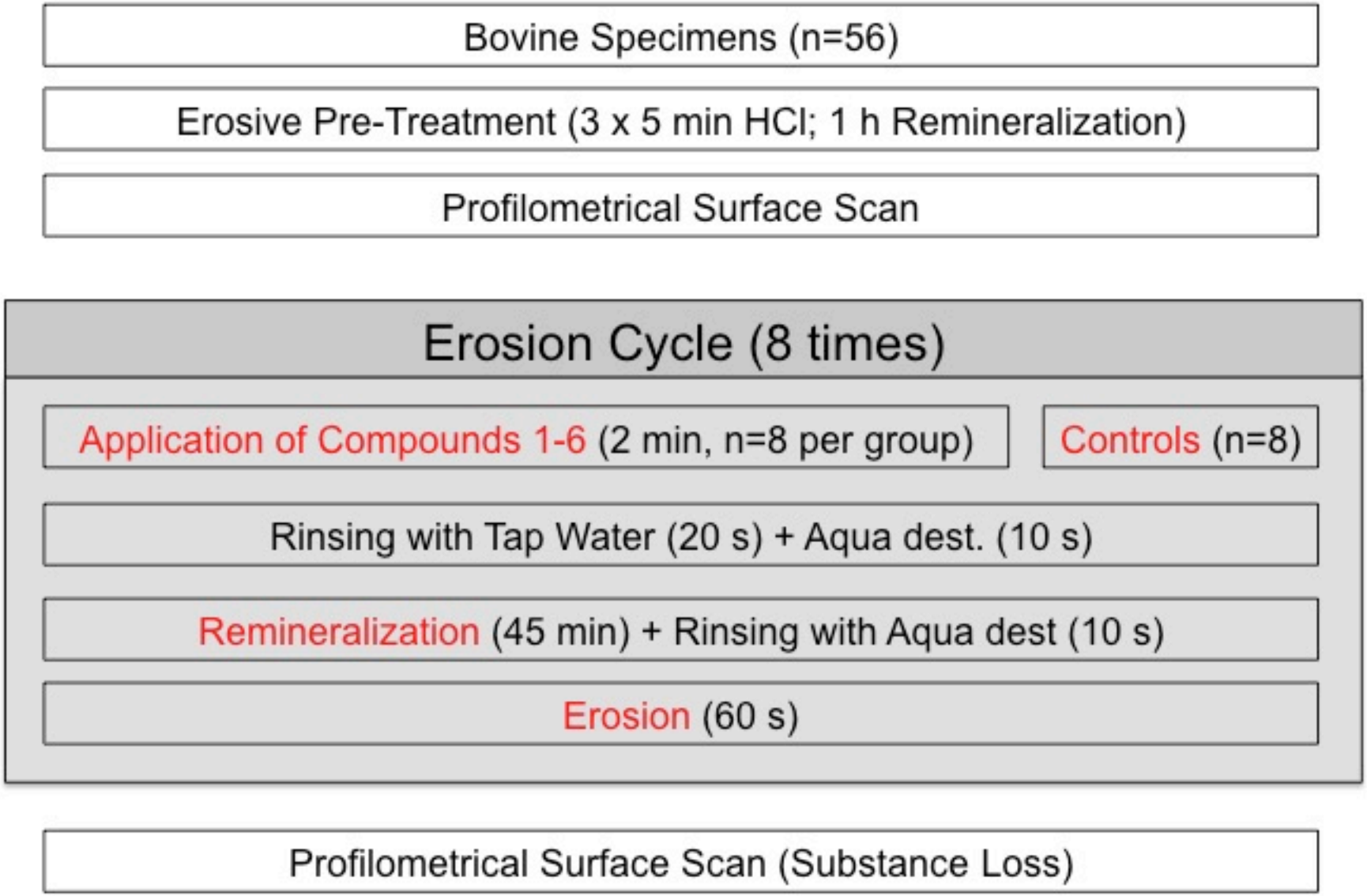
Bovine Specimens (n=56)
Erosive Pre-Treatment (3 x 5 min HCl; 1 h Remineralization)
Profilometrical Surface Scan
Erosion Cycle (8 times)
Application of Compounds 1-6 (2 min, n=8 per group)
Controls (n=8)
Rinsing with Tap Water (20 s) + Aqua dest. (10 s)
Remineralization (45 min) + Rinsing with Aqua dest (10 s)
Erosion (60 s)
Profilometrical Surface Scan (Substance Loss)

Figure 2

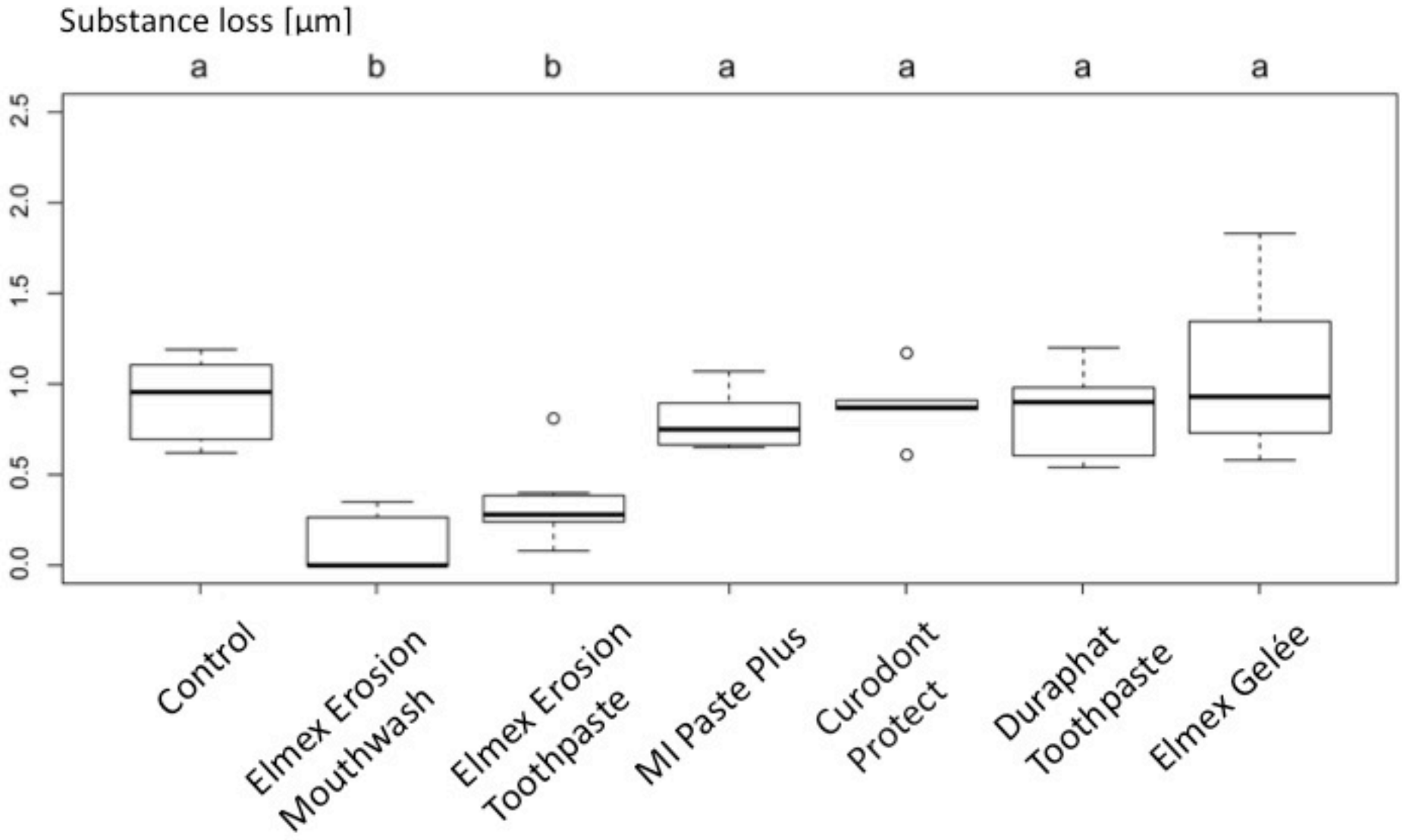
Substance loss [µm]
a
b
b
a
a
a
a
0.0
0.5
1.0
1.5
2.0
2.5
Control
Elmex Erosion Mouthwash
Elmex Erosion Toothpaste
MI Paste Plus
Curodont Protect
Duraphat Toothpaste
Elmex Gelée

Figure 3

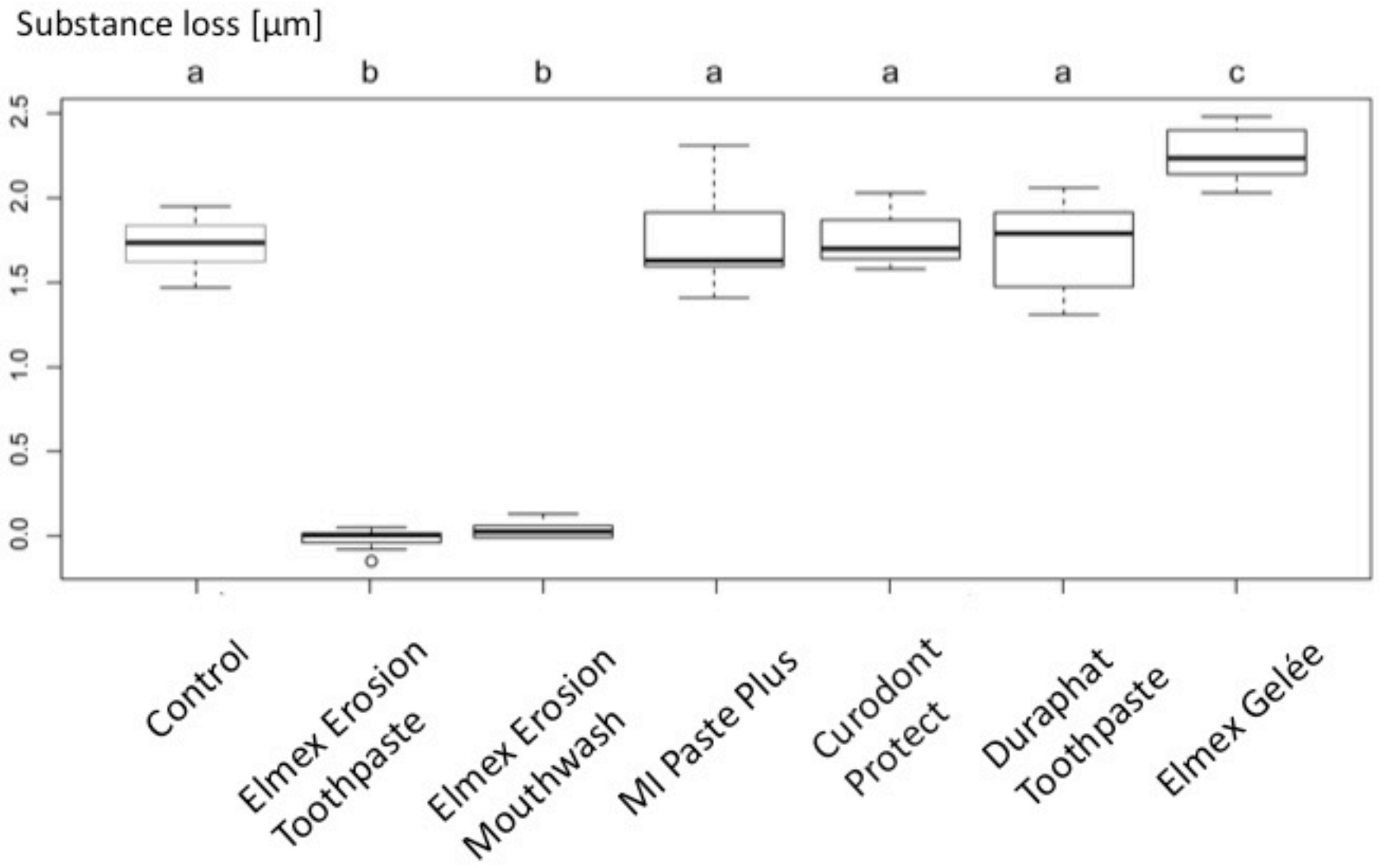
Substance loss [µm]
a
b
b
a
a
a
c
0.0
0.5
1.0
1.5
2.0
2.5
Control
Elmex Erosion Toothpaste
Elmex Erosion Mouthwash
MI Paste Plus
Curodont Protect
Duraphat Toothpaste
Elmex Gelée

Figure 4