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Abstract

Objective: This *in situ* study aimed to evaluate the protective effect of Tooth Mousse (GC) containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on human enamel erosion and to compare the difference in erosion between the anteriorly and posteriorly positioned human enamel.

Methods: This study used a 2-treatment (7 days each) crossover design with 12 healthy volunteers wearing intraoral appliances. Each appliance contained 4 human enamel specimens positioned on the buccal surfaces of the volunteers' maxillary central incisors and first molars. The specimens were intraorally treated with CPP-ACP paste or deionized water (control) for 3 min and then exposed to *in vivo* acid attacks by rinsing with 150 ml of a cola drink (4 x 5 min/day). The surface microhardness (SMH) of the specimens was measured and used to calculate the percentage of SMH loss ($\%SMH_l$). Erosion effect on enamel was also investigated by scanning electron microscopy (n = 4) at the end of study. The data were statistically analysed using two-way analysis of variance (ANOVA) and Tukey's test at a level of $P<0.05$.

Results: A significant decrease in $\%SMH_l$ was observed for the specimens of CPP-ACP group compared to that for the controls (P = 0.007). The specimens positioned posteriorly exhibited a significantly lower $\%SMH_l$ than those positioned anteriorly (P=0.033). Samples of CPP-ACP group showed fewer etching patterns than those of the control group.

Conclusions: In this *in situ* model, application of Tooth Mousse containing CPP-ACP before erosion reduced the $\%SMH_l$ of human enamel. Enamel located in different positions showed different patterns of erosion.

Clinical significance: application of Tooth Mousse containing CPP-ACP could be considered as a suitable preventive strategy against enamel erosion.

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1. Introduction

Dental erosion is defined as the loss of tooth substance caused by acids without the involvement of microorganisms [1-3]. The increasing prevalence of dental erosion worldwide has led to increased attention from both clinicians and researchers [4, 5]. Dental erosion is considered a multifactorial disease, and its increased prevalence observed in recent years has been related to the increased consumption of soft drinks [6, 7].

Currently, strategies for preventing dental erosion include fluoride application, and modification of acidic beverage, and laser irridation, etc. [8-12]. Given that most fluoridated products show only a slight preventive effect against erosion [10], extensive attempts have been made to seek alternative preventive methods. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) which has shown anti-caries potential [13, 14], has been recently considered as a potential candidate for erosion prevention [15]. However, the current literature offers contradictory findings regarding the erosion-inhibiting effects of CPP-ACP on dental enamel. Previous *in situ* studies showed a greater increase in surface microhardness (SMH) of previously eroded enamel after use of chewing gum containing 1% CPP-ACP compared to a conventional chewing gum [16, 17]. Manton et al. [18] reported that the erosivity of soft drinks after the addition of 0.2% CPP-ACP was similar to that of distilled water. Ranjitkar et al. [19] reported that the application of 10% CPP-ACP paste reduced enamel and dentin wear due to erosion and abrasion.

Similar findings were also reported by an *in vitro* study using atomic force microscopy and scanning electron microscopy [20]. Despite these positive results, some *in situ* and *in vitro* investigations found no positive effect of CPP-ACP on dental erosion [16, 21-24]. The differences in the study designs (*in vitro*, *in situ / ex vivo*, *in situ* / *in vivo*) could account, at least in part, for the discrepancy in the abovementioned reports.

The effects of test products against erosion should ideally be investigated using *in vivo* models. However, measurement of the erosive outcomes *in vivo* with acceptable accuracy is difficult. Therefore, *in vitro* and *in situ* studies with standardized conditions have been advocated for testing the effectiveness of preventive strategies against dental erosion [25]. Although most of the published studies were conducted *in vitro*, *in vitro* models cannot replicate the oral environment and can overestimate erosive changes by approximately 10-fold [26, 27]. *In situ* models allow erosion changes to be measured over time in a natural environment of saliva and pellicle. In previous *in situ* studies, specimens were eroded either *in vivo* (intraorally) or *ex vivo* (extraorally). To our knowledge, the best available method to simulate the clinical environment is an *in situ* study with *in vivo* erosion since the natural oral environment offers extra resistance to erosion compared to only the presence of the applied pellicle. A recently published study reported that surface roughness and SMH of specimens eroded *in vivo* were significantly different from those eroded *ex vivo* for 45 min [26]. However, the effects of CPP-ACP on dental erosion have

not yet been investigated using *in situ* / *in vivo* models. Moreover, dental erosion is proven to have distinctive characteristics in location based on clinical evaluation. As for intrinsic erosion, erosion is generally present on the palatal surface of the maxillary anterior teeth and occlusal surface of the mandibular posterior teeth. With regards to extrinsic erosion, the affected areas are often observed on the labial surface of anterior teeth and occlusal surfaces of the mandibular posterior teeth. [1] However, no *in situ* model has been designed to evaluate the effects of location on the erosion susceptibility of human enamel.

Thus, the aim of this *in situ* study was to determine the effects of Tooth Mousse containing CPP-ACP on human enamel subjected to *in vivo* erosion and to compare the difference in erosion between the anteriorly and posteriorly positioned human enamel. The following null hypotheses were tested: 1) the application of Tooth Mousse containing CPP-ACP would not affect the erosion susceptibility of human enamel; 2) the location of samples would not affect the erosion susceptibility of human enamel.

2. Methods and materials

This study was a single-blinded, controlled, randomized, two-treatment crossover *in situ* study, in accordance with the consolidated standards of reporting trials (CONSORT) guidelines. The overall observation period was 2 x 7 days with a washout period of 7 days. The protocol was approved by the

Ethics Committee of the local university (ref 16-FMUSS-L81) and was registered under NCT03426150. The present study was performed following the Declaration of Helsinki and the Guidelines of Good Clinical Practice. The study was conducted at the Hospital of Stomatology of the local university from March 2017 to October 2017.

2.1. Inclusion and exclusion criteria

Healthy volunteers were examined and recruited based on the following inclusion criteria: (a) at least 18 years old and in good general health, (b) absence of caries, periodontal disease, and erosion, and (c) stimulated and unstimulated physio-logical saliva flow rates >1 mL/min and >0.25 mL/min, respectively. The following exclusion criteria were applied: (a) systemic diseases or oral mucosal disorders, (b) fixed or removable orthodontic appliances or removable prostheses, (c) pregnancy, (d) known allergies to the experimental drink, (e) history of gastric regurgitation or any current use of medication causing gastric reflux or xerostomia, and (f) any condition that precluded consumption of 600 ml of cola drinks per day for 7 consecutive days.

At the screening visit, the selected volunteers gave their written informed consent to participate in the study.

2.2. Specimen preparation

Enamel blocks were prepared from extracted non-carious human third molars from 18- to 35-year-old subjects of either gender [28]. After extraction,

the teeth were stored in sealed containers with 0.1% thymol solution. Buccal enamel blocks (3 mm x 3 mm) were cut with a low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) under water cooling. The enamel blocks were further embedded with acrylic resin (Paladur, Heraeus Kulzer, Germany) using a custom-made silicone mould. The enamel surfaces were ground flat and polished using a series of carborundum discs (600#, 1200#, 2400#, and 4000#; Buehler) under water cooling. The specimens were cleaned in an ultrasound bath for 10 min after polishing. The final dimensions of the specimens were as follows: top surface 3 mm x 3 mm, bottom surface 4 mm x 4 mm, and thickness 2.2 mm. The absence of white spots and cracks on the enamel surface was confirmed with a stereomicroscope (MM400, Nikon, Tokyo, Japan). The SMH of the samples was measured using a Vickers microhardness tester (HXD-1000 TMC, Shanghai Taiming Optics Ltd., Shanghai, China) with a 50-g load and 15-s dwell time. Only samples with SMH values ranged from 310 to 360 VHN were included in the study. Prior to the *in situ* experiment, the specimens were stored in aqueous thymol solution for 2 weeks and 70% ethanol for 30 min for disinfection [29, 30].

2.3. Intraoral appliance

Maxillary and mandibular impressions of each participant were taken using alginate materials (Jeltrate Alginate, Dentsply Detrey GmbH, Konstanz, Germany). The impressions were poured with dental hard stone (Heraeus, Hanau, Germany). The lower mouthguard was fabricated with a 0.035-in-thick

soft-tray sheet (Ultradent Products Inc., South Jordan, UT, USA) and a heat/vacuum tray-forming machine (Ultraform, Ultradent Products Inc). The upper intraoral appliance was prepared in a similar manner but with 4 niches (slots) on the buccal surfaces of the central incisors and first molars (2 on the left and 2 on the right). An opening of 3 mm x 3 mm was made on each niche with a sharp scalpel (Fig. 1). The specimens were then fixed in the 4 niches with sticky wax approximately 0.5 mm beneath the appliance surface to avoid any abrasion from the buccal mucosa and tongue. The intraoral appliance was designed to protect the volunteers' natural dentition and expose the enamel surfaces of the specimens to *in vivo* acid challenges.

2.4. In situ experiment

The study used a crossover design with 2 treatment periods of 7 days each. The sequence of treatment (CPP-ACP group and control group) was randomly assigned to each volunteer. Seven days prior to and during the experimental period, the volunteers were trained to brush their teeth with a standard manual toothbrush (Crest, Procter & Gamble, Blue Ash, OH, USA) and fluoride toothpaste (Crest anti-cavity; Procter & Gamble) twice daily using a modified Bass method. The volunteers wore the upper and lower appliances and performed the *in situ* experiment under supervision between 8 a.m. and 5 p.m. in the Clinical Research Centre of the local university. During the experimental period, participants were not permitted to leave the study site.

The appliances were removed for 1 h between 11:30 a.m. and 12:30 p.m. to allow for lunch.

At the beginning of each experimental day, the intraoral appliances with samples were inserted and worn for 2 h to allow for formation of an acquired salivary pellicle on the enamel surfaces. After being dried briefly, the specimens were then intraorally treated by topical application of a fluoride-free commercial available product containing 10% CPP-ACP (Tooth Mousse, GC, Tokyo, Japan) or deionized water (control) for 3 min. Topical application of test solutions was performed with a microbrush through the openings of the niches in the appliance by a study coordinator. After rinsing with 20 ml of deionized water for 10 s, appliances were worn for 30 min, followed by a daily *in vivo* erosive challenge. The erosive challenge consisted of rinsing with cola drinks for 5 min, 4 times per day for 7 consecutive days (Fig. 2). The volunteers were instructed to rinse with 150 ml of freshly opened cola drinks (Coca-Cola, Xiamen, China) for 5 min at short intervals and then expectorated the rinse. No other food or drink was permitted while the appliance was in place. Appliances were worn for 1 h between each erosive challenge [25].

At the beginning and end of each experimental day, the appliances containing specimens were immersed in 0.2% chlorhexidine solution for 60 s and rinsed with deionized water to prevent microbial colonization on the enamel surfaces [31]. At the end of each experimental day, the appliances were placed in sealed containers in 100% humidity overnight.

2.5. Pilot study and sample size calculation

A pilot *in situ* study with 4 volunteers (2 males and 2 females, mean age 25.8 years) was performed to test the efficacy and safety of the current *in situ* regimen. The SMH were measured at baseline and at the end of study. The data was used for further sample size calculation. The sample size was calculated to obtain a difference in SMH loss of 15% between the 2 groups at the end of the study. A sample size of 11 volunteers was estimated based on a power analysis with 80% power at an alpha level of 0.05 for a two-tailed test. Assuming a dropout rate of 10%, 12 volunteers were selected for the current study.

After cleaning and drying, the central incisors and first molars of the volunteers were isolated with light body silicone material (Express XT light body, 3M ESPE, USA) to obtain replicas of the buccal surfaces. The replicas, made at the baseline and the end of study, were then analysed using a Supra 50 VP ESEM scanning electron microscope (Carl Zeiss NTS, Oberkochen, Germany) with an acceleration voltage of 10 kV at 5,000x magnification. This was done to detect the surface morphology changes of the participants' natural teeth after the *in situ* experiment.

2.6. Surface microhardness measurement

SMH values of the samples were measured at baseline and at the end of the experiment. Three indentations at intervals of 0.1 mm were made in the

centre of the sample surface. The mean SMH values were then used to calculate the percentage of SMH loss ($\%SMH_l$) using the following equation:

$$\%SMH_l=[(SMH_b – SMH_e)/ SMH_b] \times 100$$

where SMH_b is the SMH at baseline, and SMH_e is the SMH at the end of the experiment.

2.7. Surface morphology analysis

At the end of the study, 4 specimens (2 specimens located anteriorly and 2 located posteriorly) were randomly selected from each group for scanning electron microscope observation as described above.

2.8. Statistical analysis

The assumptions of equality of variances and normal distribution of errors were confirmed using the Levene test and the Kolmogorov-Smirnov test, respectively. Two-way analysis of variances (ANOVA) was used to evaluate the effects of CPP-ACP application and the sample locations and their interactions on the $\%SMH_l$. Tukey's post hoc test was performed for post hoc comparisons. The data were analysed using the SPSS statistical software package (SPSS 19.0 for Windows, SPSS, Chicago, IL, USA). All statistical analyses were performed at a significance level of 0.05.

3. Results

Similar surface morphology of the participants' replicas was observed at baseline and at the end of the study, indicating that the natural tooth surfaces remained unchanged during this *in situ* experiment.

Twelve healthy adult volunteers (mean age: 26.7 years; 5 males, 7 females) who fulfilled the inclusion criteria were recruited, and all the volunteers completed the study. A total of 96 specimens with a mean SMH value of 336.44±27.29 VHN were selected and assigned to different groups. During the study period, no specimen was lost, and no significant adverse reactions were reported by the participants.

Significant decreases in SMH were observed in both groups at the end of the study. The $\%SMH_l$ of the specimens in different locations ranged from 35.60 to 60.19 (Table 1). Significant effects were found for both surface treatment and specimen location (P = 0.007 and 0.033, respectively), but no significant interaction was found (P = 0.176). Application of Tooth Mousse containing CPP-ACP significantly enhanced the acid resistance of human enamel compared to that of the control group (38.06±5.68% vs. 52.39±10.85%). Specimens located posteriorly exhibited a significantly lower $\%SMH_l$ than those located anteriorly.

Although similar etching patterns were observed between the 2 groups, samples from the control group showed relatively greater demineralization, especially on the interprismatic portion, compared with those from the

CPP-ACP group. Samples located posteriorly revealed slightly smoother surfaces than those located anteriorly (Fig. 3).

4. Discussion

Based on the present findings, the null hypotheses that the application of Tooth Mousse containing CPP-ACP would not affect the erosion susceptibility of human enamel and that the location of specimens would not affect the erosion susceptibility of human enamel were rejected.

This study used an improved *in situ* model and was performed under strict supervision during the working hours so that the compliance of the participants could be monitored by the study coordinators. Although most published *in situ* studies have been performed using *ex vivo* erosion, the *ex vivo* erosive challenge is not fully comparable to an *in vivo* challenge because interactions between the acid and oral environment (e.g., temperature, saliva buffering) are absent. However, in the literature, only few *in situ* studies adopted *in vivo* erosion models using either palatal [28] or lower appliances [26]. The current *in situ* design not only exposed the samples to *in vivo* erosion but also protected the participants' natural teeth (as confirmed by the scanning electron microscope images of the replicas). Moreover, it enables comparisons between samples positioned in different locations. It would be theoretically possible to perform future *in situ* studies on susceptible individuals subjected to an *in vivo* erosive challenge using the present regimen. The Tooth mousse

was applied according to the manufacturer's recommendation. The 5-min erosive challenge was chosen in accordance with previous *in situ* studies [26, 32, 33]. Additionally, the participants were requested to rinse and expectorate the acidic drinks because swallowing the acidic drinks may lead to adverse events [25]. Although the current *in vivo* challenge seems irrelevant from a clinical standpoint, the actual or simulated drinking failed to provide detectable erosion effects during a reasonable period of time in our preliminary studies. Future studies with improved *in vivo* erosion protocols are needed to provide useful results.

Recently, CPP-ACP has been investigated as an alternative preventive strategy against dental erosion. However, limited information is available regarding the erosion-inhibiting potential of CPP-ACP under *in situ* / *in vivo* studies. Consistent with previous studies [19, 34], the application of Tooth Mousse containing CPP-ACP reduced surface softening and morphology change of dental enamel subjected to erosion. Although the mechanism by which CPP-ACP prohibits enamel erosion remains unclear, it has been suggested that CPP-ACP can form micelles on the tooth surface, which might restrict the acid reaction and inhibit calcium loss [32]. Poggio et al. [20] concluded that CPP-ACP paste created a layer that filled the interprism cavities and partially covered the prisms to protect against acid challenges. Similarly, fewer etching patterns, especially on the interprism portion, were found on the specimens treated with Tooth Mousse containing CPP-ACP in

the present study. As confirmed by transmission electron microscopy, after CPP-ACP application, the modified pellicle had greater electron density than the control [35]. Moreover, precipitates were evident on the enamel surface after the application of CPP-ACP paste in a previous study [22]. However, although the specimens of CPP-ACP group did show relatively less demineralization than those of control group, no precipitates were found on the enamel surface at the end of the present *in situ* experiment. This may indicate that the protective barrier after CPP-ACP application is only capable of providing protection against acidic attacks for a certain period of time. Importantly, studies have reported that CPP-ACP has no protective potential against enamel erosion [16, 21-23]. Conceivably, the large variation reported is, at least to some extent, related to the variations in the concentration of CPP-ACP (0.2%-10%), the vehicles (paste, solution, chewing gum, etc.), and the study designs. More specifically, the possible interaction of CPP-ACP with pellicle components and the salivary dilution of acidic drinks could serve as possible explanations [35, 36].

Despite the protective effects of CPP-ACP on enamel erosion, significant variation in erosion was found between the specimens positioned in the anterior region (buccal surfaces of central incisors) and the posterior region (buccal surfaces of first molars). Samples located anteriorly exhibited relatively smoother surface than those located posteriorly, which is consistent with a previous study using the palatal appliances [28]. This finding may help explain

the variation in the severity of dental erosion in different teeth. A possible explanation could be a rapid pH recovery in the posterior region compared to the anterior region after consuming acidic drinks [37, 38].

A limitation of the present study is that the enamel samples were ground and polished for SMH measurements. Thus, the current findings should be interpreted with caution since the native enamel provides innate resistance to erosive attacks [39]. Additionally, although CPP-ACP is claimed to be the only active agent of Tooth Mousse tested, the protective effects against erosion may not be solely related to CPP-ACP since the distilled water was used as a control. Future clinical studies with appropriate placebo are needed to clarify this issue.

5. Conclusion

Within the limitations of this *in situ* study, the following conclusions can be drawn:

1. Application of Tooth Mousse containing CPP-ACP effectively reduced the surface softening and morphology changes of human enamel after erosion.

2. Specimens located in the anterior region had greater erosion susceptibility than those located in the posterior region.

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Table 1: Means and standard deviations of $\%SMH_l$ at different specimen locations in the two groups.

Group	Location	$\%SMH_l$
CPP-ACP	Anterior	39.95 (8.25) [a]
	Posterior	35.60 (2.59) [a]
Control	Anterior	60.19 (7.22) [b]
	Posterior	43.91 (8.12) [a]

Different superscripted letters indicate significant differences.

Figure Legends

Figure 1. Intraoral appliances containing 4 specimens. Each niche has 1 opening for acid exposure, and the specimen was fixed 0.5 mm beneath the appliance surface to prevent abrasion from the tongue and oral mucosa.

Figure 2. Flow chart of daily erosion cycles.

Figure 3. Representative scanning electron microscopy images of the enamel specimens at different time points (5,000X magnification): A. specimen located anteriorly (CPP-ACP group); B. specimen located anteriorly (control group); C. specimen located posteriorly (CPP-ACP group); D. specimen located posteriorly (control group).