SCANNING VOL. 37, 73–81 (2015)

Interproximal Enamel Reduction: An *In Vivo* Study

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Summary: The study aimed to investigate the morphology and composition of the interproximal reduced enamel after exposition to saliva and casein phosphopeptide amorphous calcium phosphate with sodium fluoride (CPP-ACPF). Fourteen patients undergoing an orthodontic treatment with 4 premolars extractions participated to the study. Interproximal enamel reduction (IER) was performed on mesial surfaces of 3 extractive premolars for each patient while 1 served as untreated control. Premolars were assigned to 4 groups: No-S group, sound enamel as control; S-Ex group, stripped and immediately extracted enamel; S-Sal group, stripped and exposed to saliva enamel; S-CPP group, stripped enamel treated with CPP-ACPF. Teeth were extracted at different times, depending on the group they were assigned to and sliced into mesial and distal halves. Mesial surfaces were subjected to environmental scanning electron microscopy with energy dispersive X-ray spectrometry (ESEM/EDX) and to scanning electron microscopy (SEM) analysis. ESEM/EDX investigations showed no statistically significant differences in the content of calcium and phosphate between the 4 groups. SEM observations showed no difference in the morphological appearance of stripped enamel after 30 days of exposure to saliva and CPP-ACPF. Saliva and CPP-ACPF effects on stripped enamel *in vivo* showed no difference after 30 days. SCANNING 37:73–81, 2015.

Key words: stripping, ESEM/EDX, SEM, saliva, orthodontics

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Received 24 September 2014; Accepted with revision 31 October 2014

DOI: 10.1002/sca.21181
Published online 8 December 2014 in Wiley Online Library (wileyonlinelibrary.com).

Introduction

Interproximal enamel reduction (IER) is a common orthodontic clinical procedure for correction of tooth size discrepancies, crowding and misalignment (Livas *et al.* 2013).

Qualitative Scanning Electron Microscopy (SEM) evaluations showed that all the IER methods affect enamel morphology, leaving furrows and scratches and produce a significantly rougher surface compared to intact enamel (Zachrisson *et al*. 2007, 2011; Koretsi *et al*. 2014).

Although these irregularities might facilitate plaque and bacteria retention and abraded enamel appears to establish a favorable environment for caries development, many follow up studies indicated that there is not a significant clinical relationship between IER procedures and the increased susceptibility to caries (Zachrisson *et al.* 2007, 2011; Koretsi *et al.* 2014) and that it is possible to prevent mineral loss and promote remineralization (Alessandri Bonetti *et al*. 2009, 2014; Uysal *et al.* 2010).

Approaches to reducing the incidence of demineralization during orthodontic treatment have either involved decreasing the amount of plaque through rigorous oral hygiene regimens or tackling the susceptibility of enamel to demineralization with fluoride application in various forms, enamel sealants, glass ionomer cement for bonding brackets and modified appliance designs (Uysal *et al.* 2010; Zanarini *et al.* 2012; Alessandri Bonetti *et al.* 2014).

Recently, it has been suggested that the addition of sodium fluoride to the casein phosphopeptide amorphous calcium phosphate complex (CPP-ACPF) seemed to show an increased remineralization, promoting the formation of fluorapatite or fluorhydroxyapatite within the lesion in presence of bio-available calcium and phosphate ions (Oshiro *et al*. 2007; Rahiotis *et al.* 2008; Robertson *et al.* 2011).

So far, the use of CPP-ACPF has mainly been reported in *in vitro* experiments (Oshiro *et al.* 2007;

Alessandri Bonetti *et al*. 2009; Hegde and Moany 2012) but they were very limited in assessing clinical effectiveness because they are not able to reproduce the many variables that are present in individual patients such as salivary flow and constituents, dietary intake, temperature changes.

The aim of the present case-control *in vivo* study was to evaluate by qualitative (SEM) and quantitative (Environmental Scanning Electron Microscope/Energy Dispersive X-ray or ESEM/EDX) analysis the healing efficacy of CPP-ACPF paste versus saliva on the stripped enamel.

Materials and Methods

Subjects Recruitment

Fourteen patients (mean age: 15.0 ± 1.7 years) referred to the Orthodontics Department of the Universities of Bologna and Brescia, scheduled to have 4 first premolars extracted as part of their orthodontic treatment, were recruited. The present protocol was approved by the local Institutional Review Board and parents signed an adequate informed consent.

The inclusion criteria were: good oral hygiene, in terms of plaque index scores (O'Leary *et al*. '72), normal salivary parameters (in terms of flow rate >1.0 mL/min, buffer capacity with final pH: 6.8–7.5, checked by GC Saliva-Check Kit - GC Corp. Belgium) and compliance on paste application in the investigator's opinion, checked by questions at each visit and on the basis of personality characteristics (Bos *et al*. 2003).

Exclusion criteria were: a current orthodontic therapy; the presence of caries, white spots, fluorosis, cracks, fillings; any systemic disease or use of any drugs that could affect salivary composition and flow; allergy/intolerance to dairy products; no compliance on paste application.

Each patient underwent a randomized split mouth design. An envelope, containing the number of the group to which it was assigned, was given to each tooth and a blind operator (EP) chose the envelope.

Interproximal Enamel Reduction

One premolar was extracted before any treatment, representing the control of sound enamel (No-S group).

The remaining 3 premolars underwent IER procedures and were extracted at different times, depending on the group they were assigned to:

- one premolar was extracted immediately after IER procedures (S-Ex group);

- another one was extracted 30 days after IER, representing the sample exposed to saliva in the oral environment (S-Sal group);

- the last extractive element was subjected to IER procedures after the extractions of the previous premolars and MI Paste PlusTM containing CPP-ACPF (GC Europe, N.V. Leuven, Belgium) was then applicated for 30 days before being extracted (S-CPP group).

IER was performed for each patient with a cilindric bur with medium diamond grit (80 μm) (Komet, Lemgo, Germany) used at medium speed (about 30,000 rpm); polishing was made with fine Sof-Lex abrasive hand held strips (3M-ESPE, Seefeld, Germany). Posterior straight elastic separators were positioned 1 week before, to improve the access to the interproximal surfaces. The diameter at the tip of the bur served as a feeler of the amount of IER.

CPP-ACPF Application

During the experimental period, patients were instructed to smear a pea-size amount of MI Paste PlusTM using a fluoride-tray to facilitate and standardize the application. The treatment regimen of 2 min twice-daily application was employed following the manufacturer's recommendations. Subjects were asked not to rinse afterwards and not to introduce soft erosive drinks and acidic foods after the application of the product.

Samples Preparation

All the 56 extracted teeth were thoroughly cleaned from debris and soft tissues. They were sliced buccolingually into mesial and distal halves using a diamond disk bur. While the distal unstripped surfaces of each tooth were set apart, the mesial ones were collected and then conserved and fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer solution at 4°C, dehydrated in graded concentration alcohol and air dried.

In all, 56 surfaces represented the sample of this study. As already specified, they were divided into 4 groups of 14 specimens:

No-S group, sound enamel as control;

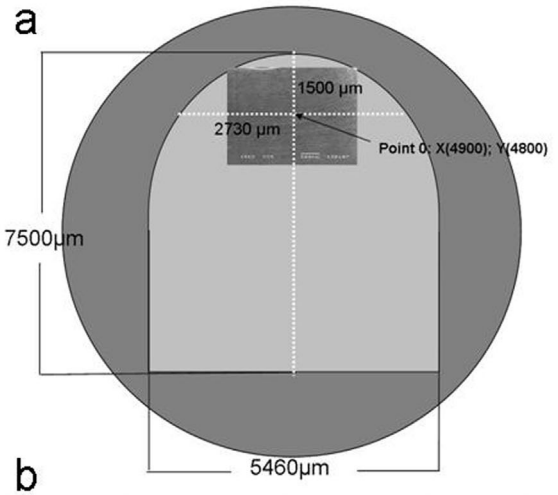
S-Ex group, stripped and immediately extracted enamel;

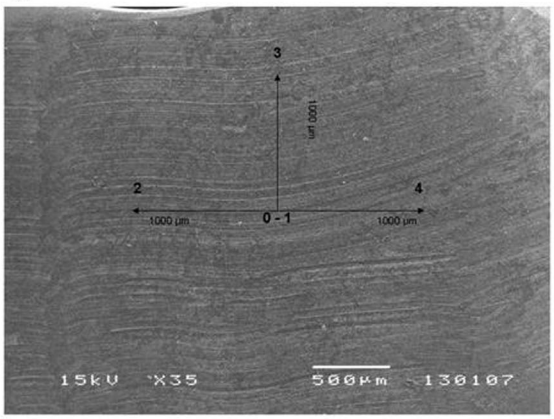
S-Sal group, stripped and exposed to saliva enamel;

S-CPP group: stripped enamel treated with CPP-ACPF.

ESEM/EDX

As the primary objective of the study was to investigate the components of enamel, in particular Calcium/Phosphorous ratio (Ca/P) as an indication of





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Standardized Micrometric Analisys

	name of analisys:	CPP-ACPF Paste on stripped enamel		
	specimen:	A3a	**group**	1
	date:	19.11.2012	KV =	15
			WD =	20
point 0:	magnification:	X35	TILT =	0
	R =	45°		
	X =	4900	Y =	4800
	n. photo	120803		

point	Y	X	n. photo	magnification
1	4800	4900	120804	500X
2	3800	4900	120805	500X
3	4800	5900	120807	500X
4	5800	4900	120806	500X
5				
6				

NOTE:

group 1 = sound enamel
group 2 = stripped and immediately extracted enamel
group 3 = stripped and exposed to saliva enamel
group 4 = stripped and treated with MIPaste enamel

Fig. 1. a) For each sample a Point 0, from which all the analysis started, has been identified and it has the following coordinates: Width (y): the center of the sample horizontally; Height (x): 1500 µm below the occlusal surface of sample. b) A 35X image made at point 0 identified the observation window (3470 × 2600 µm) for subsequent analysis. A 500X image has been acquired at the same coordinates (Point 1). Another 500X images were made at Points 2, 3, 4, identified as: Point 2, 1000 µm left from the Point 0, varying the y and keeping the x; Point 3, 1000 µm up from the Point 0, varying the x and keeping the y; Point 4, 1000 µm right from the Point 0, varying the y keeping the x. c) The Standardized Micrometric Analysis for each sample was recorded to allow repeatability in finding the same observation fields.

mineralization of dental tissue, semi-quantitative investigations by ESEM (Quanta 200, FEI-Hillsboro, Oregon)/EDX (INCA 350, Oxford Instruments, Tubney Woods, Abingdon, Oxfordshire, UK) were made; it allowed also to analyze the quantity of Carbon (C) as a component of organic matter and therefore bacteria adhering to the enamel.

The investigations were made at two different powers: 10 KeV to investigate the composition on the surface portion and 20 KeV for the analysis of the deeper underlying intact area.

SEM

Subsequently specimens were gold-palladium sputter coated (Quorum-Emitech Sc 7620 Ashford, UK) for qualitative analysis by SEM (JSM-5200; JEOL, Tokyo, Japan).

A systematic method (Marchionni *et al*. 2010) providing 4 records on predetermined points on the surface of enamel sample (as shown in Fig. 1) has been adopted for SEM and ESEM/EDX images observation and interpretation, allowing 100% repeatability in finding the same observation fields. On these points, a morphological analysis was made at 500X, 1000X and 2000X.

To evaluate the method error, intra-observer and inter-observer reliability, 5 experienced operators examined SEM images recorded at 500X as regards the enamel damages twice in a blind manner. A modification of a scoring scale, previously used to describe enamel surface after an acid attack (Nucci *et al*. 2004) was adopted and the integrity level of the enamel surface was evaluated as per the following criteria:

Score 0: The enamel surface is clean and free of scratches and grooves;

Score 1: Scratches and grooves are not very accentuated, have rounded edges and cover a portion of the surface;

Score 2: Deep furrows with rounded edges are visible over the entire surface, without debris;

Score 3: Particularly evident and deep-edged furrows are visible on the whole surface and debris are present on the enamel.

Statistical Analysis

As the primary objective of the study was to investigate the Ca/P ratio as an indication of enamel mineralization, the sample size has been assessed by www.dssresearch.com on two-tail test, in reference to the Ca/P ratio values of a previous study (Hegde and Moany 2012) and the sample size of 14 specimens for each group would give more than 80% power to the study with an α error level of 5%.

TABLE I ESEM-EDX: Ca/P ratios comparisons using one - way ANOVA followed by Tukey test

Groups	N	Ca/P ratios (Mean; SD) 10 KeV	Sig. (Anova test)	Multiple comparisons (Tukey test) Group S-Ex	Group S-Sal	Group S-CPP	Ca/P ratios (Mean; SD) 20 KeV	Sig. (Anova test)	Multiple comparisons (Tukey test) Group S-Ex	Group S-Sal	Group S-CPP
Group No-S	14	2.024 ± 0.077	$p < 0.05$	NS	NS	NS	1.904 ± 0.096	$p < 0.05$	NS	NS	NS
Group S-Ex	14	2.988 ± 0.409			NS	NS	2.301 ± 0.465			NS	NS
Group S-Sal	14	1.878 ± 0.085				NS	1.810 ± 0.086				NS
Group S-CPP	14	1.989 ± 0.090					1.943 ± 0.057				

N, Sample size; SD, Standard Deviation; Sig, Significance; NS, Not Significant.

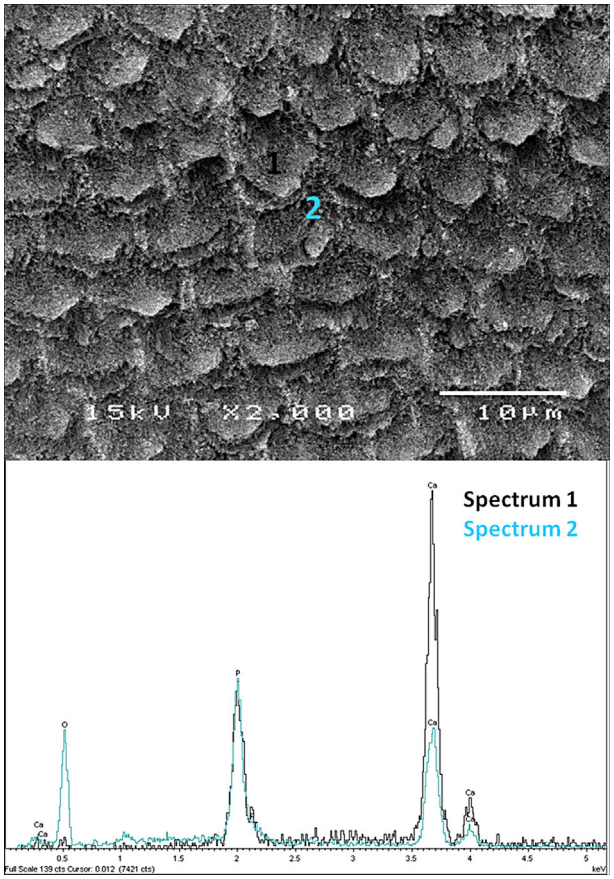

Fig. 2. Overlap of the two spectra recorded in the core (Spectrum 1) and outer of the prism (Spectrum 2) in S-Ex group showed greater quantities of Calcium in the core, as confirmed by t-test.

Data analysis was performed using Statistical Package for Social Sciences (SPSS, Version 17.0, SPSS Inc. Chicago, IL) and Excel 2000 (Microsoft Corporation, Redmond, WA). The statistical significance level was set at $p < 0.05$.

As the data were normally distributed, one-way ANOVAs were applied to compare the mean Ca/P ratios and the amount of C in the 4 groups; moreover, in the S-Ex Group *t*-test was used to compare the mean Ca/P ratios between the core and the interprismatic enamel. When

TABLE II ESEM-EDX: comparison of Ca/P ratios between the core and the interprismatic enamel in S-Ex group using *t*-test

Group	N	Ca/P ratios (Mean; S.D.) core	Ca/P ratios (Mean; S.D.) inter-rod	Sig. (*t* test) $p < 0.05$
Group S-Ex	14	2.798 ± 0.795	1.990 ± 0.216	*

N, Sample size; SD, Standard Deviation; Sig, Significance.

TABLE III ESEM-EDX: comparison of the amount of C (weight % of all analyzed elements) using one - way ANOVA followed by Tukey test for multiple comparisons

Groups	N	C (weight%) (Mean; SD) 10 KeV	Sig. (Anova test)	Multiple comparisons (Tukey test) Group S-Ex	Group S-Sal	Group S-CPP	C (weight%) (Mean; SD) 20 KeV	Sig. (Anova test)	Multiple comparisons (Tukey test) Group S-Ex	Group S-Sal	Group S-CPP
Group No-S	14	6.928 ± 2.677	$p < 0.05$	NS	NS	*	6.711 ± 3.259	$p < 0.05$	NS	NS	NS
Group S-Ex	14	3.197 ± 1.231			NS	*	5.863 ± 3.067			NS	NS
Group S-Sal	14	8.649 ± 3.280				*	6.580 ± 1.849				NS
Group S-CPP	14	26.512 ± 14.656					12.776 ± 5.399				

N, Sample size; SD, Standard Deviation; Sig, Significance; NS, Not Significant.

ANOVA test was significant, Tukey's post-hoc test was used for pair-wise comparisons between the means.

A Kruskal–Wallis test was performed to SEM scores; multiple comparisons were assessed by Tukey test.

To evaluate the method error, intra and inter-observer reliability checks were carried out using the Intraclass Correlation Coefficient (ICC $>$ 0.9 in a 95% CI).

Results

ESEM/EDX

There were not statistically significant differences among the 4 groups in regards to the Ca/P ratio (Table I); in the S-Ex group a statistically significant greater quantity of Ca was detected in the core rather than interprismatic enamel (Fig. 2, Table II).

In S-CPP group, spherical globular agglomerates showing a mineral composition similar to the one known for the MI PasteTM were observed across the entire portion of enamel surface of this group and a significantly higher quantity of C was found on the surface (Table III).

SEM

Kruskal–Wallis test showed a statistically significant difference among the different groups in regards to the scores of the observers; Tukey tests showed statistically significant differences in all between-group comparisons, except for S-Sal/S-CPP comparison (Table IV).

The morphological analysis of all the images made at different magnifications on predetermined points, according to the systematic observation method, showed:

in No-S group the surfaces appeared free of cracks, streaks and debris at 500X magnifications (Fig. 3a);

in S-Ex group enamel surfaces appeared severely damaged by mechanical abrasion and uniform parallel grooves produced by the diamond burs and loss of dental tissue were detectable (Fig. 3b);

in S-Sal group the grooves caused by drilling procedures were still visible, although less deep at 500X magnifications (Fig. 3c);

in S-CPP group the images at 500X magnifications were very similar to those from S-Sal group (Fig. 3d). At 1000X magnification a complete coating of small spherical globular agglomerates was detected across the entire portion of the enamel surface (Fig. 3e) and a thick layer of biofilm covered the grooves caused by IER (Fig. 3f).

Discussion

The Ca/P values of sound enamel recorded in this study (1.9) were comparable to those found in the

TABLE IV SEM scores: between-groups comparison using Kruskal–Wallis test followed by Tukey test

	SEM scores (Mean ± SD)					Multiple comparisons (Tukey test)					
Observer	Group No-S	Group S-Ex	Group S-Sal	Group S-CPP	Sig. (Kruskal Wallis test)	No-S vs S-Ex	No-S vs S-Sal	No-S vs S-CPP	S-Ex vs S-Sal	S-Ex vs S-CPP	S-Sal vs S-CPP
1	0.036 ± 0.09	2.654 ± 0.57	1.571 ± 0.61	1.500 ± 0.78	$p < 0.05$	*	*	*			
2	0.036 ± 0.09	2.571 ± 0.66	1.572 ± 0.60	1.500 ± 0.78					*	*	
3	0.000 ± 0.00	2.607 ± 0.54	1.607 ± 0.48	1.429 ± 0.62							NS
4	0.000 ± 0.00	2.403 ± 0.47	1.643 ± 0.45	1.464 ± 0.60							
5	0.043 ± 0.09	2.570 ± 0.43	1.679 ± 0.40	1.358 ± 0.56							

SD, Standard Deviation; Sig, Significance; NS, Not Significant.

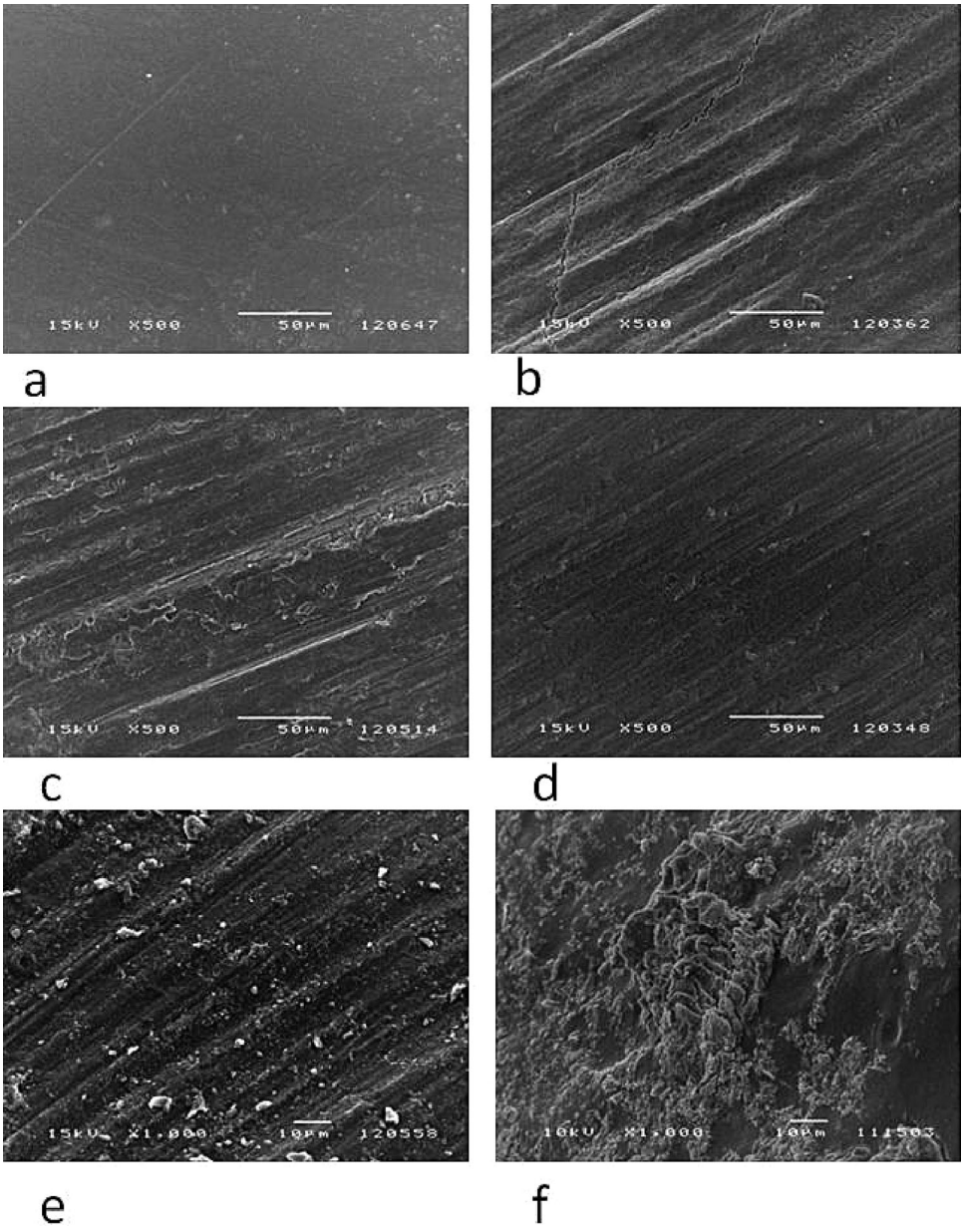

Fig. 3. a) SEM micrograph (500X): in No-S group surfaces appeared free of cracks, streaks and debris. b) SEM micrograph (500X): in S-Ex group enamel surfaces appeared severely damaged by mechanical abrasion and furrows, scratches and loss of dental tissue were detectable. c) SEM micrograph (500X): in S-Sal group the grooves caused by drilling procedures were still visible, although less deep. d) SEM micrograph (500X): in S-CPP group slight furrows and scratches were detectable on enamel surface. Small globular agglomerates covered large area of enamel. e) SEM micrograph (1000X): in S-CPP group small spherical agglomerates of paste particles covered enamel surfaces. f) SEM micrograph (1000X): in S-CPP group a thick layer of biofilm covered the stripping grooves.

literature (Arnold and Gaengler 2007; Hegde and Moany 2012).

Although the differences among the 4 groups were not statistically significant, a greater amount of Ca was recorded in S-Ex group (Table I). To investigate this finding, in this group where the prism cores were exposed as a consequence of IER, analyses were made both in the core and in the interprismatic enamel and statistically significant greater Ca/P ratios were actually discovered in the core (Fig. 2, Table II).

It was already stated that in the caries process the reduction of Ca is due to the loss of the intraprismatic substance (Xue *et al*. 2009), in the IER instead the superficial portion of the enamel prisms is removed and the core, rich in Ca, is exposed. According to the hypothesis, ESEM/EDX analysis showed greater amount of Ca at 10 KeV (external surface of reduced enamel) and less at 20 KeV (deeper enamel surface) (Table I).

At the ESEM/EDX analysis the stripped enamel composition was unaltered and compatible with the

absence of demineralization, although a statistically significant difference was recorded in SEM scores between the No-S and the S-Ex groups (Table IV); enamel surfaces immediately after IER appeared severely damaged since furrows and loss of dental tissue were detectable (Fig. 3b).

This finding suggest that, despite the injured morphology of the enamel, IER can be considered a reliable procedure, which does not predispose to caries lesions, since the regular Ca/P ratio is not compromised.

The absence of statistically significant differences between groups at ESEM-EDX analysis could indicate an analogue behavior of saliva and CPP-ACPF on stripped enamel *in vivo* after 30 days.

These data were confirmed at SEM, showing a reduced enamel damage in S-Sal and S-CPP groups, compared to S-Ex group and no statistically significant difference in SEM scores between S-Sal and S-CPP groups (Table IV).

The results of this study allowed to consider saliva a valid healing aid, able to repair within 30 days early enamel lesions caused by IER, as already stated in long-term clinical studies (García-Godoy and Hicks 2008; Tschoppe *et al.* 2010; Alessandri Bonetti *et al*. 2011; Zachrisson *et al*. 2011). The present *in vivo* study confirmed the ability of CPP-ACPF to bind bacteria within the biofilm (Reynolds *et al*. 2003; Marchisio *et al*. 2010; Hegde and Moany 2012). The C content was found in significantly higher quantities in S-CPP group on ESEM-EDX analysis at 10 KeV, while it returned not significant at 20 KeV (Table III). This finding may confirm that enamel composition didn't change and the film visible on the surface by SEM images at 1000X (Fig. 3f) is reasonably indicative of plaque presence and has nothing to do with tooth composition.

The results of this study agree with the clinical trials that showed no clinical advantage for the use of the CPP-ACPF supplementary to a normal oral hygiene (Huang *et al*. 2013) but is in contrast with those which claimed its effectiveness on remineralization of early enamel lesion (Robertson *et al*. 2011; Shen *et al*. 2011). A possible reason of this difference may be due to the short treatment application time and the clearance of the paste from the oral cavity, since the excess is spat out. Not surprisingly all the studies that highlighted the effectiveness of remineralizing paste were those where the delivery vehicle employed was either lozenges or gum, thus it was not necessary to spit out of the excess, and longer times of oral exposure and possibly retention might have occurred. In addition, these vehicles increased salivary flow, and its remineralizing effect was not differentiated from the one produced by the CPP-ACPF active ingredient (Pulido *et al.* 2008; Shen *et al.* 2011).

With regard to the specific topic of IER, the lack of *in vivo* studies about application of CPP-ACPF on the stripped enamel makes it difficult to compare our results with those available in the literature concerning acid demineralization or white spot lesions (Pulido *et al.* 2008; Beerens *et al.* 2010; Robertson *et al.* 2011; Shen *et al.* 2011; Huang *et al.* 2013).

A limitation to the present study design was the likelihood of spurious inferences that could affect the results, such as the compliance of the patients, not easily assessable (Bos *et al*. 2003) and the subject variability, in terms of oral hygiene, individual susceptibility to caries and saliva properties.

Therefore more researches need to be performed *in vivo* to provide the best clinical recommendation for the use of CPP-ACPF.

With the limitations of this study, it was concluded that no statistically significant differences were recorded in Ca/P ratio between sound and stripped enamel at semi-quantitative investigation (ESEM-EDX analysis).

After 30 days from IER, the effect of saliva *in vivo* appeared to be quantitatively (ESEM-EDX analysis) and qualitatively (SEM observations) comparable to CPP-ACPF.

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