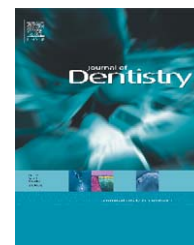


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Protective effect on enamel demineralization of a CPP-ACP paste: an AFM in vitro study

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ABSTRACT

Objectives: The aim of the present in vitro study was the evaluation of a CPP-ACP paste (Tooth Mousse) on preventing dental erosion produced by a soft drink (Coca Cola), using Atomic Force Microscopy (AFM).

Methods: Thirty extracted human central incisors free of caries were selected and divided in a treatment and a control half. The treatment halves were divided in three groups—group 1: demineralization with soft drink (4 intervals of 2 min); group 2: demineralization with soft drink (4 intervals of 2 min) plus Tooth Mousse; group 3: intact enamel plus Tooth Mousse. In groups 2 and 3 Tooth Mousse was applied for 3 min at 0, 8, 24 and 36 h. The surface of each specimen was imaged by AFM and R_{rms} values were registered.

Results: Among treatment specimens of groups 1 and 2, a statistically significant difference ($P < 0.01$) in R_{rms} values was registered: treatment of the specimens with CPP-ACP paste had a protective effect on enamel demineralization. In group 3 no statistically significant difference was registered between exposed and not exposed halves of the specimens.

Conclusions: The use of a CPP-ACP paste had a protective effect on enamel demineralization in an in vitro model.

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1. Introduction

Erosive tooth wear or dental erosion is a localized loss of the tooth surface by a chemical process of acidic dissolution without the involvement of bacteria.^{1,2} In the oral environment, tooth structure undergoes continuous demineralization and remineralization: if this balance is interrupted, demineralization will lead to a progressive deterioration of tooth structure.^{3,4}

Acids which cause dental erosion may be extrinsic or intrinsic in origin. Dietary acids are the most extensively studied aetiological agents and can be said to be the most important extrinsic factor.¹ Typical acid sources come from the diet, medications, occupational exposure and lifestyle activities.⁵ The prevalence of erosion is thought to be

increasing, reflecting the wide availability and frequent consumption of acid beverages, fruit juices, carbonated beverages, wines, sport drinks.^{6,7} The complete removal of acids from beverages can affect the palatability and stability of the product; product modification by addition or supplementation with calcium or phosphate is possible but consumers may reject the altered palatability and texture.⁷ The severity of erosion is related to several factors, including the chemical properties of the erosive medium and the frequency and method of contact between acid and tooth. It is also related to the effectiveness of the protective mechanisms in the oral environment, such as salivary composition, flow rate, buffering capacity, individual dental anatomy.¹

Casein phosphopeptides (CPP) containing the cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- have a remarkable

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ability to stabilize calcium phosphate (ACP) in metastable solution. Through the multiple phosphoseryl residues, the CPP binds to forming nanoclusters of ACP, preventing their growth to the critical size required for nucleation and phase transformation.⁸ CPP-ACP has been demonstrated to have anticariogenic activity in laboratory, animal and human *in situ* experiments.⁹ The CPP-ACP solutions have also been shown to significantly remineralize enamel subsurface lesions *in vitro*.¹⁰ CPP-ACP has been successfully incorporated into oral health products such as mouthrinses, sugar-free chewing-gums, and a sports drink to reduce enamel erosion.¹¹

Changes in tooth structure due to extrinsic factors have been widely investigated through SEM. This method requires proper specimen preparation and examination conditions: these procedures change the natural condition and/or part of the specimen structure.¹² In contrast, Atomic Force Microscopy gives images with atomic resolution with minimal sample preparation. This technique has been widely used to characterize the erosion of enamel and dentin.^{13–17} More recently, also AFM nanoindentation has been applied to the study of enamel erosion.¹⁸ Whereas the material losses (absolute erosions) have been carefully characterized, only minor attention has been devoted to the investigation of tooth surface change during erosion and demineralization.

The aim of the present *in vitro* study was the evaluation of a CPP-ACP paste on preventing dental erosion produced by a soft drink, using Atomic Force Microscopy (AFM).

2. Materials and methods

The overall experimental design is shown in Fig. 1. Specimens were prepared from 30 extracted human incisors free of caries and defects. After the extraction, the teeth were cleansed of soft tissue debris and inspected for cracks, hypoplasia and white spot lesions; they were disinfected in 5% sodium hypochlorite for 1 h and stored in artificial saliva (pH 7.0) during the whole experimentation. The specimens were cut longitudinally, with a high-speed diamond rotary bur with a

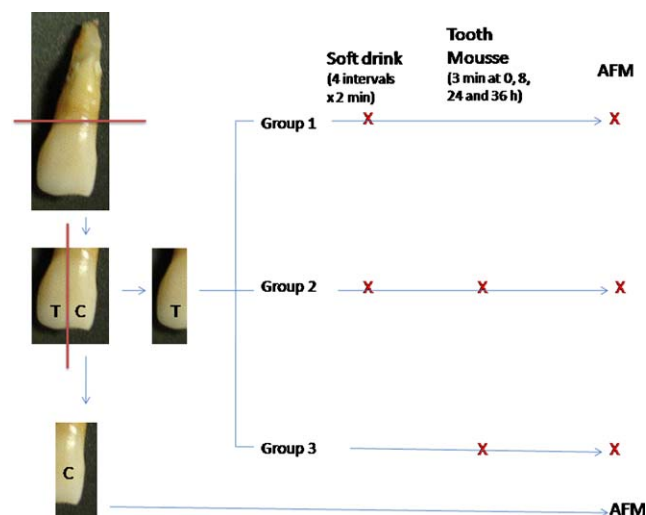


Fig. 1 – Overall experimental design.

water–air spray; one half served as a control, and the other half as treatment. The labial surfaces were ground using silicon carbide papers (grades 600–1200) under water irrigation to remove 50–100 μm and produce flat surfaces.⁷ Samples were placed into Teflon moulds measuring 10 mm \times 8 mm \times 2 mm, embedded in flowable composite resin and polymerized.

The CPP-ACP paste used was Tooth Mousse (GC Corp., Tokyo, Japan), a water based, sugar-free topical crème.

A soft drink (Coca Cola, Italy) was chosen for the demineralization process. The pH at 20 °C, buffering capacity, concentration of calcium and phosphate of the beverage were measured. All measurements were performed in triplicate and average values calculated. The samples were randomly assigned to 3 groups, each made of 10 teeth:

Group 1: Soft drink.

Group 2: Soft drink plus Tooth Mousse.

Group 3: Intact enamel plus Tooth Mousse.

The treatment specimens of groups 1 and 2 were immersed in 6 ml of the soft drink for 2 min at room temperature before rinsing with deionized water. Four consecutive intervals of the immersion procedure were carried out.¹⁹

The remineralizing agents were applied onto the surface of the specimens to just cover the enamel surface without brushing in the treatment groups 2 and 3 and wiped off with distilled water washing; the matching control specimens received no treatment. The remineralizing paste was applied to the enamel surfaces for 3 min at 0, 8, 24 and 36 h.¹⁹

After that, the remineralizing agents were removed by rinsing the enamel surface with distilled water and the samples were immersed in artificial saliva again.

2.1. Atomic Force Microscopy (AFM) observations

AFM images were collected with an Atomic Force Microscopy AutoProbe CP 100 (Themormicroscopes, Veeco), equipped with a piezoelectric scanner which can cover an area of 100 μm \times 100 μm with a range of 7 μm in the z-direction. The root-mean-square roughness, R_{rms} , was obtained from the AFM investigations by analysing, for each sample, at least 10 different film areas of 30 μm \times 30 μm with a resolution of 256 \times 256 pixels. From the analyses of the AFM height profiles, it was also possible to estimate the erosion cavities depth of the enamel surface. The data were obtained by averaging on at least 20 selected lines of the image.²⁰ The measurements were performed on the treatment specimens and on the matching controls.

2.1.1. Statistical analysis

Differences in the averaged values among the groups were analyzed by ANOVA test. Statistical difference was set at $P < 0.01$.

3. Results

The pH of the soft drink at 20 °C was 2.44; the buffering capacity was 0.0056. Concentration of calcium was 20.83 mg/ml, concentration of phosphate 175.7 mg/ml.

Table 1 – Mean roughness values (R_{rms}) obtained in the three groups on the exposed and the non-exposed specimens.

Groups	Treatment	R_{rms}	Depth (μm)
Group 1: Soft drink	Exposed	0.29 ± 0.01	0.50 ± 0.15
	Not exposed	0.05 ± 0.01	
Group 2: Soft drink + CPP-ACP paste	Exposed	0.14 ± 0.02	0.20 ± 0.09
	Not exposed	0.06 ± 0.02	
Group 3: CPP-ACP paste	Exposed	0.06 ± 0.05	0
	Not exposed	0.07 ± 0.02	

Table 1 reports mean R_{rms} values and maximum depth of the cavities obtained through the demineralization process.

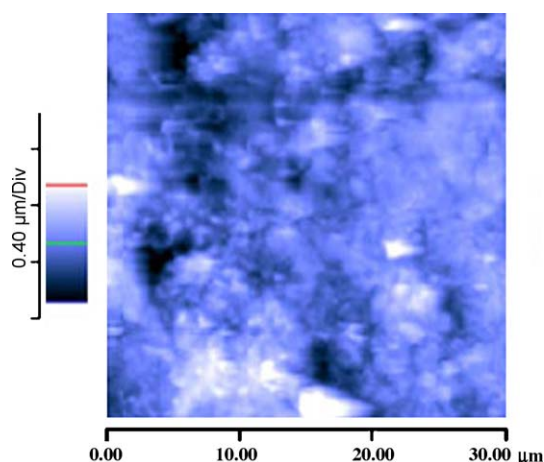
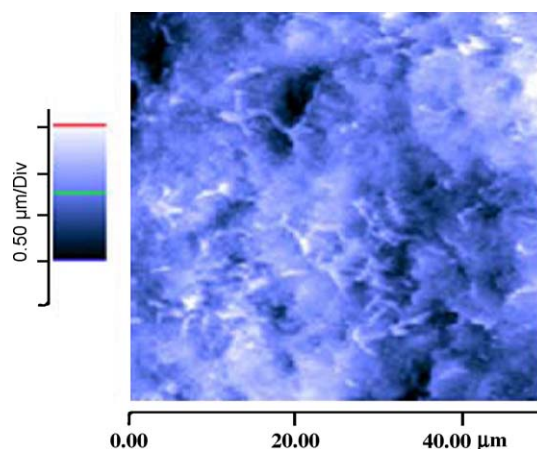
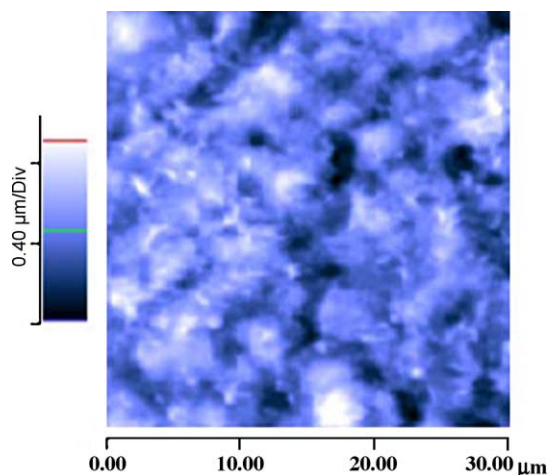
In groups 1, 2 and 3, the not exposed halves of the specimens provided similar R_{rms} values (no statistical difference), suggesting that the two groups may be comparable. Among exposed specimens of groups 1 and 2, a statistically significant difference ($P < 0.01$) in R_{rms} values was registered.

In group 3, no statistically significant difference was registered between exposed and not exposed halves of the specimens.

Fig. 2 shows an untreated specimen surface. Fig. 3 shows the demineralized surface of a specimen (group 1). Fig. 4 displays a specimen surface demineralized and then exposed to the protective effect of CPP-ACP paste (group 2). In Fig. 5, an untreated enamel specimen was exposed to CPP-ACP paste (group 3).

The surfaces of the untreated teeth appear quite smooth, except for some scratches (Fig. 2). A remarkable increase of enamel surface alterations was observed after the exposure to the acid drink, which causes a loss of material from the surface (Fig. 3). The final enamel morphology recalls a typical prismatic structure, with prism size ranging between 3 and 5 μm . As described by Silverstone *et al.*²¹ and Oshiro *et al.*,²² the etching process involves mainly the inner area of the prism creating a honeycomb-like structure.

In both groups 1 and 2, there is a statistically significant difference in R_{rms} between the exposed and the not exposed halves of the specimens. Regarding the tooth surfaces treated

**Fig. 2 – Untreated specimen surface.****Fig. 3 – Specimen surface after demineralization with soft drink (group 1).****Fig. 4 – Specimen demineralized with soft drink and exposed to CPP-ACP paste (group 2).**

with CPP-ACP paste after exposure to the soft drink (group 2), the interprism cavities appear largely filled in, with a consequent decrease of their depth. Moreover, the comparison of the exposed halves of group 2 shows that the treatment with the CPP-ACP paste causes statistically significant reductions of the R_{rms} values. Concerning the maximum depth of the cavities, the treatment with the CPP-ACP paste resulted in a statistically significant depth reduction.

4. Discussion

In the present in vitro study, AFM has been used to verify the protective effect of a CPP-ACP paste on enamel exposed to erosive action of a soft drink.

AFM was used to study tooth surfaces in order to compare the pattern of particle distribution in the outermost layer of the tooth surfaces.¹² It was found that AFM gives high-contrast, high-resolution images and is an important tool as a source of new structural information: tapping mode AFM (TM-

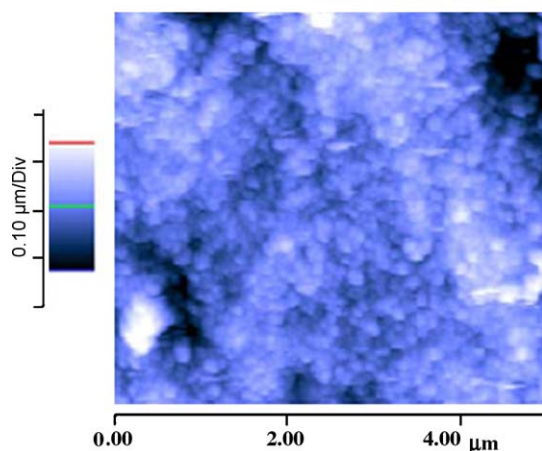


Fig. 5 – Untreated specimen exposed to CPP-ACP paste (group 3).

AFM) images treated with demineralizing solutions are able to show net differences between exposed and unexposed enamel areas.⁶ AFM was used by Hegedus *et al.*²³ to study the effects of bleaching agents on enamel, as a suitable microscopic method to analyze biological objects under natural conditions: the authors concluded that in contrast with other studies that used SEM, AFM imaged non-dehydrated enamel surfaces.

AFM nanoindentation was used to investigate the demineralization and remineralization of surface softened enamel, which provided a very sensitive measurement.^{24–26} TM-AFM investigations showed that the amount of mineral loss increased in the presence of a demineralizing solution with increasing exposure time.^{24–26}

Enamel surface is often aprismatic and more highly mineralized than enamel subsurface; however, enamel surface is completely removed by the polishing process and the resulting flat surface is as such not present in the oral cavity. This prismless enamel arises at the end of amelogenesis. Although this layer of enamel is more frequent on the surface of deciduous teeth, it can also be found on the surface of permanent teeth. It is known that the prism-free enamel is gradually worn off during mastication but it is retained in protected areas. Flat and polished specimens were used in the present study in an attempt to standardize specimens and remove natural variations in surface enamel between teeth and between different tooth sites and types, which may result in different responses to acid dissolution.¹¹ However, it should be noted that natural tooth surfaces erode more slowly than polished surfaces.²⁷ The specimens were cleaned with 5% NaOCl for 1 h, which could not alter enamel surface.¹² Even a 3-day exposure to 13% NaOCl did not alter the nanomechanical properties or the demineralization behaviour of enamel samples.²⁴

In a previous study, the chemical properties of Coca Cola and Gatorade were evaluated.²⁰ Their pH, buffering capacity, concentration of calcium and phosphate were measured at 20 °C. Their pH level was below 4, so it was to be expected that exposure to each of them resulted in a progressive loss of enamel. It was demonstrated that erosion is correlated to pH; moreover, there was a negative correlation between calcium

concentration and erosion, but no clear relationship between phosphate concentration and erosion.¹ For these reasons, the beverage with the lowest pH and highest concentration of calcium was chosen for the present in vitro study: Coca Cola.

There is a clear relationship between erosion and temperature of the beverages.²⁸ In this study, the beverage was kept at a constant temperature of 20 °C. Although erosion proceeds more slowly in vivo than in vitro owing to the protective effect of saliva and acquired pellicle, the effect of temperature can be expected to be significant.¹⁸ In order to stress their demineralizing potential, the cola drink was replenished every 2 min to ensure that it was carbonated and to reduce the buffering effect from ions dissolved from the enamel surface.¹⁹

Devlin *et al.*²⁹ demonstrated that Coca Cola reduced the mean indentation hardness of enamel in the teeth, but the hardness was partially restored with artificial saliva. Artificial saliva was not used during the erosion phase; its use would buffer the acidity of the cola drink and limit the demineralization process.¹⁹ Soft drink tested showed high erosive potential on human enamel. In the oral environment, many factors can enhance or prevent demineralization such as mineral concentration, pellicle and plaque formation, salivary factors (flow rate and buffering capacity).

Many studies described CPP-ACP properties.^{30–32} CPP-ACP increases the number of potential calcium-binding sites thereby decreasing the calcium diffusion constant. This large effect will decrease the rate of calcium loss from plaque during a cariogenic attack.^{33,34} This protective effect could be demonstrated not only for enamel but also for dentin demineralization.²²

Other studies established that CPP-ACP in a mouthwash significantly increased the level of calcium and inorganic phosphate ions in supragingival plaque with the CPP bound to salivary pellicle and to the surface of bacteria.³⁵ In the plaque biofilm, Shen *et al.*⁹ demonstrated that CPP-ACP remineralize enamel subsurface lesions in situ; Ijima *et al.*,³⁶ confirmed these results: enamel remineralized lesions were more resistant to subsequent acid challenge. Casein derivatives complexed with calcium phosphate preparations acted as preventive agents in patients with salivary gland dysfunction.³¹ The release of CPP-ACP and fluoride from a CPP-ACP-containing glass ionomer cement was associated with enhanced protection of the adjacent dentin during acid challenge in vitro.³² In a SEM in vitro study, adding CPP-ACP to a sports drink significantly reduced the beverage's erosivity, without altering the product's taste.^{7,37} The mechanisms by which CPP-ACP reduces erosive tooth wear are unclear. However the finding that CPP-ACP increases hardness of enamel eroded by cola drink implies that its erosion-inhibiting potential probably involves remineralization action.²⁷

It is well known that the localization of ACP at the tooth surface buffers the free calcium and phosphate ion activities, thus helping to maintain a state of supersaturation which depresses demineralization and enhances remineralization of the enamel.³⁵ In that study CPP could still be detected on the tooth surface 3 h after the consumption of xilitol-gum containing CPP-ACP. According to a previous study and to the present study, the action of the CPP-ACP paste cannot

involve a remineralization process, but a protective action against acid attack. From the point of view of the surface properties, the addition of the CPP-ACP paste gives origin to a composite material which can be more or less selectively eroded by the H^+ ions of the drink depending on the chemical properties of the solution.²⁰ The treatment of the teeth surface with the CPP-ACP paste causes the formation of a layer that fills the interprism cavities, and partially covers the prisms for a long time, thus preventing a following acid attack.

5. Conclusions

Under the limitations of the present in vitro study, it can be concluded that the application of CPP-ACP paste is effective on preventing dental erosion produced by a soft drink.

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