

## The effect of casein phosphopeptide-amorphous calcium phosphate on chemical-induced enamel erosion: an in vitro study with ESEM analysis

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**AIM:** Chemical exogen acids effect can be considered in terms of the loss of hard tissue of teeth or potential loss. When the remineralization does not antagonize the demineralization, the dissolution of hard tissues arises. Significant among physical processes is degeneration in enamel structure due to the chemical processes of acidification and alkalinization. There are also several underlying determinants of enamel erosion. The enamel erosion induced by chemical exogen acids causes the dissolution of the hard tissues of teeth. The aim of this study was to analyze the erosive effect of three different soft drinks on the enamel surface and the potential remineralizing effect after applying casein phosphopeptide-amorphous calcium phosphate.

**MATERIALS AND METHODS:** Fifteen human third molar teeth specimens were analyzed to assess the effects of the topical cream containing 10% w/w casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) nanocomplexes on enamel teeth lesions. Morphological characterization of teeth was performed using Environmental Scanning Electron Microscope (ESEM). In addition, the surface of specimens was examined before and after immersion into three recipients containing three different soft drinks and after the remineralization process for comparison.

**RESULTS:** Environmental Scanning Electron Microscopy analysis showed enamel morphology alterations after acidic soft drink exposure and superficially repair of teeth enamel after remineralizing treatment.

**CONCLUSION:** This in vitro study demonstrated that the casein phosphopeptide-amorphous calcium phosphate treatment was effective in remineralizing demineralized subsurface lesion enamel in vitro after having caused alterations by exogen acids.

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Dental erosion can be defined as the chemical dissolution of tooth minerals resulting in the loss of hard tissues but without the involvement of bacteria. Chemically-induced enamel erosion is a multifactorial condition and is recognized as being triggered by exogenous acid exposure. Several diet agents have been associated with enamel erosion. The consumption of the adverse diet causes characteristic abnormalities of hard tissues of teeth, primarily with the dissolution. It is well known that this lesion results from the dissolution of the tooth by extrinsic or intrinsic acids or chelator acids when the surrounding aqueous phase is undersaturated concerning tooth minerals (1-7). The exogenous acid introduction in the oral cavity is the leading cause of dental erosion as a result of a chemical reaction between hydrogen ions in the acid with dental hydroxyapatite  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  that promote the release of mineral ions ( $\text{Ca}^{2+}$ ,  $\text{OH}^-$ ) and  $\text{PO}_4^{3-}$  (8-9).

Furthermore, the enamel demineralization causes a substantial decrease in microhardness (10-17), increasing the vulnerability of the surface to mechanical impacts (18). The enamel erosion influences the occurrence of cervical lesions. Indeed, when the enamel is exposed to chemical insult, a process defined as "softening" begins, consisting of the loss of a mineral layer extending a few micrometres below the surface (19). In the early stages, this process is reversible. If the softening evolves, it results in complete loss of enamel. The high consumption of acidic beverages as diet components in childhood explains the most prevalence of these lesions among children (18-21). The consumption of acidic drinks has changed through the decades, increasing exponentially (22-26). There is strong evidence that the chemical composition and properties of the demineralizing solution are implicated in the dental erosive process caused by acidic drinks (27-30). Indeed, conventional soft drinks also consist of titratable acidity and chemical preservatives. In addition to lower pH value, these agents increase the erosive potential of these beverages. In other risk factors, this phenomenon is associated with a high incidence and progression of dental erosions, mainly in children and adolescents

(31-33); this may compromise the dentition and lead to expensive restorations in adulthood (34-37). Therefore, interest in early child development and recognition of its lifelong impacts grows. Preventive measures to counteract enamel erosion are imperative.

One result, in recent years, has been a substantial increase in numerous treatment regimens, including a new formulation of toothpaste containing casein phosphopeptide – amorphous calcium phosphate (CPP-ACP), which studies in vitro have demonstrated to be effective in inhibiting cariogenic activity and reducing demineralization of the altered tooth surfaces. The casein phosphopeptide – amorphous calcium phosphate (CPP-ACP) is a bioactive agent with a base of milk. It has been demonstrated that the anticariogenic property of CPP-ACP due to its ability to stabilize calcium and phosphate in an amorphous state, also due to the presence of two cluster sequences in CPP, Ser (P)-Ser (P)-Ser (P)-Glu-Glu from casein (38-41). Moreover, ACP separates from CPP in an acidic environment, thereby enhancing salivary calcium and phosphate concentrations (39-43). Furthermore, CPP can become constant with the level of ACP in saliva by inhibiting the precipitation of calcium and phosphate and stabilizing the calcium level. Our study aimed to analyze the potential remineralizing of paste containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) after demineralization process induced by exogenous acids, using a well-known chemical process. Three predisposing factors (soft drinks) were selected based on available data present in the literature (44-52).

## MATERIALS AND METHODS

Fifteen third human molar teeth were included in the study. All were extracted for orthodontic reasons, and written informed consent was obtained from all participants to use their teeth for scientific purposes. Inclusion criteria were teeth caries lesions free. Biological samples were handled by one of three different procedures before ESEM (Environmental Scanning Microscope Quanta 200, FEI Company, Philips Electron Optics, Eindhoven, Netherlands) observation. Analysis of specimens for

morphological characterization was performed using an ESEM, set in low vacuum mode (120 Pa), at pressures that were ca. 0.6 Torr lower than the pressure of maximum brightness. The ESEM technology allows the analysis of biological samples in their natural state and without metal coating (Danilatos 1988, Goldstein et al. 1992, Johnson et al. 1993). The instrument allows a resolution of 3.0 NM @ 30kV, with a high signal to noise ratio both in high/low vacuum and ambient mode. The acceleration voltage (200V÷30 KV) and the filament current (up to 2µA) are constantly adaptable. Stehr and Oestlund (2000) showed that ESEM is an essential tool in studying the surface condition and acceptable machining deformations without the risk of artefacts in specimen preparation. This microscope was equipped with the following facilities: SED Everhart-Thornley secondary electrons detector, LFD (large field secondary electrons detector, GSED (secondary gaseous electrons det., in ESEM mode), BSED/CLD detector for photocathodoluminescence electrons, Thermostatic Peltier heating/cooling stage (20°-5°C) (in ESEM mode) with external chiller, Eucentric goniometer stage with R = 360° continuous and repeatability: 2µm, Digital video recording.

#### *Preparation protocol for ESEM*

Before the treatments, samples were handled by the following procedure: (1) the teeth specimens were thoroughly cleaned of all residual debris with mechanical instruments and then stored in physiological saline solution for three weeks; subsequently, each sample was mounted on stubs with double-adhesive. No metallization of the samples was necessary. (2) The teeth were untreated to evaluate the enamel surface before the effect of exogen acids; (3) the chamber conditions were set at 5° and 6.0 Torr (humidity in the specimen chamber was about 85%); and (4) the samples were observed and photographed. Therefore, after undergoing ESEM observation, all specimens were divided into three groups and then immersed in different acid solutions for thirty minutes a day for 7 days to simulate a typical daily assumption of soft drinks. In Group A, each specimen was immediately washed with normal saline and then immersed into individual containers of Coca Cola (having phosphoric acid) with PH 2,53. After washing with normal saline in group B, the teeth were placed into containers of RED BULL (having citric acid) with PH 3,41. Samples of Group C were rinsed with sterile saline and then immersed into individual containers of orange juice (having

citric acid about 0.2-0.004 M) with PH 3.5. Subsequently, each sample was treated with casein phosphopeptide-amorphous calcium phosphate for three minutes for 10 days to evaluate the remineralizing effectiveness. After each treatment, the teeth specimens were cleaned and washed with distilled water and then stored in normal saline until further treatment was stored in physiological saline solution up to the time they were analyzed. Therefore, morphological analysis was performed. Samples were analyzed using secondary electrons with SS Detector (Solid Stake detector) or LFD (Large Field Detector).

## RESULTS

### *In vitro morphological ESEM evaluation*

The images obtained from the treated samples at different conditions were compared with images observed after treatment with casein phosphopeptide-amorphous calcium phosphate. Before the treatments, samples were handled by the following procedure: to prepare these samples, (1) the teeth specimens were thoroughly cleaned of all residual debris with mechanical instruments and stored in physiological saline solution for 3 weeks; (2) the deposition of a conductive layer on the specimens was not necessary. Samples were mounted on stubs with double adhesive. The teeth were untreated to evaluate the effect of the exogen acids on the enamel surface; (3) the chamber conditions were set at 5°C and 6.0 Torr (humidity in the specimen chamber was about 85%); and (4) the samples were observed and photographed using an SR stereomicroscope (Carl Zeiss AG, Oberkochen, D-73446, Germany). The images obtained from the treated samples in different solutions were compared with images observed after treatment with casein phosphopeptide-amorphous calcium phosphate. Teeth were examined at magnifications of (×100, ×250, ×500 and ×1000) with the ESEM. All teeth specimens were shown at the ESEM about the same appearance.

The enamel is more evident than the root to the rear-scattered electrons or the secondary electrons. Representative ESEM images of the retrodiffused electrons, at the low empty ESEM, of the teeth before treatment with the soft drinks were revealed. The ESEM image from Group A showed the regular enamel prisms' ends. The enamel surface was fairly

uniform, with longitudinal grooves and fracture lines. The morphological analysis showed a jagged root, specifically at the dental-enamel junction, and the level can be either jagged or uniform. The ESEM image from group A showed standard prisms' ends with few surface deposits and no surface enamel roughness after immersion into Coca Cola. The ESEM image from group B showed surface deposits with mild enamel surface roughness after casein phosphopeptide-amorphous calcium phosphate treatment. The ESEM image from group C showed low surface deposits and no surface roughness. The ESEM image from group D showed severe enamel surface roughness with no surface deposits. The ESEM showed deep fissures and damaged enamel prisms in the sample treated with Coca Cola. The ESEM image from Group B, treated with RED BULL, showed surface deposits with mild enamel surface roughness. The ESEM image from group C showed scarce surface deposits and no surface roughness. The ESEM image from group D showed severe enamel surface roughness with no surface deposits. After treatment with Coca Cola, at a magnification of 500 $\times$ , the dental surface showed numerous extended fractures on the enamel, with more uniform portions and others more susceptible to the erosive action of the drink. The prisms were damaged, showing more eroded than the sheaths, with more uniform portions and others more susceptible to the effect of the drink. At a low magnification of 250 $\times$ , before the treatment with Coca Cola, the surface of the enamel appeared sufficiently uniform, with some slight longitudinal groove and some lines of surface fracture. The root occurred at the ESEM with a frayed surface, especially near the amelocementitious junction and some fracture lines. The treatment with casein phosphopeptide-amorphous calcium phosphate revealed the almost total absence of scratches and grooves, filled and levelled. After treatment with casein phosphopeptide-amorphous calcium phosphate, it was shown clearly how the level of the prisms of the enamel was settled, occluding the cavities produced by the erosive action of Coca Cola. After treatment with Red Bull, the ESEM sample presented an accentuated erosive damage; the surface was more eroded at the enamel level, with newly formed fracture lines. Taking as a reference the same

surface, treated first with Red Bull and then with casein phosphopeptide-amorphous calcium phosphate, we noted slight improvements, especially on the enamel, with the presence of casein phosphopeptide-amorphous calcium phosphate deposits, which had the characteristic of chemically binding to it. Near the root, we saw the casein phosphopeptide-amorphous calcium phosphate bound to the surface, and the fracture lines occurred with less thickness than in the previous image. Before being treated with Red Bull, the surface had depressions of the prisms in specific sites; in others, the homogeneous glaze was observed. Near the amelocementitious junction, the appearance of the surface was frayed. In order to analyze an area of the larger surface at lower magnification, it is highlighted how the Red Bull does not have uniform effects on the prisms but produces irregular excavations. Analyzing at the same magnification, the same surface area, following the treatment with Red Bull and subsequently with casein phosphopeptide-amorphous calcium phosphate, we found how erosion defects were reduced, showing a more compliant surface. After the treatment with Red Bull, at a higher magnification, the enamel presented porous. The drink changed the appearance of the surface, producing irregular excavation of prisms and generalized microlesions. After the treatments with Red Bull and casein phosphopeptide-amorphous calcium phosphate, it was possible to notice the improvement of the enamel surface. The change in its appearance highlighted the reparative action of the casein phosphopeptide-amorphous calcium phosphate.

At a magnification of 500 $\times$ , the casein phosphopeptide-amorphous calcium phosphate treated surfaces appeared covered by a uniform layer of material. Enamel micro-cavities are filled. Before being treated with 100% orange juice, the sample was presented to the ESEM with a fringed pattern near the enamel-cement junction, with marked soft wear at the level of the enamel, where longitudinal and horizontal furrows prevailed, associated with abrasions with hard materials, and a slight presence of prism depressions. The root, because of its composition more susceptible to external insults yet presented jagged already departing. There were numerous microfractures, particularly accentuated at the cement-enamel

junction and when the same site was brought to 500x magnification to better highlight erosion from the juice. Depressions of the prisms already present were noted but aggravated by the drink's erosive effect, which also damaged the formation of new furrows.

## DISCUSSION

It is generally accepted that the physicochemical properties of exogenous chemical soft drinks increase the risk of teeth enamel erosion. Reynolds and co-workers demonstrated that formulations of professionals containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) could significantly affect the remineralizing process. (10-17, 53, 54) The authors found that increasing the duration of exposure to casein phosphopeptide-amorphous calcium phosphate increased the levels of calcium and phosphate ions and promoted the remineralization of enamel subsurface lesions *in situ* (55-61). Based on a comparison of the physicochemical properties of the various remineralizing agents, the authors showed the synergistic effect of 2% CPP-ACP in conjunction with 1100 ppm fluoride in reducing the enamel dissolution because of the formation of CPP-stabilized amorphous calcium fluoride phosphate, which results in the increased incorporation of fluoride ions into plaque (62-64). Similar findings have also been reported by Cai et al. in a randomized, controlled, double-blind trial showing that CPP-ACP significantly reduced the caries experience when the fluoride was added (65). The concentration employed in this study reflects the level of agent required to exert a clinically beneficial effect on the enamel surface when applied topically (66-68).

Furthermore, work is required on these systems to make a definitive comparison of the effect of solvent volatility and penetrant lipophilicity upon their penetration into the skin (34-39, 69). Microradiography of the remineralized lesions demonstrates that the 2% CPP-ACP paste generated homogenous remineralization of the lesion. This effect is attributable to the ability of the CPP to concentrate and fix the ions at the tooth surface in the correct molar ratio [Ca:PO<sub>4</sub>:F = 5:3:1]. In several different studies, the effectiveness of CPP-ACP technology

has been demonstrated in promoting the levels the remineralization of enamel subsurface lesions via increasing the level of calcium and phosphate ions in supragingival plaque (70-78). Barbour *et al.* demonstrated that CPP-ACP bound to bacterial and produced a reservoir of bioavailable calcium ions (79). In another randomized clinical study, Lussi et al. examined 152 visible white-spot lesions on 60 incisors and canines immediately after orthodontic debonding (80). They were assigned to different remineralizing treatments, which comprised a tooth cream containing CPP-ACP with a fluoride dentifrice or a daily topical application of a 0.05% sodium fluoride mouthwash in conjunction with fluoride dentifrice (81-90). The lesions were observed, and the authors reported a significant difference between the treatment ( $p < 0.01$ ), registering a regression of about 64% of white spots for the CPP-ACP group in comparison with the fluoride group, which reported a 23% of regression (91-134).

The present *in vitro* study demonstrated that the casein phosphopeptide-amorphous calcium phosphate treatment was effective in remineralizing demineralized subsurface lesion enamel *in vitro* after having caused alterations by exogen acids.

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