

C. Bertoldi*, A. Lucchi**, D. Zaffe***

University of Modena and Reggio Emilia, Modena, Italy.

*Department of Surgery, Medicine, Dentistry and Morphological Sciences with Transplant Surgery, Oncology and Regenerative Medicine Relevance

**Private Practice, Modena Italy

***Department of Biomedical, Metabolic and Neural Sciences

e-mail: davide.zaffe@unimore.it

Effects of soft-drinks and remineralising treatment on teeth assessed by morphological and quantitative X-ray investigations

ABSTRACT

Aim To morphologically and chemical-physically analyse both the surface and the subsurface of enamel undergoing soft-drink demineralisation and remineralisation treatment.

Material and methods Fifteen human premolars were split and immersed in saline or three popular soft drinks, as demineralising agent, 15 minutes per day, for seven days at room temperature. Half of drink-processed teeth was then treated with casein phosphopeptide-amorphous calcium phosphate, as remineralising agent, for an additional seven days. The surface morphology was evaluated by stereomicroscope and scanning electron microscope (SEM). Teeth were then re-embedded and sectioned, and analysed under SEM and X-ray microprobe.

Results Drink-processed teeth showed root pigmentation, opacification and deterioration of the superficial enamel. The enamel surface resulted greatly furrowed after drink processing, and apparently restored after remineralising treatment. However, in tooth sections, SEM showed always a subsurface demineralisation of dentine and enamel, in particular

at the cemento-enamel junction, also after remineralising treatment. The remineralising agent produced a partial remineralisation of the subsurface enamel, sometimes statistically significant, but not in hydroxyapatite stoichiometry.

Conclusion Soft-drink erosion impaired not only the surface but also the subsurface enamel. The applied remineralising treatment, yielding some effects on surface and subsurface enamel reversing basically the decalcification process.

Keywords Dental hard tissues, Remineralisation, SEM, Soft drink demineralisation, X-ray microanalysis.

Introduction

The childhood diet habits can greatly influence the dental hard tissue health. The dissolution of the mineralised structure of the teeth occurs with the substantial contribution of acid contact, which may come from intrinsic sources (e.g. gastroesophageal reflux) or extrinsic (e.g. food, drink, fruits, dental plaque of cariogenic bacteria, etc.) [Lussi et al., 2004]. So, an acid environment plays an important role in the development of the lesions of the dental tissues. Drinks could have a major role in tooth dissolution since the main average consumption of soft drinks in U.S. people was more than 200 L/year, and the value greatly increases in adolescent boys [Buyer, 2009]. Acidic drinks, such as soft drinks and energy/sports drinks, contain high levels of H⁺ ions reducing the pH of the oral cavity. This could lead to erosion in which the tooth enamel is subjected to the mobilisation of calcium and phosphate ions, leading to its dissolution.

Bader et al. [1996] found that drinking fruit juices more than once a day and a low salivary buffering capacity were correlated with increased non-carious cervical lesions (NCCLs). The NCCLs are pathologies often observed in relation to gingival recession [Bertoldi et al., 2011], involving loss of dental tissue at the cervical third of the dental crown and subjacent root surface, through processes unrelated to caries, periodontal disease and without the involvement of micro-organisms [Bertoldi et al., 2012; Grippo et al., 2012]. Bergström and Eliasson [1988] reported up to 85% NCCLs prevalence in 21–60 year-old patients.

NCCLs characteristics make a clear separation of causes from effects difficult during clinical investigations. Few studies considered the chemical degradation of the surface (erosion) as a key factor in the development of NCCLs. Few studies were conducted to evaluate the NCCL-inducing activity of each of the different pathodynamic mechanisms, in particular the acidity [He et al., 2011],

and the potential harmful action of widely used food and drinks is almost unknown. However, understanding erosion pathodynamic could help us in defining correct behaviours and more effective strategies of prevention and intervention. Besides, carious process beginning was also associated with demineralisation [Reynolds, 2009].

The early stages of caries seem to be reversible: at an early stage a remineralisation process, involving the diffusion of calcium and phosphate ions into the lesion, can restore the lost structure [Reynolds, 2009; Hedge and Moany, 2012; Ferrazzano et al., 2007; Ferrazzano et al., 2011].

To treat NCCLs and their pathodynamic erosive cause, a remineralising treatment could also be virtually able to reverse completely the ion deprivation due to acidic fluids. Several remineralising agents, such as ozone, sugar substitutes, fluorides, hydroxyapatite, calcium sodium phosphosilicate, casein phosphopeptides, have been proposed to heal defective hard dental tissues [Rao and Malhotra, 2011]. The remineralising agents have been applied after demineralisation treatment [Ferrazzano et al., 2011; Wu et al. 2010] to analyse the remineralisation process, whereas the remineralising agents were mixed with soft drinks to study their possible prevention of erosive enamel damage [Ferrazzano et al., 2007; Ferrazzano et al., 2012; Wegehaupt et al., 2011].

A central problem to be solved is to achieve a complete and homogeneous remineralisation of the lesion to restore the affected areas to the original chemical and mechanical properties. Almost all studies on enamel demineralisation and remineralisation have been performed analysing the enamel surface by several methods (SEM, profilometry, circular polarisation, microhardness, etc.), without a true elemental assay of the hard dental tissues. Contrary to most researchers, Reynolds [2009] and Iijima et al. [2004] performed the microradiography of artificially demineralised sections to analyse the de/remineralisation effect, without a true quantitative estimation of calcium and phosphorus content in the demineralised layer.

The aim of this study was to evaluate both the surface morphology, and the subsurface chemical composition of human enamel subjected to soft drinks, and after remineralising agent treatment, using the scanning electron microscope and X-ray microanalysis, to analyse the development and healing of hard dental tissue erosive lesions in teeth of 18-28 year-old subjects, the major drink consumer.

Materials and methods

Sample preparation

Fifteen premolars of 18-28 year-old patients, extracted for orthodontic reasons in a private dental office, were used to analyse the effects of soft drinks on enamel. Teeth were caries free, and without restorations nor observable cracks. The extracted teeth were immediately

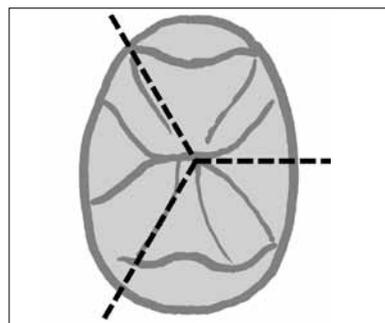


FIG. 1 Occlusal aspect of a premolar tooth. The broken lines correspond to the longitudinal cuts performed to slice the tooth.

polished and stored in a dark environment in 47.5% ethanol at room temperature until use.

Teeth were longitudinally cut (Fig. 1) to obtain 3 almost equal "crown-sections", each assigned to one of the 3 groups: A) control saline only (15 samples), B) soft drink only and C) soft drink and remineralising agent. The samples of group B and group C were subjected to three drinks much beloved by young people:

- Coca-Cola classic (Coca-Cola HBC Italia, Milano, Italy), pH 2.3 [Seow and Thong, 2005], 5 + 5 samples (CC teeth);
- Gatorade, orange flavour (PepsiCo Beverages Italia Srl, Milano, Italy), pH 2.9 [Seow and Thong, 2005] sport drink without artificial flavour, 5 + 5 samples (G teeth);
- Red Bull, (Red Bull Srl, Milano, Italy), pH 3.1 [Seow and Thong, 2005] plain caramel energy drink, 5 + 5 samples (RB teeth).

The remineralising agent used was a professional paste for dental hygiene containing casein phosphopeptide-amorphous calcium phosphate, CPP-CAP (GC Tooth Mousse, GC Italia srl, S. Giuliano Milanese - Milan, Italy).

Samples of group A were subjected to saline. Samples of groups B and C were subjected to the drinks for 15 minutes per day for 7 days at room temperature, to simulate a discontinuous but prolonged drink assumption. After drinks processing, the GC Tooth Mousse, following the manufacturer's instructions, was applied on teeth of group C for 4 minutes, twice a day for 7 days.

Group A processing

Each sample was completely immersed in 50 ml of fresh saline for 15 minutes at room temperature. Samples were then rinsed in distilled water and stored in the dark for the remaining 23 hours in saline containing 15 mM sodium azide as bacteriostatic agent (all reagents are from Sigma-Aldrich, St. Louis, MO, USA). The processing was repeated for seven days. Each sample was then washed with distilled water and stored in saline containing 15 mM sodium azide for 7 additional days. At the end of the 14th day, each sample was carefully rinsed with distilled water, and air dried.

Group B processing

Each sample was completely immersed in 50 ml of the corresponding soft drink for 15 minutes at room

temperature. Samples were then rinsed and stored in the dark for the remaining 23 hours in saline containing 15 mM sodium azide. The procedure was repeated for seven days. Each sample was then washed with distilled water and stored in saline containing 15 mM sodium azide for 7 additional days. At the end of the 14th day, each sample was carefully rinsed with distilled water, and air dried.

Group C processing

Each sample was completely immersed in 50 ml of new soft drinks for 15 minutes at room temperature. Samples were then rinsed and stored in the dark for the remaining 23 hours in saline containing 15 mM sodium azide. The treatment was repeated for seven days. At the end of the 7th day, the application of the GC Tooth Mousse was performed using a stainless steel spatula for 7 days, twice daily for 4 minutes. After each GC Tooth Mousse application, the sample surface was washed with distilled water and stored in saline containing 15 mM sodium azide for about 12 hours. At the end of the 14th day, each sample was carefully rinsed with distilled water, and air dried.

Microscopy and X-ray microanalysis

Samples were photographed using a SR stereomicroscope (Carl Zeiss AG, Oberkochen, D-73446 Germany) plus reflex digital camera (Optikam B2, Optikam s.r.l., Ponteranica BG, Italy).

Each sample was then attached to an aluminium stub (Philips) using a biadhesive tape and a conduction band of colloidal silver liquid between the root of the tooth and the aluminium stub was applied. After gold-sputtering, samples were analysed under scanning electron microscope (SEM) Quanta 200 (FEI, Eindhoven, The Netherlands) at 20 KV in the high vacuum (lower than 10⁻⁴ Torr) using the backscattered detector.

Samples were afterwards embedded in PMMA without infiltration, to avoid medium penetration inside tooth

cavities. The PMMA blocks containing the samples were sectioned along the longitudinal axis of the tooth using a diamond saw microtome (SP1600, Leica Microsystem, Nußloch, D-69226 Germany), to obtain 2 mm-thick sections of the embedded crown and root. The surfaces of tooth sections were then ground using 800 and 1200 grit silicon-carbide paper, polished with alumina, washed with distilled water and finished with a polishing cloth to obtain a shiny surface. Sections were then attached to an aluminium (Philips) stub using a biadhesive tape and, without any gold-sputtering, analysed under SEM Quanta 200 (FEI) at 20 KV in the low vacuum (0.53 Torr) using the backscattered detector, and analysed by X-ray microprobe (INCA, Oxford instruments, Abingdon, United Kingdom), EDS system, using a fragment of pressed CaHPO₄.H₂O as a standard reference. In each tooth, X-ray analyses were performed 5 times in each site.

Primer of Biostatistics 6th Ed. Software (New York, USA, 2005) was used for statistical analysis. Comparisons were performed by means of the nonparametric Kruskal-Wallis test followed by the Student-Newman-Keuls multiple comparison test [Bertoldi and Zaffe, 2012]. The null hypothesis H₀ was rejected for a critical significance level of P < 0.05.

Results

The stereomicroscope examination of teeth highlighted differences after processing with the three drinks (Fig. 2). Compared to 5 teeth (controls), CC teeth showed a pigmentation of crown and, particularly, root (Fig. 2B).

The step of enamel produced by drinks allowed a clear identification of the anatomic cementum-enamel junction (CEJ), not easily detectable in S teeth at the stereo microscope (Fig. 2A). G teeth showed an irregular enamel opacification, an almost untouched

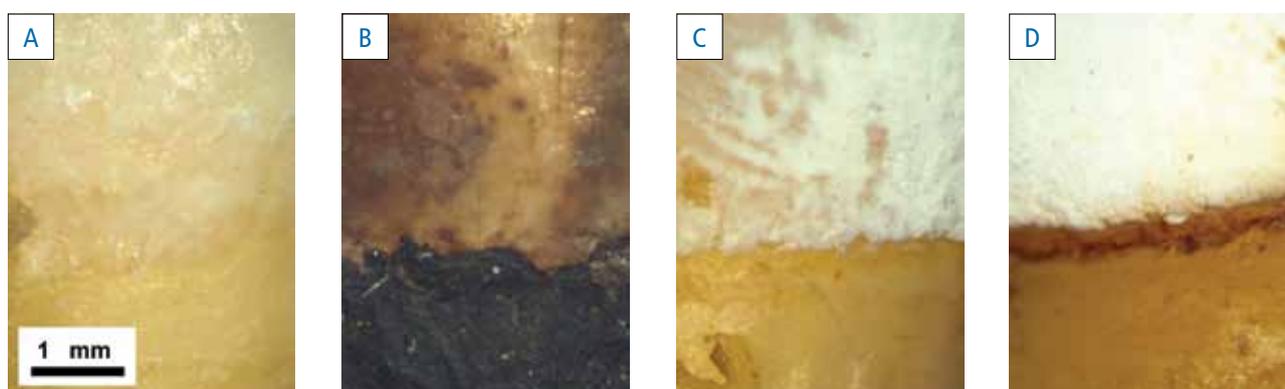


FIG. 2 Stereomicroscopic images of the teeth subjected to A, saline (S teeth); B, Coca Cola (CC teeth); C, Gatorade (G teeth); D, Red Bull (RB teeth), 15 minutes-per-day, for 7 days. Drink-treated teeth (B-D) showed a variable degree of dentine pigmentation, particularly deep in CC teeth (B). The enamel resulted darkened in CC teeth (B), irregularly opacified (whitened) in G teeth (C) and uniformly opacified in RB teeth (D). The cementum-enamel junction (CEJ) was scarcely distinguishable in S teeth (A), whereas it became clearly discernible in the drink-processed teeth (B-D) where the enamel rose on the dentine (the step of enamel).

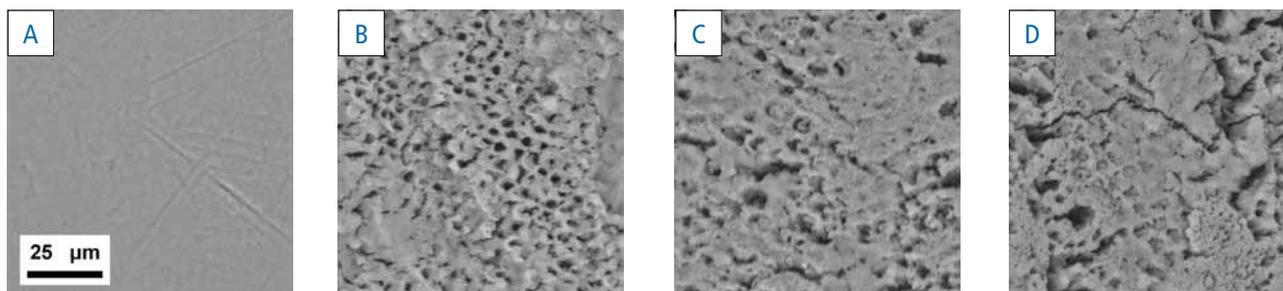


FIG. 3 Back-scattered SEM images showing the enamel surface at the cervical third of teeth processed with A, saline (S teeth); B, Coca Cola (CC teeth); C, Gatorade (G teeth); D, Red Bull (RB teeth), 15 minutes-per-day, for 7 days. The enamel of S teeth (A) had a smooth surface, with few very small pits and furrowed by some shallow grooves. The enamel of CC teeth (B) showed a rough surface of an irregular aspect: some zones had enamel prism removal, surrounded by others smeared with a non-uniform material. The enamel of G teeth (C) showed a spotted surface with smeared parts, partial prism removal, and excavations due to prism and interprismatic material erosion. The enamel of RB teeth (D) showed a surface with smeared parts, partial prism removal, and large excavations and deep cracks.

root, and an enamel surface discrepancy (step) at CEJ (Fig. 2C). RB teeth showed an almost uniform enamel opacification, enamel step and root aggression at CEJ (Fig. 2D). The following remineralising treatment did not produce substantial changes of the aspect of teeth, if we excluded a slightly lesser staining and enamel greater translucence.

The SEM examination of the enamel surface at the cervical third showed great modifications after processing with the three drinks (Fig. 3). The surface of CC teeth enamel appeared irregularly shaped with parts covered by smear materials and others which a clear aggression of prisms (Fig. 3B). The surface of G teeth enamel showed prism aggression, parts covered by smear materials, and the presence of cavities due to the prism, sheath and interprismatic material erosion (Fig. 3C). The surface of RB teeth enamel appeared covered by smear materials and grooved with several large pits (Fig. 3D).

The enamel surface appeared at SEM greatly modified after Tooth Mousse treatment (Fig. 4), which produced the greater modifications in CC teeth, and the minor modifications in G teeth. The subsequent Tooth Mousse treatment filled almost all the excavation produced by the drink on the enamel surface (Fig. 4A), particularly in CC teeth. The prisms' drink aggression appeared filled with amorphous material (Fig. 4A), and only the outer portion of few prisms resulted unfilled. The cavities previously formed by the drink action were abundantly reduced in depth in RB teeth (Fig. 4C), whereas the greater cavities in G teeth were highly unfilled in the superficial parts (Fig. 4B).

At a low magnification, the SEM examination of the sections showed the presence of a peripheral continuous demineralised layer of CC teeth enamel and an almost untouched dentine (Fig. 5B-L). In G teeth, the cervical-third enamel showed a peripheral irregular demineralised layer, and some dentine aggressions (Fig. 5C-L). In RB teeth, the cervical-third enamel showed a peripheral continuous demineralised layer, thicker than

that of CC teeth enamel, and dentine aggressions (Fig. 5D-L). At a great SEM magnification, the demineralised layer of the cervical-third enamel appeared different in size and morphology in the sections of teeth subjected to the three drinks (Fig. 5C-H). The demineralised enamel of CC teeth had an almost uniform thickness of 60-65 µm, an outer profile with small indentations, and a linear inner boundary (in contact with the sound enamel). Some small cleft, starting from the

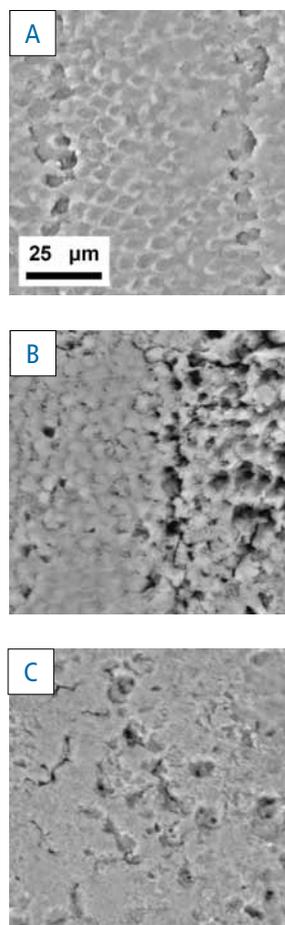


FIG. 4 Back-scattered SEM images showing the enamel surface at the cervical third of teeth subjected to A, Coca Cola (CC teeth); B, Gatorade (G teeth); C, Red Bull (RB teeth) 15 minutes per day, for 7 days, then treated with GC Tooth Mousse, for additional 7 days. The remineralising treatment by GC Tooth Mousse almost completely filled the cavities of the CC teeth enamel (A). Many cavities remain unfilled at the enamel surface of G teeth (B). An intermediate behaviour, with some small and shallow cavities, was recorded at the enamel surface of RB teeth (C).

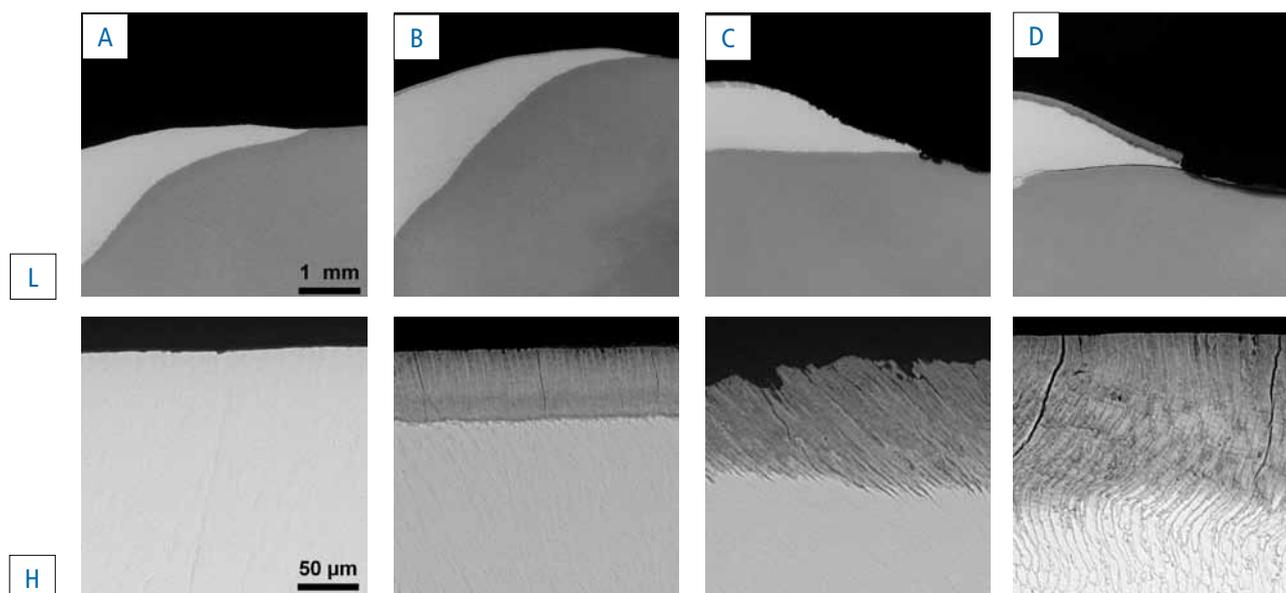


FIG. 5 Back-scattered SEM images of polished sections (L, low magnification; H, high magnification) showing the CEJ enamel and dentine aspect of teeth subjected to: A saline (S teeth); B, Coca Cola (CC teeth); C, Gatorade (G teeth); D, Red Bull (RB teeth), 15 minutes-per-day, for 7 days. Contrary to ST (L-A), drink-treated teeth showed dentine aggression and a peripheral demineralised layer of enamel: thinner in CC teeth (L-B), irregularly thick in G teeth (L-C) and thicker in RB teeth (L-D). Contrary to S teeth (H-A), the peripheral demineralised layer of enamel of CC teeth (H-B) showed a nearly uniform thickness, an indented surface by small cavities and crossed to the sound enamel by thin cracks. The layer appeared highly irregularly thick in G teeth (H-C). Its surface was greatly indented by large cavities from which some cracks deepened. In RB teeth (H-D) the layer was uniform but very thick, about 150 µm, and furrowed by large cracks from its poorly indented surface to the sound enamel.

external, crossed the layer reaching sometimes the inner boundary (Fig. 5B-H). The demineralised enamel of G teeth had an uneven thickness of 50-110 µm, a disorderly-shaped outer profile with wide excavations, an irregular-shaped inner boundary, and cleft crossing the layer up to the sound enamel (Fig. 5C-H). The demineralised enamel of RB teeth had an almost uniform thickness of 150 µm, an outer profile with few indentations, an irregular-shaped inner boundary, and cleft crossing the layer up to the inner boundary (Fig. 5D-H). The treatment of teeth with the GC Tooth Mousse did not produce changes of the morphological appearance of demineralised layers.

At a greater magnification, the SEM examination of sections in CEJ region showed demineralised layer of both enamel and dentine, but the almost same behaviour for the three drinks (Fig. 6). The knife-edge shaped enamel appeared cut off, and the demineralised layer (up to 50 µm) of the dentine extended a little way along the dentinoenamel junction (Fig. 6). The GC Tooth Mousse treatment did not produce CEJ morphological restoration in tooth sections.

The X-ray microanalysis confirmed the loss of phosphorus and calcium, and a small increase of carbon, in the demineralised layer with respect to the sound enamel (Figure 7). The quantitative analysis, performed using a fragment of pressed $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ as reference, highlighted a statistically significantly greater loss of calcium and a lower loss of phosphorus in the

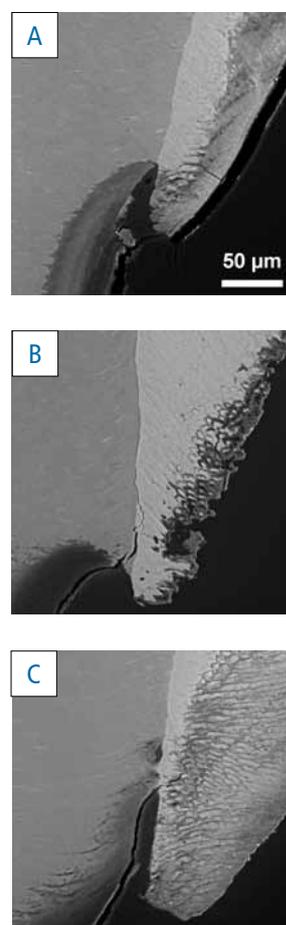


FIG. 6 Back-scattered SEM images of polished sections, at, showing the CEJ enamel and dentine aspects of teeth subjected to A, Coca Cola (CC teeth); B, Gatorade (G teeth); C, Red Bull (RB teeth), 15 minutes per day, for 7 days. Drink-processed teeth showed suddenly cut off the knife-edge shaped enamel at CEJ (enamel step), with the thinner peripheral demineralised layer in CC teeth (A), irregularly thick in G teeth (B) and thicker in RB teeth (C). The demineralisation of the dentine produced not only a layer up to 50 µ thick, but also the disappearance of a part of the tissue supporting the enamel at CEJ (enamel step).

demineralised layer of the cervical enamel subjected to the three drinks (Table 1). The loss of calcium (wt) was 17.5÷33% for CC teeth, 33÷41% for G teeth, and 20÷30% for RB teeth; the loss of phosphorus (wt) was 10÷25% for CC teeth, 28÷34% for G teeth, and 14÷25% for RB teeth; the consequent decrease in Ca/P rate (wt/wt) was 8÷10% for CC teeth, 6÷10% for G teeth, and 7÷9% for RB teeth. The treatment with the GC Tooth Mousse did not increase the mineral content of all sites of the demineralised layer (Table 1). A mineral increase was found only in G teeth, where both calcium and phosphorous increased 1.2 fold in the site 3, without reaching the initial value before the drink processing. In both drink and drink + tooth mousse teeth, the lower calcium and phosphorous content of sites 1, 2 and 3 was statistically significant against the corresponding values of site 4, after the nonparametric Kruskal-Wallis test followed by the Student-Newman-Keuls multiple comparison test. Contrarily, a statistical significance was found comparing the calcium content of site 2, and calcium and phosphorus contents of site 3 of G teeth and G + tooth mousse teeth, after Kruskal-Wallis + Student-Newman-Keuls test (Table 1).

Discussion

The acid action on tooth hard tissues directly causes erosion. The term erosion is used in dentistry to define the loss of dental hard tissues by chemical action, not involving bacteria [Grippo et al., 2012; Perez Cdos et al., 2012]. However, a large part of the cariogenic effect, especially in its initial stages, is based on the acid action [Reynolds, 2009]. The erosion is defined by the Standards of the American Society for Testing and Materials (ASTM) as “the progressive loss of a material from a solid surface due to mechanical interaction between that surface and a fluid, a multi component fluid, impinging on liquid and solid particles”.

Since it is accepted that caries and NCCLs have a multifactorial aetiology, the relative contributions of the

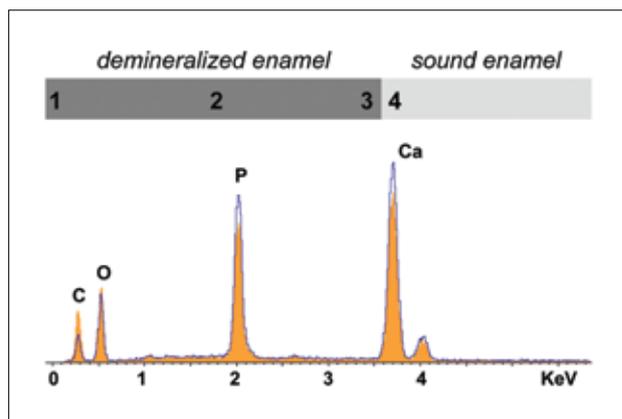


FIG. 7 Site diagram of the peripheral enamel analysis of teeth subjected to soft drinks and representative X-ray microanalysis graphs.

X-ray microanalyses were performed in the following sites: 1) at 10 µm from the surface; 2) in the center of the layer; 3) 10 µm before the boundary with the sound enamel; 4) 10 µm after the boundary (in sound enamel). A 100 µm thick layer was considered for S teeth.

The blue X-ray graph pertained to the sound enamel (site 4) and the orange graph to the site-3 enamel of a CC tooth. Notice the Ca and P reduction, but C and O increase.

various aetiological factors remain unclear for NCCLs [Grippo et al., 2012]. So, in our study, we considered the isolated effects of single erosion pathodynamics taking into account the tooth examined area. In order to exclude that the possible differences in range of properties between the different tooth sides might act as a disturbing variable, we split each tooth in three parts, so as to analyze the same area both in test and control samples.

It has been reported that any food substance with a critical pH value of less than 5.5 can become a corrosive agent that could demineralise teeth. The mineral phase is lost from the enamel, resulting in the formation of a lesion [Reynolds, 2009]. This can occur as a result of consuming highly acid foods and drinks such as citrus

site	Control			CC teeth						G teeth						RB teeth					
	A group			B group			C group			B group			C group			B group			C group		
	Ca	P	Ca/P	Ca	P	Ca/P	Ca	P	Ca/P	Ca	P	Ca/P	Ca	P	Ca/P	Ca	P	Ca/P	Ca	P	Ca/P
1	37.4	17.7	2.11	25.6*	13.4*	1.91*	26.3*	13.1*	2.01	22.5*	11.9*	1.90*	22.1*	12.1*	1.83	26.5*	13.6*	1.96*	25.4*	13.5*	1.88
2	37.5	17.8	2.11	27.8*	14.6*	1.91*	27.9*	14.7*	1.90	25.4*	12.9*	1.98*	26.8§	13.6*	1.97	28.5*	14.5*	1.97*	27.6*	14.2*	1.95
3	38.0	18.1	2.10	31.4*	16.2*	1.95*	31.5*	16.1*	1.96	24.0*	12.3*	1.96*	29.8§	14.8§	2.01	28.9*	15.0*	1.93*	28.8*	15.4*	1.87
4	38.1	18.1	2.11	37.7	17.8	2.11	37.8	18.0	2.10	38.0	18.1	2.11	37.9	18.0	2.10	37.5	17.8	2.11	37.8	17.9	2.11

A group = Saline treated teeth ; B group = Drink treated teeth; C group = Drink + GC Tooth Mousse treated teeth;
 * = p < 0.05 after Kruskal-Wallis test followed by the Student-Newman-Keuls multiple comparison test (against the corresponding content of site 4).
 § = p < 0.05 after Kruskal-Wallis test followed by the Student-Newman-Keuls multiple comparison test (against the content of the corresponding site of B group).

TABLE 1 Mean values of X-ray microanalysis of treated teeth in the sites indicated in the diagram of figure 7.

fruits, carbonated soft drinks, sucking on sour candies and acid mouthwashes also could be implicated [Perez Cdos et al., 2012; Jensdottir et al., 2005].

Various methods had been used to study the effect of soft drinks on enamel: AFM [Ferrazzano et al., 2007; Poggio et al., 2013], chemical dissolution [Jensdottir et al., 2005], microhardness [Rios et al., 2009; Seow and Thong, 2005; Van Eygen et al., 2005] profilometry [Barbour et al., 2006; Fujii et al., 2011; Hooper et al., 2005], SEM [Borjian et al., 2010; Brown et al., 2007; Ferrazzano et al., 2011; Ferrazzano et al., 2012; Owens and Kitchens, 2007; Torres et al., 2010]. Almost all these methods merely performed superficial analyses, with the exclusion of microhardness that penetrates the enamel, but up to 5 μm maximum [Seow and Thong, 2005]. The chemico-physical method we used to analyse both surface and subsurface, allowed us to investigate thoroughly the demineralisation and remineralisation mechanisms.

The analysis of tooth sections was indeed performed using different methods in different studies of enamel remineralisation [Reynolds, 2009; Shen et al., 2001], and cervical region demineralisation [He et al., 2011; Ferrazzano et al., 2011]. Enamel demineralisation was induced by Reynolds [2009] and Shen et al. [2001] using lactic acid in studies on casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) remineralisations. The microradiographic study revealed a subsurface demineralisation of enamel over 100 μm thick. He et al. [2011] used a pH 4.5 solution up to two days to induce CEJ demineralisation and found enamel and dentine demineralisation.

The temperature of drinks used in tooth processings greatly varied: 4° [Torres et al., 2010], room temperature [Van Eygen et al., 2005], 37° [Brown et al., 2007; Ferrazzano et al., 2012; Owens and Kitchens, 2007], or 75°C [Barbour et al., 2006]. Our tooth processing, at room temperature, roughly meant to simulate the effect of a cold drink warmed up in the mouth.

The time of teeth processing with the drinks was also very variable in the literature: 3 minutes, single treatment [Lussi et al., 2000], 5 minutes, 3 times a day, for 60 days [Torres et al., 2010], 20 minutes, 1 to 3 times a day, repeated five times [Van Eygen et al., 2005], 72 hours, single treatment [Jensdottir et al., 2005], 14 days, single treatment [Owens and Kitchens, 2007]. Our results, related to 7 days, 15 minutes per day, soft drink treatment, confirmed both the superficial and the subsurface demineralisation.

Contrary to He et al. [2011], who performed an almost continuous exposition of teeth to the acid solution for 1 or two days (with a change of the solution after 24 hours), our discontinued processing would simulate the drink action on the teeth of an average drinker. This discontinued tooth processing (15x7 minutes, for a total sum of 1 hour and 45 min) is significantly shorter than the 25 hours (90,000 seconds) of enamel exposure

per year, calculated for a heavy drinker by Owens and Kitchens [2007].

Our results highlighted several effects of drinks on teeth. CC teeth showed macroscopic enamel and dentine pigmentation. G and RB teeth had enamel opacification and macroscopic CEJ aggression. These effects, beyond a deep aesthetic modification, were exclusively the results of the drink aggression of the tooth surface. These aggressions were clearly shown by SEM analysis on the enamel surface, but the analyses of tooth sections clearly highlighted not only the different way of enamel aggression by the tested drinks, but also the dentine demineralisation and erosion at CEJ, which undermined the stability of the cervical enamel, as recorded by He et al. after tooth demineralisation at pH 4.5 [He et al., 2011].

Beyond the different size of shape of the demineralised layer of the enamel produced by the tested drinks, X-ray microanalysis highlighted an almost common behaviour of teeth subjected to the three drinks. All processed teeth lost both calcium and phosphorus, but not in a similar stoichiometric rate: calcium was lost more than phosphorus. This does not merely produce a lower Ca/P rate (1.91-1.98 w/w) with respect to the estimate Ca/P rate (2.11, close to the theoretic 2.15 w/w) of the sound enamel hydroxyapatite, but probably corresponds to a crystallographic change. The Ca/P rate would correspond to that of $\text{Ca}_3(\text{PO}_4)_2$ (Ca/P rate, w/w = 1.93), i.e. the superficial layer of enamel was turned from the crystalline form into an amorphous one. This structural change implies a deep reduction of the chemical and mechanical characteristics of enamel, strictly tied to the theoretical pathodynamic basis of NCCLs [Grippio et al., 2012].

Several recent clinical studies observed that gingival recession and NCCLs were often paired to form a complex pathology that must be treated in a not yet completely defined way [Pecie et al., 2011]. Furthermore, the abrasion pathodynamic pattern is commonly considered also in the ethiopathology of gingival recession. These clinical observation could find sustain in our experimental study. If CEJ was exposed to aggressive fluids, erosion processes could become synergic to gingival recession leading to dentogingival critical situation in a short time.

Our work, on the effect of three acid drinks, emphasizes the need of further studies to control the intake of potentially erosive foods, and the stringent preservation of dentogingival health. Furthermore, our study seems to draw particular attention to activities immediately after acid drink assumption. Tooth brushing immediately following the acid food intake can synergetically favor NCCLs by abrasion of a weakened hard tissue. A compatible pathodynamic could not be excluded even in cariogenesis although with respective different levels of effectiveness. Even the early carious lesions were reversible by a remineralisation process

involving the diffusion of calcium and phosphate ions into the lesion to restore the lost structure at the early stage [Reynolds, 2009; Hedge and Moany, 2012]. The diffusion of calcium and phosphate ions, through the protein/water-filled pores of the carious surface into the body of the enamel lesion, was required by the remineralisation process. Once in the body of the enamel lesion, these calcium and phosphate species increased the activities of Ca^{++} and PO_4^{---} . However, to obtain the formation and crystallisation of hydroxyapatite, the ion activity should be made in a compatible stoichiometry thereby increasing the level of saturation with respect to hydroxyapatite.

Our study highlighted that the erosion was even able to affect the deepest parts of the dental hard tissues by inducing substantial alterations in tissue properties. So, any hypothetical remineralising agent should ensure efficiently the availability of calcium and phosphate ions into the body of the hard tissue lesion. This property could be the limiting factor for enamel remineralisation to occur. Thus, the process of surface crystallisation or critical size cluster forming must be avoided to allow the diffusion of ions in depth [Reynolds, 2009; Hedge and Moany, 2012]. In the body of the lesion, the need of a delivery system for bioavailable calcium and phosphate ions was conjectured.

The different means now available in reversing the erosion of enamel and dentine had positive but not determinant effects [Pecie et al., 2011]. Even the use of fluoride drugs does not seem to impact significantly, especially in deep areas of the erosive lesions [Reynolds, 2009]. The non-invasive treatment of early caries lesions by remineralisation has the potential to be a major advance in the clinical management of the disease, within the stoichiometry needs [Reynolds, 2009]. Even the use of amorphous calcium phosphate (ACP - $\text{Ca}_3(\text{PO}_4)_2 \cdot n\text{H}_2\text{O}$) as remineralizing agents seemed to prove effective. ACP could play a special role as a precursor to bioapatite and as a transient phase in biomineralisation, but ACP nuclei formed spontaneously in neutral and alkaline supersaturated calcium-phosphate solutions. So, ACP was converted readily to stable crystalline phases such as products and thus could hardly reach the subsuperficial areas [Reynolds, 2009].

Differently from other used remineralising agents, the casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) would possess chemical requirements to produce better results [Ferrazzano et al., 2007; Hedge and Moany, 2012; Iijima et al., 2004; Pecie et al., 2011; Reynolds, 2009]. The remineralisation process involved diffusion of calcium and phosphate ions through the protein/water-filled pores of the carious surface enamel into the body of the enamel lesion. Furthermore, dissociation of the CPP-bound ACP should be facilitated by the acid generated during enamel remineralisation. This would explain why CPP-supported, metastable

calcium phosphate solutions were such efficient remineralizing solutions, as they would consume the acid generated during enamel lesion remineralisation by generating more calcium and phosphate ions, thus maintaining their high concentration gradients into the lesion. By stabilising calcium phosphate in a metastable solution, the CPP could facilitate high concentrations of calcium and phosphate ions, which could then spread into the enamel subsurface lesion [Hedge and Moany, 2012].

In our study, we used the CCP-ACP as a remineralising agent. We observed a mineral smear on the surface, filling almost all the cavities produced by drinks. However, we did not follow the morphological resolution of the demineralised layer after the CCP-ACP use. The X-ray microanalysis revealed the Ca/P rate did not reach the original hydroxyapatite value (2.1 wt/wt), and a statistically significant mineral content increased only in GT teeth, that is the teeth showing the greater mineral deprivation. Moreover, these increases were recorded in the deeper parts of the demineralised layer. Our results agree with those of Hedge and Moany [2012] which found the remineralisation of completely demineralised enamel by superficial X-ray microanalysis, not in enamel sections. Contrary to Hedge and Moany [2012] we analyzed the sections of the mineralised enamel recovering significant increment of the mineral content after CCP-ACP use only in a portion of the treated teeth. This result, together with the lacking of the Ca/P rate increment, could suggest a positive action of the CCP-ACP, but with a resolution of the erosive pathology with long time. Our study was performed to observe the results of an erosion and remineralising activity for a period of time similar for the two treatments. It is likely that the remineralising activity needed a longer time of application before reaching complete results. However, within the limits of this study, we did not find a Ca/P stoichiometric rate increase reaching that of the hydroxyapatite. A subsequent series of studies concerning timing and modalities of application using our model of study should be performed to warrant our supposition on CCP-ACP action.

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Conflicts of interest

Authors declare that they have no conflict of interest.

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