Surface and Subsurface Erosion of Primary Enamel by Acid Beverages over Time

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This study evaluated the influence of a cola-type soft drink and a soy-based orange juice on the surface and subsurface erosion of primary enamel, as a function of the exposure time. Seventy-five primary incisors were divided for microhardness test (n=45) or scanning electron microscopy (SEM) analysis (n=30). The specimens were randomly assigned to 3 groups: 1 - artificial saliva (control); 2 - cola-type soft drink; and 3 - soy-based orange juice. Immersion cycles in the beverages were undertaken under agitation for 5 min, 3 times a day, during 60 days. Surface microhardness was measured at 7, 15, 30, 45 and 60 days. After 60 days, specimens were bisected and subsurface microhardness was measured at 30, 60, 90, 120, 150 and 200 μm from the surface exposed. Data were analyzed by ANOVA and Tukey's test (α=0.05). Groups 2 and 3 presented similar decrease of surface microhardness. Regarding subsurface microhardness, group 2 presented the lowest values. SEM images revealed that after 60 days the surfaces clearly exhibited structural loss, unlike those immersed in artificial saliva. It may be concluded that erosion of the surfaces exposed to the cola-type soft drink was more accentuated and directly proportional to the exposure time.

Key Words: primary teeth, enamel, erosion, aprismatic layer.

INTRODUCTION

Over the last decades, the decline in the prevalence of dental caries in the world population has been accompanied by a remarkable increase in the incidence of non-carious lesions, such as dental erosion, that lead to an irreversible loss of tooth structure, characterizing tooth wear.

Dental erosion is defined as the pathological, chronic, localized and painless loss of hard tooth tissue resulting primarily from non-bacterial chemical attack, and usually involving acid substances (1). It may be caused by intrinsic factors (e.g.: bulimia, anorexia, and other gastrointestinal disturbances with frequent gastric acid reflux) (2) and/or extrinsic factors, such as the consumption of beverages and acid fruit juices, acid filling of a chewing gum, as well as the exposition to acid contaminants (3,4) and a new type of erosive challenge caused by a sour sweet, a kind of candy which contains organic acids, particularly lactic, citric and malic acid, in order to developed the characteristic sour flavor (5).

Over the last decades, there has been a significant worldwide increase in the consumption of acid beverages, such as soft drinks and ready-to-use fruit juices. Furthermore, soft drinks placed in nursing bottles have been increasingly consumed and earlier introduced to the diet of young children.

Several *in vitro* methods have been proposed to

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evaluate dental erosion, including microhardness tests (6,7), which are directly related to alterations in the mineral content of dental hard tissues. The literature is still scarce on articles investigating the aspects regarding the erosion in deciduous teeth. In addition, to the best of our knowledge, no study has yet used primary teeth without removing the aprismatic enamel layer. This layer may influence the behavior of the solutions because it is less permeable than the underlying enamel. Therefore, the purposes of this study were: to evaluate *in vitro* the influence of 2 types of beverages frequently ingested by children on the surface and subsurface erosion of primary enamel, by means of mineral loss determined by a microhardness test, as a function of the exposure time; and to observe the surface morphology after the experimental period using scanning electron microscopy (SEM). The erosion potential of popular beverages is important for clinical guidelines regarding beverage consumption practices and development of potentially "safer" beverages, especially for children.

It was hypothesized that: 1. there is no difference in the erosive effect between the beverages studied (a soy-based orange juice and a cola-type soft drink); 2. there is no difference in the erosive effect with respect the exposure time; 3. there is no difference in enamel demineralization regarding the depth measurements.

MATERIAL AND METHODS

This study was approved by the Research Ethics Committee of the Ribeirão Preto Dental School, University of São Paulo, Brazil (process #2003.1.1079.58.6).

Experimental Design

This study was designed as randomized complete blocks and comprised 75 specimens. The variation factors were: beverages, with 3 levels [2 experimental (cola-type soft drink and soy-based orange juice) and one control (artificial saliva)]; exposure time, with 5 levels (7, 15, 30, 45 and 60 days); and depth, with 6 levels (30, 60, 90, 120, 150 and 200 µm from enamel surface).

Quantitative response variables were percent surface Knoop microhardness change (%SMH) and Knoop microhardness number (KHN), in kgf, at different depths from enamel surface, i.e., subsurface microhardness. SEM was used to observe the effects of beverages on primary enamel surface morphology.

Selection of Teeth

Healthy human primary central incisors were obtained from the tooth bank of the Ribeirão Preto Dental School, University of São Paulo, and immersed in a 2% formaldehyde solution (pH 7.0) for 30 days. Teeth were cleaned with pumice-water slurry using Robinson bristle brushes in a low-speed handpiece. Then, they were examined with a stereomicroscope (Nikon Inc. Instrument Group, Melville, NY, USA) at 10× magnification to discard those with cracks, fractures or structural abnormalities that could interfere in the results. Thirty teeth were randomly separated for SEM analysis and the remaining destined for microhardness test.

Preparation of Specimens and Initial Surface Microhardness Measurements

The teeth allocated for microhardness test had their roots, when present, removed at the cementoenamel junction with the water-cooled diamond saw of a precision sectioning machine (Miniton; Struers A/S, Copenhagen, Denmark). Each crown was fixed with plastic wax in the central orifice of an acrylic plate, with its buccal surface faced upwards using a parallelometer (ElQuip, São Carlos, SP, Brazil) to keep the flattest region of the buccal face (incisal third) parallel to the plate. Next, the crowns were stabilized with red wax.

The tooth/plate sets were rendered acid proof by coating them with 2 layers of cosmetic nail polish (Colorama Maybelline Ultra Duração; Cosbra Cosméticos Ltda, São Paulo, SP, Brazil), leaving uncoated a circular enamel area (3 mm in diameter) on the flattest region of the buccal surface. Prior to the initial microhardness test, specimens were immersed in artificial saliva for 24 h at 37ºC. Initial Knoop microhardness was assessed on the uncoated enamel area using a microhardness tester (Shimadzu HMV-2000; Shimadzu Corporation, Kyoto, Japan). Settings for load and penetration were 50 g and 5 s. The uncoated enamel area of each specimen received 5 penetrations as follow: a central penetration and four 500-µm-spaced penetrations apart from the center toward right, left, up, and down directions. An average microhardness value was calculated for each specimen, and after an overall mean value was obtained from all averages. The specimens that presented averages 10% higher or lower than the main value were discarded, as well as those with an individual standard deviation 10% above

or below the average, i.e., among the 5 penetrations. Based on this criterion, 45 specimens were selected for microhardness test and their averages were considered as initial surface microhardness values.

Immersion Cycles in the Beverages

After the initial microhardness measurements, 15 specimens were allocated at random to each group: artificial saliva - control (KH2PO4, K2HPO4, KCl, NaCl, MgCl2.6H2O, CaCl2.2H2O, NaF, sorbitol, nipagin, nipasol, carboxymethylcellulose (CMC), water/ Laboratory of Pharmaceutical Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil), cola-type soft drink (Coca-Cola; Cia. Fluminense de Refrigerantes, Porto Real, RJ, Brazil) and soy-based orange juice (Ades Orange; Unilever Bestfoods Brasil Ltda., Pouso Alegre, MG, Brazil). The 30 teeth initially separated for SEM analysis (not embedded in acrylic plates and having all surfaces exposed) were randomly added to the same groups and jointly submitted to the respective immersion cycles.

The pH of the commercially available beverages used for the immersion cycles (the cola-type soft drink containing phosphoric acid and the soy-based orange juice containing citric acid) was measured with a digital pH meter (Analion AN2000 Microprocessado, Ribeirão Preto, SP, Brazil) at 4ºC (Coca-Cola = 2.35; Ades Orange = 3.86). The titratable acidity was determined once before the immersion cycling protocol by assessing the amount of 5 N sodium hydroxide needed to raise the pH of 500 mL of the beverage to 7.0, which was 3.7 mL and 2.6 mL for cola-type drink and Ades® Orange, respectively.

The following immersion cycling protocol was adopted to simulate a considerable number of intakes: the specimens were immersed for 5 min in 75 mL of the beverage under agitation by a magnetic stirrer (Fanen, São Paulo, SP, Brazil) 3 times a day with 4-h intervals between the immersion cycles, during a 60 days experimental period. After each immersion cycle, the specimens were washed with distilled water, gently dried with gauze and maintained in 15 mL of artificial saliva at 37ºC until the next immersion cycle. The control specimens were kept in artificial saliva during the course of the experiment (60 days), with daily change of the solution.

In order to maintain an acceptable level of carbon dioxide in the cola-type drink, a new can was opened for every 5-min immersion time in the beverage, and the unused drink remaining in the can was discarded. The unused orange juice remaining in the package was discarded at the end of each day. Artificial saliva was kept at $37 \pm 1°C$. The beverages were used at their usual consumption temperatures (approximately 4ºC).

Percent Surface Microhardness Changes (%SMH)

Surface microhardness of the uncoated enamel area was measured at 7, 15, 30, 45 and 60 days after the continuous and systematic repetition of the daily immersion cycles. Microhardness measurements were performed as described for the initial values. %SMH was calculated as a percentage of the initial microhardness measurements, using the following equation: <u>initial microhardness – final microhardness</u> ×100

Initial Subsurface Microhardness Measurements

Subsurface microhardness was tested at the end of the 60 days experimental period. The specimens were included in autopolymerizing resin blocks (JET; Clássico Artigos Odontológicos, São Paulo, SP, Brazil), which were bisected longitudinally in a sectioning machine (Minitom; Struers A/S, Copenhagen, Denmark), resulting in 2 sections *per* tooth. The sections were wet ground in a polishing machine (DP-9U2, Struers A/S) with #1500-grit silicon carbide paper (Norton/ Saint-Gobain Abrasivos Ltda, Guarulhos, SP, Brazil) and polished with a 1 μm alumina paste (Struers A/S) to obtain a smooth surface for microhardness measurements. The measurements were made at the distances of 30, 60, 90, 120, 150 and 200 μm from the surface exposed to the beverages. Three 100-μm-spaced linear penetrations were performed for each distance to obtain a mean value.

Statistical Analysis

All statistical procedures were performed using a statistical software (NCSS/PASS Dawson edition; NCSS, Kaysville, UT, USA) at a significance level of $\alpha=5\%$. Data showed normal and homogeneous distribution, thus, microhardness values were analyzed by two-way ANOVA and Tukey's LSD multiple-comparison test with time/beverage and beverage/depth as study factors. For surface microhardness data, %SMH was used for the

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factors beverage and time. For subsurface microhardness data, KHN means at the different depths were analyzed separately, comparing the beverages.

SEM Analysis

Two teeth from each group were retrieved at the 7th, 15th, 30th, 45th, and 60th days of immersion cycles and maintained in distilled and deionized water at 4ºC. SEM analysis was performed at the 60th day of the experiment. The following protocol was undertaken: the specimens were immersed for 10 min in a ultrasonic cleaner (T-1449-D; Odontobrás Ind. e Com., Ribeirão Preto, SP, Brazil) containing distilled water; posterior dehydration in an ascending ethanol series (25%, 50%, 75%, 95% and 100%); mounting on stubs; sputter-coating with gold and analysis in a scanning electron microscope (Philips XL30 FEG-SEM; Philips Electron Optics, Eindhoven, Holland), operating at 20 kV. The entire buccal surface of each tooth was scanned and the most representative images were recorded at magnifications of 8,000× and 20,000×. The SEM analysis was intended to provide a visual and illustrative comparison of the specimens and hence no statistical analysis was performed.

RESULTS

Percent Surface Microhardness Change (%SMH)

%SMH means, and standard deviations, as a function of exposure time to the beverages are displayed at Table 1.

Comparison of the beverages showed that the soy-based orange juice produced a statistically similar decrease in microhardness as that of the cola-type soft

drink and significantly different from that of artificial saliva, which caused an increase in microhardness.

With respect to the exposure time, there was minimal, non-significant alteration up to the 30th day for all groups, and thereafter a significant accentuation of microhardness loss at 45 days, being even more evident at 60 days.

The time x beverage interaction showed that artificial saliva produced a gradual and significant gain in surface microhardness up to 30 days, occurring stabilization thereafter. For the soy-based orange juice, there was a loss of microhardness, which remained stable up to the 30th day and increased significantly ($p<0.05$) at 45 days, being more accentuated after 60 days ($p<0.05$) of immersion. The cola-type soft drink produced gradual, statistically significant alteration in microhardness at all times, except for 15, and 30 days, at which statistically similar results were observed.

Subsurface Microhardness

KHN and standard deviations, as a function of the distance from enamel surface are displayed at Table 2.

There was statistically significant difference between the cola-type soft drink and the soy-based orange juice regarding subsurface microhardness. The control group showed a statistically difference from the other groups, with a higher microhardness.

Regarding the depths, the surface microhardness was similar to that measured at 90 μm and 120 μm and significantly different from the others, occurring a significant gradual increase in microhardness with the

Table 1. Percent surface microhardness change (%SMC) means and standard deviations as a function of exposure time to the beverages.

Time	Artificial saliva	Ades® orange	Coca-Cola®
7days	- 2.18 (7.53)c	4.73 (3.25)de	2.35 (5.97)d
15 days	- 12.04 (4.15)b	4.67 (3.97)e	8.33 (7.2)ef
30 days	- 20.01 (5.22)a	5.29 (2.95)e	8.29 (5.11)ef
45 days	- 20.86 (5.74)a	11.85 (3.66)f	15.06 (4.74)g
60 days	- 20.7 (5.23)a	17.67 (3.83)gh	20.08 (4.71)h

Different letters indicate statistically significant difference at 5%.

Table 2. Knoop microhardness number (KHN) and standard deviations as a function of enamel depth.

Time	Artificial saliva	Ades® orange	Coca-Cola®
Surface	307.43 (1.62)a	209.86 (2.67)f	204.14 (1.46)f
30 μm	233.14 (4.98)e	211.14 (2.27)f	205 (1.15)f
60 μm	240.43 (4.12)e	222.86 (2.85)e	213.57 (2.64)e
90 μm	249.14 (4.14)d	234.86 (2.67)d	224.86 (5.21)d
120 μm	258.71 (0.95)c	243.43 (5.19)c	235.43 (5.71)c
150 μm	269.29 (5.15)b	253.71 (2.36)b	248.29 (6.45)b
200 μm	271.29 (2.14)b	266.43 (1.51)a	268 (1.53)a

Same letters indicate statistical similarity - intragroup comparison. Colored cells indicate statistical similarity - intergroup comparison.

increase of distance.

The beverage x depth interaction showed that the beverages presented similar results only at the depth of 200 μm. For the control group, the surface microhardness presented significant higher values than the subsurface (30 μm) and the other distances. Regarding the soy-based orange juice, there was lower surface microhardness, which was similar to the 30 μm distance and significantly lower than the other distances, being recorded a gradual and significant increase up to the distance of 200 μm. The cola-type soft drink had similar behavior to that of the soy-based orange juice, except for the distances of 60 up to 120 μm.

SEM Analysis

Panels of SEM micrographs of enamel immersed in the beverages and artificial saliva are presented in Figures 1 to 3.

The SEM images of the cola-type soft drink group showed an increasing alteration of surface enamel as the exposure time increased. At 7 days, there was an accentuated demineralization of the entire surface, being more pronounced on the interprismatic portion (Fig. 1A). Throughout the experimental period, there was gradual demineralization, with apparent loss of minerals (Figs. 1B to 1E). After 60 days of immersion
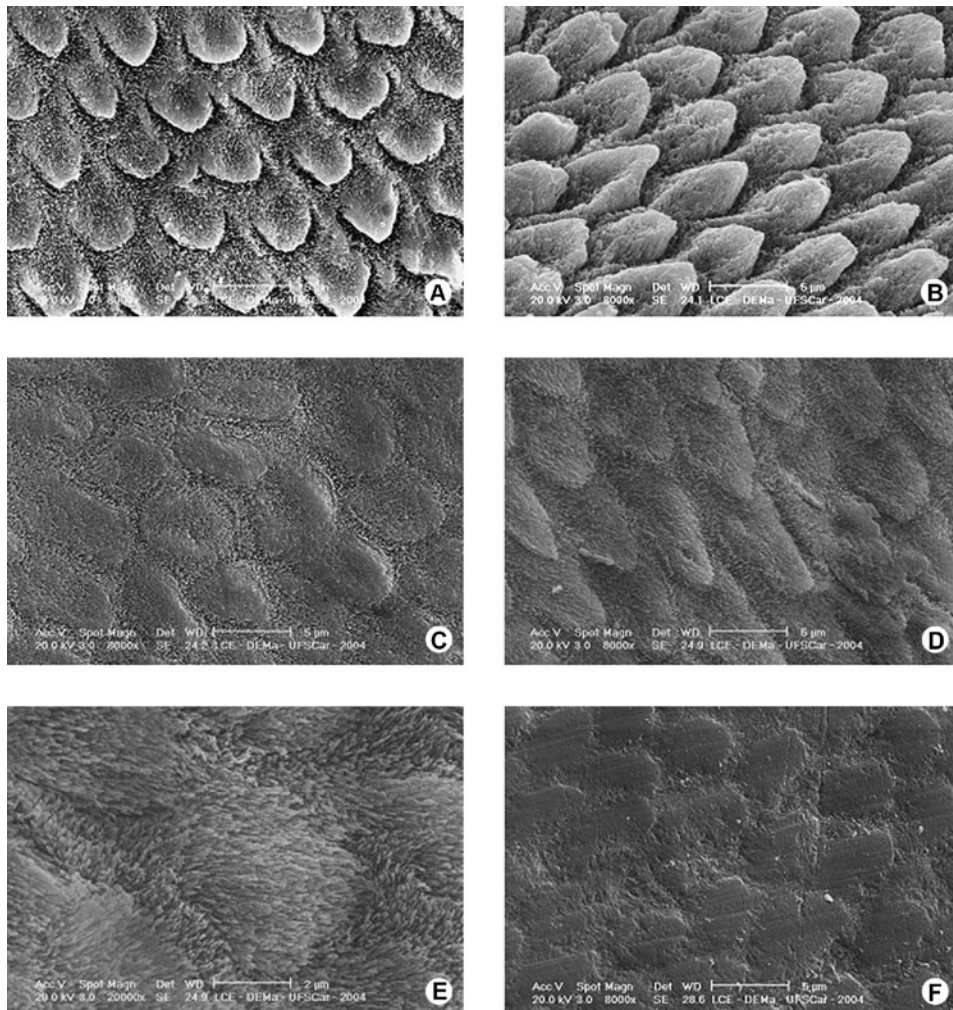

Figure 1. Enamel immersed in the cola-type soft drink. A = After 7-day immersion, there was great demineralization, especially on the interprismatic portion. B = After 15-day immersion, there was accentuated demineralization of the interprismatic region, exposing the enamel prism heads. C = After 30-day immersion, there is generalized demineralization. D = After 45-day immersion, enamel is widely demineralized. E = Detail of the enamel prism head after 45 days of immersion. F = After 60 days of immersion, the enamel exhibited an evident structural loss.

cycles, the surface clearly exhibited structural loss and was planned and worn. At the end of the experiment (60th day), the enamel prisms of the specimens exposed to

the beverages were hardly identifiable (Fig. 1F), unlike those of the specimens immersed in artificial saliva, in which the enamel of the aprismatic layer presented with
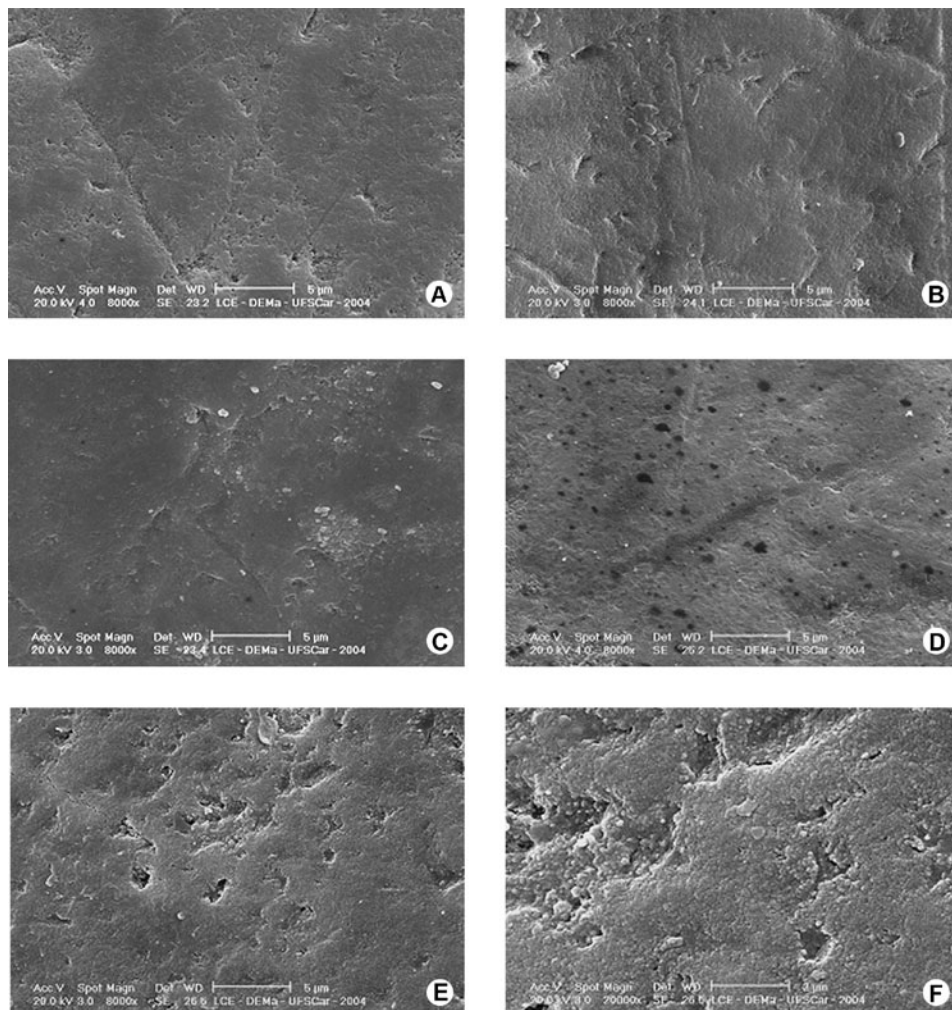

Figure 2. Enamel immersed in the soy-based orange juice. A = After 7-day immersion. B = After 15-day immersion. C = After 30-day immersion. D = After 45-day immersion. E and F = After 60-day immersion, an extremely irregular surface with small enamel depressions is observed (arrows).
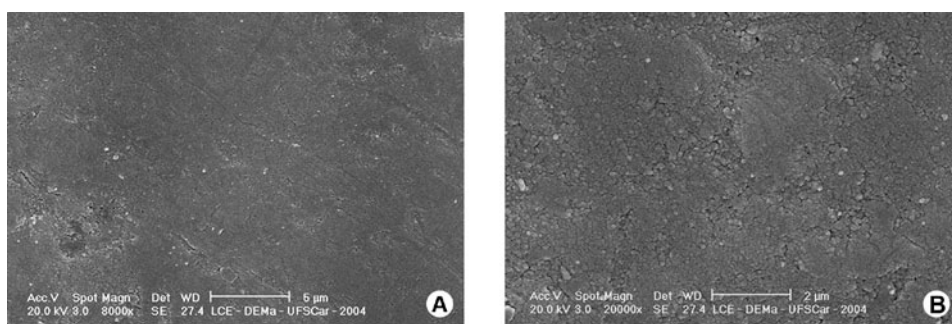

Figure 3. Enamel immersed in artificial saliva. A and B - After 60 days of immersion.

an amorphous aspect (Fig. 3A).

The specimens exposed to the soy-based orange juice presented a similar surface to that of the control group and an intact enamel aprismatic layer up to the 15th day of immersion (Figs. 2A to 2D). After 60 days, the enamel surface of the specimens in this group was irregular (Figs. 2E and 2F) and differed from that of the control specimens (Fig. 3B). However, there was no region with exposure of enamel prisms.

DISCUSSION

The findings of the present study showed that the beverages employed (Ades® Orange and Coca-Cola®) can potentially erode the enamel of primary teeth after successive immersion cycles. The erosive effect was confirmed by a softening of the enamel surface, which presented a decrease in Knoop microhardness, resultant from the mineral loss caused by acid beverages intake (4,8-11).

Both acid beverages tested in this study had a similar erosive effect on surface enamel, but cola-type soft drink caused greater demineralization in depth, which might be attributed to some of its characteristics, such as pH, buffering capacity, calcium, fluoride and phosphate contents, type of acid, and its titratable acidity (10,12). The greater the titratable capacity of the beverage, the longer it takes saliva to neutralize the acid (10). In addition, the erosive potential could be based on the degree of saturation of hydroxyapatite and fluorapatite, by determining the pH, calcium, phosphate and fluoride content of a beverage (13).

Regarding surface microhardness changes, it was observed that microhardness decreased after 15 days and, despite the difference in their acid composition, both beverages had similar behavior at all time points. In addition, there was an increase in surface microhardness loss by both beverages as a function of time. It is assumed that long-term consumption of acid beverages may soften dental hard tissues (14). A direct correlation between the prevalence of erosion and consumption of soft drinks was previously demonstrated (10). Similar results have been reported by a previous study (14), which found an increase in erosion, as measured by mineral loss and lesion depth, with increase of exposure time.

The SEM analysis showed a progressive destruction of the enamel ultrastructure with the increase of exposure time, mainly specimens immersed in cola-type soft drink. This enamel dissolution may have occurred due to the loss of the protective glossy enamel surface, which is more resistant to rapid dissolution (14). The specimens exposed to the soy-based orange juice presented perceptible surface alteration only after the 45-day immersion cycle, which probably occurred due to its low buffering capacity and to the presence of amorphous aprismatic enamel on tooth surface. The findings of a scanning electron microscope study (15) of the enamel surface of primary teeth immersed in different acidic beverages showed that enamel prism demineralization increased with the increase of exposure time, being more accentuated in the specimens exposed during 12 h than in those immersed in the solutions during 15 min. A gradual mineral loss has also been reported by Maupomé et al. (7) after exposing primary teeth enamel to cola-type soft drink.

In the present study, the aprismatic enamel layer was not removed in order to reproduce as reliably as possible the conditions that actually occur in the mouth. However, this fact may have influenced the behavior of the solutions because this layer is less permeable than the underlying enamel. Intact tooth surfaces have been shown to soften at slower rates than ground tooth surfaces, being less soluble as well (14). Therefore, it is likely that ground enamel respond differently from intact enamel to the exposure to beverages.

The goal of undertaking subsurface microhardness measurements was to assess the capacity of penetration of the beverages into primary enamel. Hall et al. (16) evaluated the remineralizing effect of saliva by microradiography analysis using longitudinal enamel and dentin sections exposed to an erosive challenge with different solutions. This methodology differed from that of the present study in which only the intact enamel surface was exposed to the beverages and longitudinal sectioning of the specimens was made at the end of a 60-day period of immersion/agitation cycles. Using microradiography and image analysis, Amaechi and Higham (17) observed that 1-h immersion in orange juice produced eroded enamel lesions in bovine incisors, characterized by a shallow crater with subsurface demineralization, reaching a depth of 75 μm. Therefore, potentially erosive solutions act in depth and the present study was aimed at evaluating how depth the erosive action of the tested beverages could reach. Both soy-based orange juice and cola-type soft drink altered enamel surface and subsurface (30 μm), with a gradual increase in microhardness up to the distance of 150 μm from the exposed enamel surface. The cola-type

soft drink group presented lower microhardness means, which demonstrates a more aggressive behavior of this beverage. Soy-based orange juice and cola-type soft drink groups presented similar microhardness means to each other only at the distance of 150 μm and only at the distance of 200 μm both groups presented similar results to those of the control group (artificial saliva), which indicates that only at that point there was no more interference of the beverages on primary teeth enamel microhardness.

The analysis of the bulk of eroded enamel lesions caused by cola-type soft drink presented lower microhardness, although the orange juice had citric acid in its composition. This fact may be explained by the analysis of the titratable acid content of the solutions, which indicates the need of a greater amount of sodium hydroxide to neutralize the action of cola-type soft drink compared to soy-based juice, and may explain the different demineralization behavior of these solutions. Additionally, according to information from the manufacturer, the soy-based juice employed presents calcium in its composition (13.5 mg/100 mL), which may decrease the erosive potential of citric acid. Previous studies (8,18) reported that the addition of calcium, phosphate and fluoride ions to acid beverages was effective in reducing their erosive potential.

Differently from the present study, Sanchez and Fernandez De Preliasco (19) found that Ades Orange soy-based juice had lower pH and higher buffering capacity than cola-type soft drink. However, the beverages took the same time to be neutralized by saliva in patients with eroded enamel lesions. These findings demonstrate that dental erosion occurs when there is an imbalance between the consumption of the beverage and the organism's protective capacity, more specifically the buffering capacity of saliva, and not only due to the type of the acid present in the composition of the beverages (20).

Artificial saliva containing calcium, phosphate and fluoride was used as a control because it has been proved to exert the same remineralizing effect as that of fresh human saliva (17,20). This fact was confirmed in the present study as the control specimens exhibited a gradual microhardness gain up to the 30th day and stabilization thereafter. This stabilization may be explained by the saturation on enamel surface. The analysis of the eroded lesion at different depths revealed that the surface presented higher microhardness than the subsurface, probably because of the incorporation of minerals to the outer surface.

Another important factor was the agitation of the solution during immersion of the specimens because it has been shown that (6,7,13) immersion under agitation increases the degree of erosion produced by acid beverages on primary and permanent teeth enamel.

Although all experimental steps of this study were conducted in a judicious manner and strictly according to the protocol, *in vitro* and *in situ* studies have limitations, and therefore their results cannot be extrapolated to clinical conditions. Dental erosion associated with consumption of acidic beverages is a potential oral health concern. Thus, in view of the increasing soft drinks and ready-to-use fruit juices among children, further research should be undertaken to widen the scopes and knowledge in this research field.

Based on the results of this study, it may be concluded that the null hypothesis were rejected since the cola-type soft drink and the soy-based orange juice reduced surface and subsurface microhardness of primary enamel. Longer exposure to the beverages resulted greater alteration of enamel microhardness. Changes in enamel microhardness at different depths were evident for both beverages up to 150 μm, and the cola-type soft drink produced more accentuated primary enamel alteration than the soy-based orange juice.

RESUMO

Este estudo avaliou a influência de um refrigerante a base de cola e um suco de laranja a base de soja na erosão da superfície e subsuperfície do esmalte do dente decíduo, em função do tempo de exposição. Setenta e cinco incisivos decíduos foram divididos para o teste de microdureza (n=45) ou para a análise em microscópio eletrônico de varredura (MEV) (n=30). Os espécimes foram alocados aleatoriamente em 3 grupos: I - saliva artificial (controle); II - refrigerante a base de cola; e III - suco de laranja a base de soja. Ciclos de imersão nas bebidas foram realizados sob agitação durante 5 min, 3 vezes ao dia, durante 60 dias. A microdureza superficial foi mensurada aos 7, 15, 30, 45 e 60 dias. Após 60 dias, os espécimes foram seccionados e a microdureza subsuperficial foi mensurada aos 30, 60, 90, 120, 150 e 200 μm. Os dados foram analisados pelos testes ANOVA e Tukey (α=5%). Os grupos II e III apresentam uma diminuição similar da microdureza superficial. O grupo II apresentou menores valores de microdureza subsuperficial. As imagens de MEV revelaram que após 60 dias as superfícies mostraram perdas de estruturas claramente identificadas, diferentemente das superfícies imersas em saliva artificial. Pode-se concluir que a erosão das superfícies expostas ao refrigerante a base de cola foi mais acentuada e diretamente proporcional ao tempo de exposição à bebida.

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