Adding Casein Phosphopeptide-amorphous Calcium Phosphate to Sports Drinks to Eliminate In Vitro Erosion

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Abstract

Purpose: Enamel erosion can occur with frequent consumption of sports drinks. The purpose of this study was to determine a minimal concentration of casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP) which when added to a sports drink would eliminate such erosion in vitro.

Methods: Human enamel specimens were immersed in: (1) the sports drink Powerade; (2) Powerade plus 4 concentrations of CPP-ACP (0.063%, 0.09%, 0.125%, 0.25%); or (3) double deionized water. Windows of test and control enamel were profiled, and the enamel surface characteristics were examined under scanning electron microscopy (SEM).

Results: The pH of test solutions increased and the titratable acidity decreased with increasing CPP-ACP concentrations. Erosive step lesions occurred in specimens immersed in Powerade (mean depth=38.70kA±5.60), which were eliminated by the addition of CPP-ACP to Powerade at all test concentrations except 0.063% CPP-ACP. Microscopic surface irregularities on test enamel were observed, apparent as adherent granules or globules. These may represent redeposited mineral phases following mobilization of calcium and phosphate from CPP-ACP. Tasters in a taste panel could not distinguish Powerade from Powerade plus 0.125% CPP-ACP.

Conclusions: Adding casein phosphopeptide-stabilized amorphous calcium phosphate to the sports drink Powerade significantly reduced the beverage's erosivity without affecting the product's taste. (*Pediatr Dent.* 2005;27:61-67)

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Dental erosion (also known as corrosion) is a chronic loss of dental hard tissue, which is chemically removed by acid and/or chelation without bacterial involvement.[1] Typical acidic sources come from the diet, medications, occupational exposure, and lifestyle activities.[2] The prevalence of erosion is thought to be increasing, reflecting the wide availability and frequent consumption of acidic beverages, fruit juices, carbonated beverages, wines, and sports drinks.[3-6]

The consumption of sports drinks by athletes, recreational users, and adolescents has increased with heavier promotional advertising.[7,8] By formulation, sports drinks are palatable beverages that prevent dehydration, supply carbohydrates, and replenish lost electrolytes.[6] Sports drinks have a low pH, high titratable acidity, and may be viscous. Their erosive potential is increased when they are consumed during periods of dehydration and low salivary flow, as can occur with exercise.[5]

The erosive potential of sports drinks also depends on the acid type or food acid incorporated in the formulation and the concentrations of calcium, phosphate, and fluoride.[9,10] The complete removal of acids from beverages is possible, but this affects the palatability and stability of the product and is not a realistic expectation.[11] Product modification by addition or supplementation with calcium or phosphate is also possible, but consumers may reject the altered palatability and texture.[10-14]

In a previous in vitro study from the present laboratory, 3 popular sports drinks (Powerade, Gatorade, and Sports Plus) were shown to be erosive.[15] Supplementation of Powerade with casein phosphopeptide-stabilized amorphous calcium phosphate (2% CPP-ACP)—a milk

protein—with or without pH modification, eliminated erosion in vitro.[15]

The purposes of this in vitro study were to:

1. determine a minimal concentration of CPP-ACP added to Powerade that could prevent enamel erosion;
2. assess any effect on the modified Powerade's taste;
3. examine the treated enamel's surface.

Methods

Enamel specimen preparation

Specimens were prepared from 15 extracted human third molars free of caries and defects. The teeth were stored in thymol solution. Buccolingual segments (2-mm thick) were cut midcoronally with an internal annulus saw microtome (Leitz 1600, Ernst Leitz, Wetzlar, Germany) under water irrigation, and were sectioned mesiodistally, providing 2 specimens per tooth. Specimens were assigned randomly into 6 groups (5 specimens per group) and embedded in epoxy resin (EPOFI Epofix, Radiometer, Copenhagen, Denmark) in molds (1x1x1.5 cm).

Enamel specimens were then machine-polished (Struers, Rotopol-21, Copenhagen, Denmark) with silicon carbide paper (grades 600 to 1,200) under water irrigation to remove 50 to 100 μm and produce flat surfaces. Control areas were covered with nail polish; a 1 mm square test window was left exposed and demarcated by scalpel cuts to assist in specimen orientation under SEM.

Adding CPP-ACP to sports drinks

A commercially available, red berry-flavored sports drink, Powerade (PA; Coca-Cola, Sydney, Australia) was purchased from a local supermarket. Casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP, batch no. 850143, Bonlac, Melbourne, Australia) was added at concentrations of 0.063%, 0.09%, 0.125%, and 0.25% CPP-ACP. These test solutions were made by adding 0.5 grams of CPP-ACP powder to 200 mL of PA and preparing serial dilutions. The manufacturer indicates that 100 mL of PA contains 31.9 calories, 6 grams of sucrose, 2 grams of maltodextrose, 25 mg of sodium, 14 mg of potassium, citric acid, and food acid (no. 330). A previous study found that PA contains 36.3 μg/mL calcium, 13.15 μg/mL phosphate, and 24.16 μg/mL fluoride.[15] The solution for control specimens was double deionized water (DDW).

A duo-trio test[16] was used to assess if a taste panel (20 staff volunteers who all provided informed consent) could distinguish the taste of PA from that of PA plus 0.125% CPP-ACP at room temperature. The study was approved by the Human Research Ethics Committee of the University of Melbourne. This test was suitable for use with untrained tasters.[16] In this test, samples of both solutions were used randomly as reference samples. Each taster tasted the reference sample and then 2 coded samples (one of which matched the reference sample). The taster was asked to identify which sample matched the reference sample; if uncertain, he/she was asked to indicate if the response was a guess. Tasters rinsed between each sample with tap water. The minimum number of correct responses for 20 tasters in the duo-trio test was 15 (ie, a response rate of 15 or greater rejects the assumption of no difference between samples).[16] The critical level for alpha was chosen as 0.05.

Study design

Aliquots (50 mL) of each solution were agitated in a water incubator at 37°C for 5 minutes. The resin blocks were added, agitated for 30 minutes, then rinsed in DDW. The varnish was removed gently with acetone. The enamel surfaces were examined under magnification (X20, stereomicroscope), then profiled (Alpha-Step 250 Tencor surface profilometer, Tencor Co, Mich). The profile stylus was a 60° cone rounded to a spherical tip (0.2 μm radius) which profiled the surface with a 0.5-mg tracking force. Each specimen was profiled 3 times (total=15 profiles per group), and the depth of erosion was recorded at the deepest point.

Specimens were then prepared for SEM by sputter-coating with gold (Edwards, S150B, West Sussex, England) and examined at a range of magnifications (SEM XL 30 FEG, Philips, The Netherlands). The test and control areas were classified as etch patterns corresponding to Types 1, 2, or 3 of Silverstone.[17] The digital images were recorded and viewed using Corel Photo Paint 8 (version 8.0, Corel Corp, Austin, Tex, USA).

Measurement of pH and titratable acidity of solutions

Test and control solutions were measured in triplicate (3 aliquots per solution) for pH and titratable acidity (PHM 84 Research pH Meter, TTT 80 Titrator, ABU Autoburette, Radiometer, Copenhagen, Denmark). The volume of freshly prepared 0.1 M potassium hydroxide required to raise the pH of a 5-mL aliquot to 7 at 37°C was used as the measure of titratable acidity.

Data analysis

Numerical data were entered in spreadsheets and analyzed statistically using SPSS (version 10.0.5) for Windows and Excel (SPSS Inc, Chicago, Ill, USA). The mean values for pH and titratable acidity of test and control solutions were compared using one-way ANOVA at a critical level for alpha of 0.01 (incorporating Bonferroni's correction due to multiple statistical testing of the same data). Mean depths of erosive steps were compared using one-way ANOVA at a critical alpha level of 0.05.

Results

Test and control solution characteristics

Visual examination of the test solutions following specimen immersion showed that all remained berry red in color and free of precipitate. The mean values for pH of test solutions increased with increasing concentrations of CPP-ACP, from 2.709 for PA to 3.903 for PA plus 0.25% CPP-ACP (Table 1). Although none of the differences

Table 1. Values (Mean±SD) for pH, Titratable Acidity, and Depth of Erosion Step for Test Solutions and Double Deionized Water

Solutions	pH mean (±SD) (N=3 aliquots per solution)	Titratable acidity (mL KOH) as mean (±SD) (N=3 aliquots per solution)	Depth of erosion (kA) mean (±SD) (N=15 scans per group)*
Powerade	2.709 (0.012)†	1.83 (0.15)§	38.70 (5.60)‖
Powerade+0.063% CPP-ACP‡			
	3.068 (0.002)†	1.61 (0.03)	17.98 (3.05)‖
Powerade+0.09% CPP-ACP	3.273 (0.008)†	1.63 (0.02)	4.31 (0.64)¶
Powerade+0.125% CPP-ACP	3.403 (0.002)†	1.47 (0.03)†	3.35 (1.65)¶
Powerade+0.25% CPP-ACP	3.903 (0.002)†	1.36 (0.06)†	1.94 (0.65)¶
Double deionized water	6.544 (0.007)§	—	2.53 (0.67)¶

*15 scans=3 scans for each of 5 specimens per group.
†Mean values which did not differ with statistical significance.
‡Casein phosphopeptide-amorphous calcium phosphate.
§Mean values, which differed with statistical significance.
‖Mean values, which differed with statistical significance.
¶Mean values, which did not differ with statistical significance.

between these means were statistically significant, all differed with statistical significance from the pH of DDW ($P<.01$; Table 1). The mean values for titratable acidity of test solutions decreased with increasing concentrations of CPP-ACP, from 1.83 mL (of 0.1 M potassium hydroxide) for PA to 1.36 mL for PA plus 0.25% CPP-ACP.

Although the differences between the mean values for PA plus 0.125% CPP-ACP and PA plus 0.25% CPP-ACP did not differ with statistical significance, the mean values for these 2 solutions containing CPP-ACP were both significantly lower than for PA (one-way ANOVA, $P<.01$; Table 1).

Of the 20 tasters in the duo-trio taste test, 14 tasters responded without guessing (10 gave correct responses, 4 gave incorrect responses), and 6 tasters guessed responses (3 were correct, 3 were incorrect). Correct and incorrect responses for each reference solution were distributed equally, indicating there was no bias. Considering guessed responses as incorrect, 10 tasters matched the samples correctly. Recognizing that at least 15 correct identifications are required for a conclusion of statistical significance,[16] any taste difference between PA with and without the addition of 0.125% CPP-ACP was considered to be indistinguishable.

Enamel surface characteristics

Examination under a stereomicroscope showed that all specimens immersed in PA or PA plus 0.063% CPP-ACP appeared etched and stained berry red, with a loss of surface gloss. Specimens immersed in PA plus 0.09% CPP-ACP or PA plus 0.125% CPP-ACP were stained berry red with less etching apparent and little loss of surface gloss. Specimens immersed in PA plus 0.25% CPP-ACP were stained red, but appeared unetched and glossy. Specimens immersed in DDW appeared unstained, unetched, and glossy.

Specimens immersed in PA showed the greatest erosion step depth (mean=38.70±5.60 kA; Table 1), significantly exceeding the mean erosion step depths of specimens in all other test solutions and in DDW ($P<.05$; Table 1). The mean erosion step depth due to PA was reduced by approximately half (to 17.98±3.05 kA) by adding 0.063% CPP-ACP. Adding CPP-ACP in concentrations of 0.09%, 0.125%, or 0.25% resulted in a further statistically signifi-

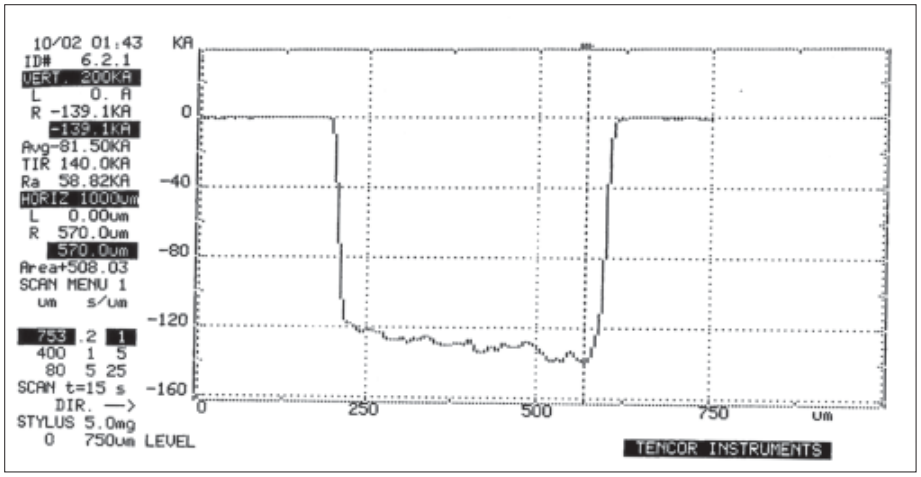

Figure 1. Representative surface profile scan for an enamel specimen immersed in Powerade. The erosion depth is shown on the vertical axis (in kiloangströms). The erosive step's length is shown on the horizontal axis (in μm). This profile scan shows the stylus' path following an erosive step from control enamel (far left) across eroded enamel to control enamel (far right). The straight vertical lines on the scan's left and right borders represent the undercut eroded areas inaccessible to the stylus for recording.

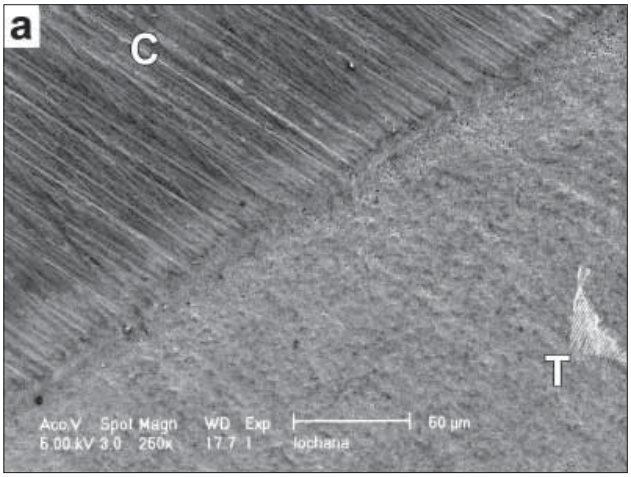

Figure 2A. Scanning electron microscope image of enamel specimens immersed in Powerade under 250X magnification, illustrating the erosive step between test and control areas of enamel. Image taken at boundary of test (T) and control (C) areas.

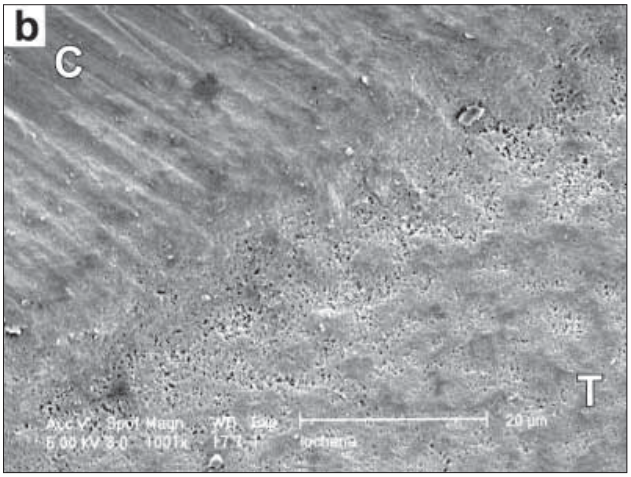

Figure 2B. Specimen in Powerade under 1,000X magnification, illustrating step between test and control areas of enamel.

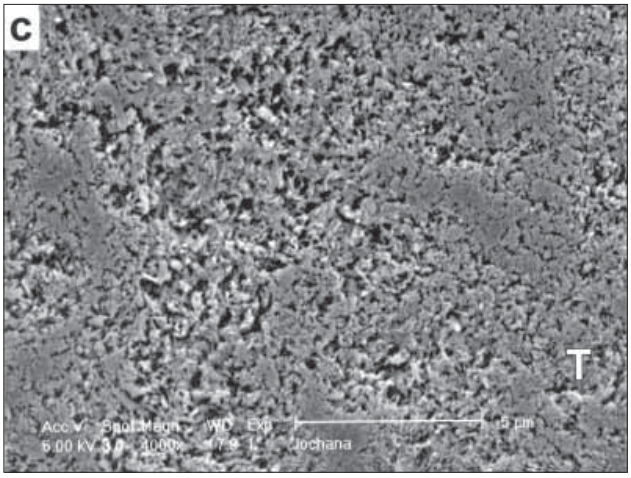

Figure 2C. Specimen in Powerade under 4,000X magnification, illustrating the irregular etch pattern.

cant reduction in the mean erosion step depths in comparison with specimens immersed in PA or PA plus 0.063% CPP-ACP ($P<.05$; Table 1). The differences in the reductions in erosion step depths, however, did not differ between these 3 groups ($P<.05$; Table 1), suggesting no further advantage at the 2 higher concentrations.

The typical shape of an erosive step lesion is shown as a profile scan in Figure 1. The profile scans of specimens immersed in PA showed sharp steps with convex floors. Since the stylus could not access undercut areas of erosion at the lesion's margin, the erosive step's periphery was depicted in straight vertical lines in the scans. Specimens immersed in PA plus 0.063% CPP-ACP showed shallow steps. Erosion steps were not apparent in specimens immersed in PA plus CPP-ACP in concentrations of 0.09%, 0.125%, or 0.25% or those immersed in DDW.

SEM of specimens immersed in PA confirmed clearly demarcated erosion steps between test and control enamel. Undercut regions not profiled by the stylus (due to inaccessibility) were apparent at the periphery. Polishing lines occurred on control areas only. At 250X magnification (Figure 2A), the lesion floor showed predominantly Type 2 etching, with scattered areas of porous enamel (Type 3). The inter-rod areas appeared porous at 1,000X magnification (Figure 2B). At 4,000X magnification, the enamel rods were differentially etched and irregular (Figure 2C).

The erosion steps between test and control enamel were indistinct in specimens immersed in PA plus 0.063% CPP-ACP. At 600X magnification, test enamel showed areas of etched enamel interspersed with unaffected enamel and indistinct enamel prism orientation. At 1,000X magnification, the etched enamel surface in test areas was very irregular and devoid of any particular etch pattern. Superficial rod-shaped particles were noted in these areas at 4,000X magnification.

Test and control areas were not clearly demarcated by erosion steps in specimens immersed in PA plus 0.09% CPP-ACP, and polishing lines were apparent on both test and control enamel. Test enamel showed superficial irregularities—including porosities—but no particular etch pattern, at 150-600X magnification. The irregular surface was finely amorphous at 4,000X magnification, including zones with globular features and indistinct enamel prisms.

Erosion steps were not apparent between test and control areas of enamel in specimens immersed in PA plus 0.125% CPP-ACP; polishing lines were apparent on both test and control areas at 150X magnification (Figure 3A). Discrete surface irregularities were noted at the junction of test and control enamel at 600X magnification (Figure 3B). Enamel cores were etched in isolated areas, and surface irregularities were generally distributed over all test enamel at 1,200X magnification. These irregularities appeared to be adherent granules or globules at 4,000X magnification (Figure 3C).

Polishing lines were seen on both test and control enamel in specimens immersed in PA plus 0.25% CPP-ACP; surface irregularities were sparse on the test enamel

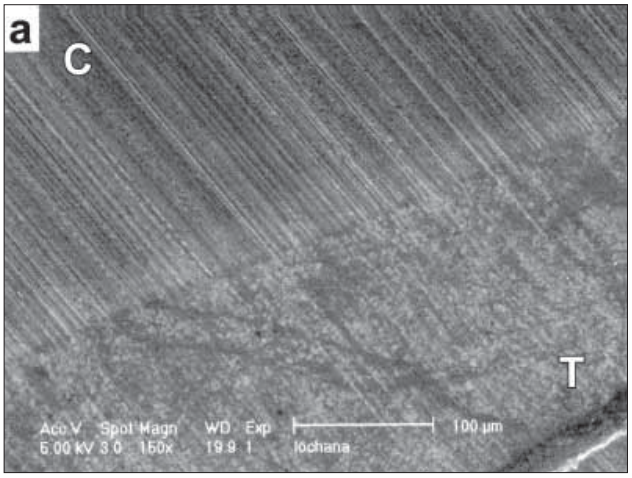

Figure 3A. Specimen in Powerade plus 0.125% casein phosphopeptide-amorphous calcium phosphate under 150X magnification, illustrating continuous polishing lines from control (C) to test (T) areas and absence of erosion step.

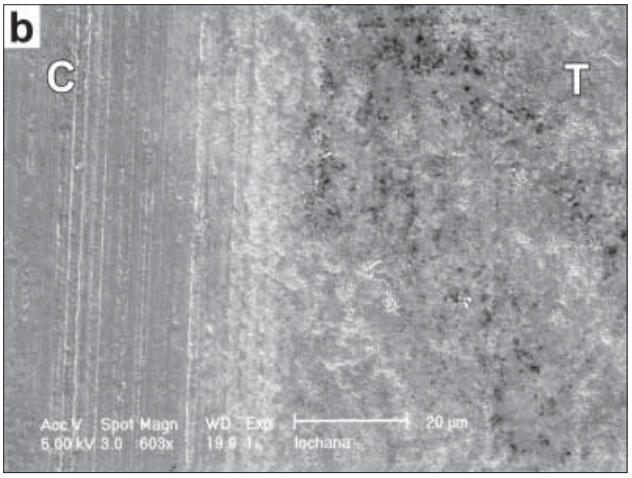

Figure 3B. Specimen in Powerade plus 0.125% casein phosphopeptide-amorphous calcium phosphate under 600X magnification, illustrating discrete areas of surface irregularities.

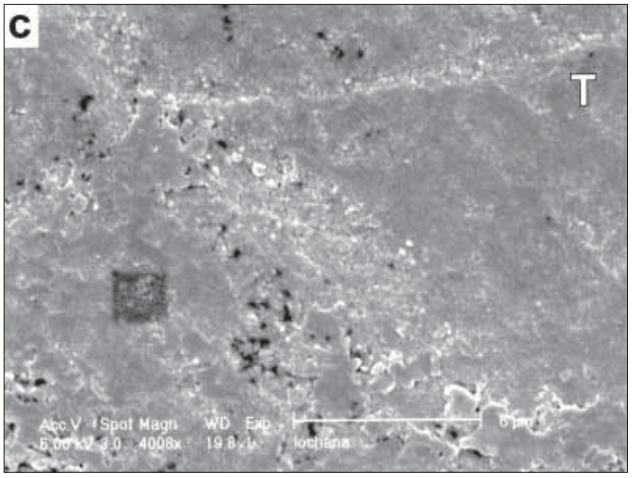

Figure 3C. Specimens in Powerade plus 0.125% casein phosphopeptide-amorphous calcium phosphate under 4,000X magnification, illustrating surface globules over enamel test areas.

with etching limited to a few isolated areas at 600X and 1,200X magnifications. Smooth enamel surfaces with polishing lines were noted for specimens immersed in DDW; these specimens were free of erosion steps and did not show surface irregularities at 100X magnification and at greater magnifications.

Discussion

The present in vitro study confirmed the erosive potential of the sports drink studied. The erosion observed, however, may not be representative of actual enamel changes in the intraoral environment. In vitro studies lack the erosion-protective factors found intraorally, where biological factors in vivo may reduce the erosive potential of acidic drinks. These factors include:

1. pattern of beverage consumption (eg, slow sipping, swishing with delayed swallowing, sipping via a straw);
2. salivary flow rates;
3. salivary buffering capacity;
4. pellicle formation;
5. tooth surface's chemical composition;
6. effects of orofacial musculature on promoting fluid flow within the mouth.[10,18-21]

The citric acid in acidic drinks can be modified by incorporating calcium, phosphate, and fluoride to exert a significant and protective effect against enamel erosion.[22] The present in vitro study showed that the enamel erosion caused by Powerade could be eliminated by adding 0.09% to 0.25% CPP-ACP. To date, techniques to determine the erosive potential of drinks have not been standardized and technical differences complicate comparisons between studies. Variables requiring standardization include factors which affect surface dissolution, such as:

1. source of enamel used (eg, human or bovine enamel, deciduous or permanent teeth, powdered hydroxyapatite);
2. experimental conditions (eg, temperature, specimen agitation, exposure time, concentration of test solutions);
3. methods of surface visualization and evaluation.[14,18]

In the present study, enamel specimens were standardized by being taken from buccolingual sections cut midcoronally. Surfaces can also vary in erosion susceptibility; for example, prismatic enamel is more susceptible to erosion than aprismatic enamel, which erodes irregularly.[1,23] Third molars are likely to contain aprismatic enamel, where it can average 16 to 45 µm in thickness on buccal and lingual surfaces.[24]

A drink's erosive potential also depends on viscosity—the more adhesive the drink is to the tooth surface, the greater the erosion potential.[5] In the present study, all specimens immersed in Powerade stained berry red (despite rinsing in DDW). This demonstrated the product's viscosity and confirmed previous observations which compared the viscosity of PA with 2 other sports drinks (Gatorade and Sports Plus) and with Coca Cola.[15]

Adding 0.09 to 0.25% CPP-ACP to PA could eliminate the erosive steps produced by immersion in PA alone. This addition, however, resulted in surface enamel changes. A sequential or progressive dissolution of enamel prisms occurs in vitro as the erosivity increases—developing initially in the prism sheaths, then in the prism cores, and finally in the interprismatic substance.[25,26] Following dissolution of the sheaths, the prisms' heads and tails dissolve and, finally, all prismatic structure disappears. Specimens immersed in PA alone in the present study showed a consistent etch pattern with peripheral dissolution, while the prism cores remained intact.

Variable etch patterns were seen in specimens immersed in PA plus 0.063% to 0.25% CPP-ACP. Zones of intact enamel within the test area were interspersed with superficial adherent irregularities, appearing as granular or globular deposits that increased with greater CPP-ACP concentrations.

It is speculated that these may represent enamel crystals, repair crystals, or surface deposits formed subsequent to demineralization or remineralization processes. Alternatively, the deposits could be redeposited mineral phases following mobilization of high concentrations of calcium and phosphate held in proximity with the enamel surface by CPP-ACP.[27] Others have observed superficial calcium fluoride globules to a depth of about 40 μm in enamel following the in vitro application of fluoride solutions.[28,29] The surface irregularities could also represent remineralization of the smear layer created during specimen polishing. Further studies are required to elucidate the nature of the surface enamel and the associated surface irregularities.

The technique used in this study included enamel surface polishing before immersion in the test solutions. Such polishing before profiling allows the measurement of structural loss produced by limited amounts of acid.[10] The polishing may remove surface fluorapatite, revealing subsurface hydroxyapatite which is more susceptible to erosion than an intact surface.[10,18,21] Large crystallites and enamel high in concentrations of carbonate and fluoride are removed, exposing more uniform hydroxyapatite and allowing formation of more uniform erosive lesions.[21] Therefore, the extent of erosive lesions formed in vitro may exceed those expected in vivo.

As the CPP-ACP concentration was increased in PA, the pH increased and titratable acidity decreased. Although the pH values observed were all below those critical for the dissolution of hydroxyapatite and fluorapatite, it is speculated that the erosion was limited by the high concentrations of calcium and phosphate that were present. Titratable acidity (representing the total ionized and unionized hydrogen ions) is considered a better indicator of erosion potential than pH, which is the concentration of dissociated hydrogen ions.[5,30] The lower titratable acidity and the raised pH associated with the addition of CPP-ACP may have limited the extent of the erosion.

As pH falls, CPP-ACP dissociates to form calcium and phosphate ions, thereby minimizing the pH drop and limiting demineralization. Since CPP-ACP can act as a reservoir for calcium and phosphate ions and maintains these ions in a state of supersaturation with respect to enamel. Because of this, CPP-ACP decreases demineralization and promotes remineralization of enamel.[12]

The erosive potential of acidic drinks can be reduced by supplementation with calcium, phosphate, or milk products.[10,11,14,22,31,32] The reaction follows the Law of Mass Action, which states that "the rate of a chemical reaction is proportional to the concentration of the reacting substances present at any given time."[11] In the current situation, the rate of enamel mineral dissolution is proportional to the concentration of calcium and/or phosphate present. Consequently, the rate and progression of enamel demineralization are decreased by the presence of the reaction products of enamel dissolution. Since the transformation of ACP to crystalline calcium phosphate is inhibited by the presence of phosphopeptides in CPP-ACP, calculus formation is inhibited.[33]

This study was a preliminary attempt at product modification. The effect on product taste was assessed using the duo-trio taste test, which is appropriate for a small panel of untrained testers.[16] The test is one of overall difference and is preferred over taste acceptability tests because it: (1) is easily conducted; (2) needs few subjects; and (3) requires no training. Other taste tests require large sample sizes or formal training.[16] Ten of the 20 taste testers could not distinguish PA alone from PA plus 0.125% CPP-ACP. Furthermore, 10 tasters correctly distinguished a difference, but commented that the samples were very similar in taste. Since a difference of 15 responses is required for statistical significance, it was concluded that the 2 drinks did not differ in taste. The palatability of the modified product should be evaluated further with trained taste testing panels.

Conclusions

It is concluded that:

1. The sports drink Powerade can erode human enamel.
2. The sports drink's color, clarity, and taste were all unaffected by adding 0.125% of casein phosphopeptide-amorphous calcium phosphate.
3. With increasing concentrations of casein phosphopeptide-amorphous calcium phosphate, the erosive potential decreased. This was associated with an increase in pH and decrease in titratable acidity of the modified sports drink.
4. Adding casein phosphopeptide-amorphous calcium phosphate at concentrations of 0.09% to 0.25% eliminated the erosion step between test and control enamel, but resulted in microscopic surface irregularities present as adherent granules or globules.

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