Development and evaluation of a low-erosive apple juice drink with Phosphoryl-Oligosaccharides of Calcium

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This study aimed to evaluate effectiveness of Phosphoryl-Oligosaccharides of Calcium (POs-Ca) added to apple juice on enamel erosion. Five juices were prepared by adding 0%, 0.5%, 1%, 1.5% or 2% POs-Ca to commercial apple juice, and subjected to Visual Analogue Scale (VAS) taste evaluation and pH and calcium/inorganic-phosphates analyses. To evaluate erosion, polished bovine enamel blocks were immersed in each juice for 5 or 60 min (n=20). Enamel surface loss (SL) and roughness (Ra) were also analyzed. VAS indicated acceptable taste for juices containing up to 1% POs-Ca. POs-Ca addition resulted in increased pH (3.61–3.88), calcium (0.95–25.10 mM), and inorganic-phosphate (1.77–20.44 mM). After 5 min, 0% juice resulted in significant erosion (p<0.05). However even after 60 min, no significant increase was found in Ra and SL compared to water (control) for 1.5–2% juices (p>0.05). Addition of 1–1.5% POs-Ca could significantly reduce enamel erosion by apple juice maintaining an acceptable taste.

Keywords: Erosion, Beverages, pH, Enamel, Phosphoryl-Oligosaccharides of Calcium

INTRODUCTION

Dental erosion is a chronic loss of dental hard tissue due to chemical removal of minerals by acid and/or chelation without bacterial involvement1). Typical acidic sources come from the diet, medications, occupational exposure, gastroesophageal reflux, and lifestyle activities2). The prevalence of erosion is thought to be increasing particularly in adolescents4,5), most probably reflecting the wide availability and frequent consumption of acidic beverages, fruit juices, carbonated beverages, wines, and sports drinks6). Previous studies have indicated that low pH beverages cause erosion to enamel and dentin7,8). The pH of a beverage is clearly an important factor in the erosive potential of a product; however, this is not the only factor to be considered when evaluating its effect on tooth erosion7,9). Thus, the chemical attributes, such as concentrations of calcium and phosphate, type and amount of acid in the solution, pH value and temperature at which the juice is consumed, are all relevant to the erosivity of beverages2,3).

Current interest in prevention of dental erosion has led to an increasing attention to the ways in which erosive products might be potentially modified. Several methods are known for reducing the erosive potential of soft drink; such as adding calcium, phosphate, and some new additives derived from natural resources8,9,14-18). The most common methods are increasing the pH by adding calcium-and/or phosphate-containing salts8,14). Product modification by addition or supplementation with calcium or phosphate is a simple possibility; however, consumers may reject the altered palatability, taste and texture8,19,20).

Phosphoryl Oligosaccharides of Calcium (POs-Ca), which is produced by enzymatic digestion of potato starch, has been known as an effective vehicle of calcium and phosphate. This compound has a high solubility in water, and therefore could be added to a chewing gum (Ezaki Glico, Osaka, Japan)21). In an in situ study, Kitasako et al.22) showed that the POs-Ca containing chewing gums could significantly contribute to remineralization and recrystallization within enamel subsurface lesions, compared to a placebo gum. Under in vitro studies, it was shown that POs-Ca was not assimilated by a cariogenic bacterium such as Streptococcus mutans and that it prevented the fall of pH by metabolism of sucrose in plaques23). Because of these advantageous properties, POs-Ca may be a desirable natural compound to improve the formulation of soft drinks with reduced erosive potential.

Combinatory investigation of the effects of calcium concentration, pH values and taste may facilitate the formulation of an improved soft drink that has a reduced erosive potential while maintaining its taste. Therefore, the aim of this study was to evaluate apple juice drink with added POs-Ca. In this study, apple juice was investigated as one kind of commercially marketed and widely consumed juice. The drink taste, pH value, the concentrations of calcium/inorganic phosphate and the erosive potential of each experimental juice were evaluated. The null hypothesis tested was that there are no differences in the degree of acid dissolution of polished enamel surface before and after adding POs-Ca into apple juice.
MATERIALS AND METHODS

Adding POs-Ca to apple juice
Phosphoryl-Oligosaccharides of Calcium (POs-Ca, batch no. 850143, Ezaki Glico, Osaka, Japan) in the form of powder was added at concentrations of 0.5%, 1%, 1.5% and 2% by weight to a commercially available apple juice (Youji-Ringo, Ezaki Glico, Osaka, Japan). The manufacturer indicates that 100 mL of apple juice contains 12.5 grams carbohydrate and 0.35% malic acid. The experimental apple juices were randomly coded to ensure blind analysis with respect to the various tests performed.

Drink taste test
Visual analogue scale (VAS) test was used to assess the taste of 5 apple juices with 0% to 2% POs-Ca by a taste panel (30 staff volunteers who all provided informed consent). The protocol was approved by the Human Research Ethics committee of the Tokyo Medical and Dental University (#495). Tasters were first asked to drink the unaltered (0% POs-Ca) juice, to get familiar with the taste. After that they drank each of the 5 juices in a random order, and evaluated the taste by marking an analogue scale ranging from 0 to 100 which was printed horizontally on a sheet of paper. Tasters were blind to the juices, and rinsed their mouth between each tasting with 10 mL mineral water. This researcher, who assessed the VAS data, was also blind to the juices, consistent with the design of a double-blind study.

Measurement of pH
The pH values of the experimental apple juices were measured directly using a hand-held electronic pH meter (Checkbuff, Horiba, Kyoto, Japan). After calibration using the supplied standard solutions of pH 4.0 and 7.0, 0.25 mL of each juice was placed onto the pH-sensitive electrode to measure the pH value within 30 s. The pH values of each sample juice were evaluated 3 times and the average was recorded.

Chemical analysis
Each juice sample was analyzed by two methods. Calcium was determined using atomic absorption spectrophotometry (SOLLAR M6, Thermo Fisher Scientific, Kanagawa, Japan) at a wavelength of 422.7 nm with 1% hydrochloric acid and 5% strontium chloride solution. Inorganic phosphate concentration was determined using inductively coupled plasma mass spectrometry (Vista-Pro, Varian Technologies Japan, Tokyo, Japan) with 1% hydrochloric acid solution. The spectrometry was performed at a wavelength of 213.6 nm. The chemical composition with respect to calcium/inorganic-phosphates of each sample juice was evaluated 3 times and the average was recorded.

Preparation of the enamel specimens
Freshly extracted, non-damaged bovine incisors were used for preparation of enamel samples. The teeth were thoroughly cleaned with a rotary brush from plaque and residual periodontal tissues. Enamel-dentin blocks (approximately 7 mm×7 mm×2 mm) were prepared using a low-speed diamond saw (IsoMet; Buehler, Lake Bluff, Illinois, USA) under water-cooling. The surface of the sample block was then ground flat and sequentially polished using an automatic lapping machine (ML-160A; Maruto, Tokyo, Japan) with slurries containing diamond particles 6, 3, 1 and 0.25 μm in size under running water. At each fine polishing sequence, the polished enamel samples were cleaned ultrasonically (Micro Cleaner; TOESCO, Kanagawa, Japan) in ion-exchanged water (pH 7.0) for 5 min to remove any traces of the polishing procedure. The blocks were coated with a tape leaving an approximately 5×5 mm window of enamel.

Erosive challenge
Enamel specimens were subjected to erosive challenge in each of the juices or double deionized water (DDW, pH 6.1) as control solution. After cleaning in ultrasonic cleaner, the samples were rinsed in distilled water for 30 s, dried by a three-way air syringe and subjected to the erosive challenge. The enamel block was exposed to 10 mL of each solution, poured in a small plastic case (35 mm×35 mm×13 mm) in a shaking machine (Personal-11, Taitec, Saitama, Japan, 120 rpm). To evaluate erosion, the values of the enamel surface loss (SL), the surface characteristics/morphological changes and arithmetical average roughness (Ra) were measured. SL of each sample was measured by a contact stylus surface profilometer (SSP). Moreover, the surface characteristics/ morphological changes and Ra were evaluated by the Focus variation 3D scanning microscopy (FVM). SSP was conducted on one half, and FVM was performed on the other half of each sample surface area. The SSP side was divided into two areas as shown in Fig. 1. The first one was evaluated after 5 min exposure and the second one was evaluated after 60 min.

The specimens were exposed to each solution for a total of 60 min. After the first 5 min, the specimen was removed, washed in distilled water, air-blown and subjected to the SL and Ra measurement. The coating tape was partially removed to expose the original level of enamel for SL measurement. Then, the sample was tape-coated again and returned to the erosive challenge for the remaining 55 min; finally, the surface characteristics were measured again.

Surface characteristics/morphological changes
An FVM (Infinite Focus I G4 Microscope, Alicona Imaging, Graz, Austria) was used to capture the 3D topography of the eroded enamel surfaces. The operating principle of focus-variation technique combines the small focus depth of an optical system with vertical scanning, and has been added to the latest ISO standard for classifying surface texture methods.

In order to measure Ra, a rectangular area (143×100 μm) was determined on each sample surface. FVM was also used to capture 3D images of these areas using the 100× objective at a vertical resolution of 20 nm. Ra value was measured along 4 lines in each selected area and the...
Fig. 1 Five specimens for each experimental group were prepared.

For the measurements using SSP and FVM, each enamel window was divided into two areas. One half of each surface was assigned to SSP for SL measurement, and the other half to FVM for capturing surface characteristics, morphological changes and evaluating Ra. The SSP side was divided into two areas; for the measurement after 5 min exposure and for after 60 min exposure measurement. After each erosive challenge, the masking tape was removed and the contact stylus of SSP traced a straight line 4 mm in length. Four tracings were performed for each specimen. For FVM measurements, a rectangular area (143×100 μm) on the enamel surface was evaluated before the erosive challenges as the baseline. After 5 and 60 min exposure to each drink, the same area was evaluated again.

average of all values was calculated for 5 specimens per one experimental group. Therefore, in all, 20 lines per group were measured and evaluated for Ra (n=20). The measurements were performed at the baseline (before erosive challenges), and after 5 and 60 min exposure on the same area of each 5 specimen.

Surface loss measured by profilometry
Prior to the measurements, the tape was removed from the reference area with a scalpel; the samples were carefully checked for remnants or damage. An SSP (XP-200, Ambios Technology, CA, USA) with a stylus radius of 2.5 μm, vertical resolution of 2 nm and stylus load of 1 mg was used to measure the mean SL. The stylus traveled at a velocity of 0.1 mm/s perpendicular to the specimen surface, across the reference and exposed areas for a tracing of 4 mm in length. SL of each traced line was recorded as the average vertical difference (Z values) between the original surface and 3 random points on the line through the exposed surface. SL was measured along 4 lines after each erosive challenge on each of the 5 specimens and the average of all values was recorded (n=20).

Statistical analysis
The differences of mean VAS among the experimental apple juices were statistically analyzed by Wilcoxon signed-rank test with Bonferroni correction, since Kolmogolov-Smirnov test indicated a significant non-normal distribution in each experimental group. The differences of mean SL and Ra among the 5 min and 60 min erosive challenges in each solution were statistically analyzed by two-way repeated measures ANOVA, as the data showed a normal distribution. The two factors analyzed were the concentrations of POs-Ca and immersion time. For each factor, t-test with Bonferroni correction was performed. Statistical significance in SL and Ra analysis was set in advance at a 0.05 probability level (α=0.05) which was adjusted by the Bonferroni correction to indicate difference in multiple-comparisons with (p<0.0033) as statistically significant. All analyses were performed by using SPSS statistical package for Windows version 17.0 software (Chicago, Illinois, USA).

RESULTS

Drink taste test
The VAS averages of each juice were shown in Fig. 2. According to VAS average, the taste of drinks containing up to 1% POs-Ca was not statistically different from the juice with no added POs-Ca. This result indicated an acceptable taste for juices with up to 1% POs-Ca. In the case of higher concentration of POs-Ca, the volunteers frequently stated that the taste was not acceptable since it “lacked flavor” and was “bitter”.

pH value and chemical analysis
The pH values and the mean concentrations of calcium and phosphate among the experimental juices are shown in Fig. 3. The pH value gradually increased with increased percentage of POs-Ca, with remarkable changes from 1%. Likewise, Ca/inorganic-P contents increased in a linear trend as more POs-Ca was added.

Surface characteristics/morphological changes
The 3D images of 0%, 0.5%, 1%, 1.5%, 2% POs-Ca and DDW samples before erosive challenge and after 5 or 60 min erosive challenge are shown in Fig. 4 and Fig. 5. After only 5 min, 0% POs-Ca juice showed morphological changes on the polished enamel surface. 0.5% POs-Ca samples also showed dramatic morphological changes after 60 min erosive challenge, while there were just slight visible changes in surface structure of samples exposed to 1.5% or 2% POs-Ca juice or DDW.

Surface loss and roughness
The SL values after erosive challenges and Ra values before and after erosive challenges obtained from the SSP or FVM are shown in Table 1. SL and Ra showed a similar statistical trend. Compared to DDW, 0% and
The VAS averages of each juice tasted by 30 volunteers.
The groups connected by a horizontal line to the 0% POs-Ca are not significantly different in taste from the original apple juice (p>0.05).

0.5% POs-Ca groups showed significantly different SL and Ra after 5 min erosive challenge; the trend continued up to 60 min (p<0.05). On the other hand, after 5 min of erosive challenge, there were no significant differences for 1%, 1.5% or 2% POs-Ca compared to DDW (p>0.05). Moreover, after 60 min erosive challenge, SL and Ra of enamels exposed to 1.5% or 2% POs-Ca were not significantly different from DDW (p>0.05).

**DISCUSSION**

Experimental techniques to determine the erosive potential of drinks have not been standardized, and technical differences complicate comparisons among various studies. Variables requiring standardization include factors which affect surface dissolution, such as source of enamel used (e.g. human or bovine enamel, deciduous or permanent teeth), experimental conditions (i.e. temperature, specimen agitation, exposure time, concentration of test solution, etc) and methods of surface visualization and evaluation. Bovine teeth were used in this study since it has been well known that their individual structural differences are small. The exposure time of 5 min was chosen because it was
comparable to the clearance time of acid in the mouth, and is thought to have a better physiological relevance than longer exposure time \(^{26}\). Moreover, in case of toddlers or children, the exposure of teeth to commercially available juice may be longer than just 5 min; as they may not be able to drink as quickly as adults, and may also fall asleep while drinking or with the juice still remaining in their mouth. The erosive potential of acidic juice is also increased when the drinks are consumed during periods of dehydration and low salivary flow, as can occur with sleeping time. Therefore, a longer exposure time of 60 min was also included in the study design.

On the other hand, it should be noted that the in vitro experiments such as the current study lack the influence of the numerous variables encountered in the oral environment, most of which would provide a protective effect against acid erosion of enamel\(^{2}\). The specimens in this study were not covered with a salivary pellicle, which has been shown to reduce the dissolution rate of enamel\(^{27}\). Nevertheless, some studies suggest that even after 5 min of exposure to citric acid, the effect of the pellicle would be lost\(^{28}\). The superficial enamel layer is a matured and prismatic enamel, which has the highest resistance against erosion; once this protective surface is lost, the exposed enamel is more prone to dissolution even under mild acidic conditions\(^ {29}\). It should be noted that in order to test standard enamel surface and minimize difference between baselines in this study, fine-polished enamel specimens were employed; these surfaces possibly showed more erosion than in situ or in vitro situation.

SL has been conventionally evaluated on enamel as an indication of acid-erosion extent using surface profilometry\(^{7,10}\). Moreover, optical methods for 3D surface analysis have recently gained popularity\(^{30}\). The analysis by FVM employed in the current study was performed directly by optical imaging, causing no damage to the sample surface. Ren et al.\(^{30}\) performed morphological characterization on enamel surface exposed into soft drinks using this technique. Fujii et al.\(^ {31}\) suggested that compared with SSP, non-contact assessment by FVM could not detect extremely small Ra changes at early stage of erosive challenge. Moreover, a strong correlation was found between FVM and SSP for Ra measurement\(^ {31}\). FVM could obtain the 3D images and Ra on the same samples and at the same areas among different exposure time because of its non-damaging measurement mechanism. The 3D images provided additional information on enamel surface topography and characteristics, such as the well-known honey comb acid-dissolution patterns (Figs. 4 and 5).

The eroded surfaces were visually roughened and became less reflective of light as compared to the baseline surface. At 60 min erosive challenge, the dissolution of samples treated with juices with 0% POs-Ca, 0.5% POs-Ca was clearly visible. The 3D images of more than 1.5% POs-Ca samples at 60 min erosive challenge showed slight changes in surface structure, so that the prismatic appearance of enamel was just visible. It is noteworthy that even for DDW, some surface changes were apparent on the 3D images after 60 min erosive challenge. Given the mild acidity of DDW (pH 6.1), this phenomenon was considered to be associated with dissolution of polished bovine enamel surface by DDW\(^{32}\). Bovine enamel is more porous than human enamel, hence, less resistant to acid diffusion and dissolution tends to progress rapidly\(^{33,34}\).

<table>
<thead>
<tr>
<th>SL (μm)</th>
<th>Before Immersion (baseline)</th>
<th>Ra (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>60 min</td>
</tr>
<tr>
<td>0%</td>
<td>2.19</td>
<td>11.58</td>
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<tr>
<td></td>
<td>(±0.76)</td>
<td>(±1.14)</td>
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<tr>
<td>0.5%</td>
<td>1.13(^a)</td>
<td>4.86</td>
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<tr>
<td></td>
<td>(±0.57)</td>
<td>(±0.83)</td>
</tr>
<tr>
<td>1%</td>
<td>0.80(^a,b)</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>(±0.66)</td>
<td>(±0.68)</td>
</tr>
<tr>
<td>1.5%</td>
<td>0.34(^a,b)</td>
<td>0.43(^a,c)</td>
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<tr>
<td></td>
<td>(±0.22)</td>
<td>(±0.23)</td>
</tr>
<tr>
<td>2%</td>
<td>0.27(^a,b)</td>
<td>0.43(^a,c)</td>
</tr>
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<td></td>
<td>(±0.17)</td>
<td>(±0.32)</td>
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<tr>
<td>DDW (Control)</td>
<td>0.28(^a,b)</td>
<td>0.42(^a,c)</td>
</tr>
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<td></td>
<td>(±0.22)</td>
<td>(±0.20)</td>
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Within each measurement, in each row, values indicated by the same superscript capital letter are not significantly different; in each column, values indicated by the same superscript lowercase letter are not significantly different (\(p>0.05\)).
The morphological changes in the control group also demonstrated that even when the dissolution occurred at very low rates, FVM 3D imaging was a powerful tool in evaluating surface topography associated with enamel erosion.

The rate of enamel mineral dissolution is proportional to the concentration of calcium and/or phosphate present, and degree of saturation with respect to enamel minerals in the solution. Consequently, the rate and progression of enamel demineralization in a solution is decreased by the presence of the reaction products of enamel dissolution, such as calcium and phosphate. It has been shown that calcium ions are detached from the hydroxyapatite surface followed by phosphate ions. The effect of these reaction products was reflected in the rate of enamel surface loss or roughening in 0% and 0.5% POs-Ca groups, where an intensive effect was detected after 5 min of erosive challenge. On the other hand, during the remaining 55 min of exposure, the SL and Ra values did not continue to increase at the same pace. Further research such as the degree of saturation with hydroxyapatite of each sample should be required for comparison and discussion on results of Ra and SL within 5 and 60 min.

Dietary acids present in low pH soft drinks are presumably the major erosive ingredients. Numerous such acids exist, but those most commonly found in manufactured acidic beverages alone or combined include citric, malic, lactic or phosphoric acids. The acids in soft drinks can be modified by incorporating calcium, phosphate, and fluoride to exert a significant and protective effect against enamel erosion. Malic acid is the main acid in the commercially available apple juice. Hughes et al. suggested that malic acid was less erosive than citric or lactic acid at pH 3.8, while at lower pH, lactic acid was more erosive than either citric or malic acid. In the present in vitro study, adding 1.5% to 2.0% POs-Ca slightly increased the initial pH of apple juice to reach approximately 3.8, and the enamel erosion caused by the juice could be decreased. A pH value of 5.5 has been proposed as a critical pH value for enamel dissolution; however, the results of current study confirm that other factors can influence the dissolution of enamel at pH values far below the proposed critical pH.

The mechanism for the effect of the added calcium must in part relate to the dynamics of the acid dissolution effect. POs-Ca is a calcium compound that can act as a reservoir for calcium ion in the solution which dissociates to form calcium ion at low pH. It is likely that this effect would maintain calcium ion in a state of saturation with respect to enamel apatite, and therefore limit enamel demineralization. In this process, phosphate ions are also released from POs-Ca into the solution; while an elevated level of phosphate in the solution should decrease dissolution rate of the phosphate-containing apatite at low-pH, further in vitro studies are needed to confirm the relationship between free phosphate ions and erosive loss of enamel.

The effect on product taste was assessed using the VAS test in this study; it has been previously shown that VAS was a reliable tool to assess taste preference. This test was suitable for use with untrained tasters. However, the palatability of the modified product should be evaluated further with trained taste testing panels. In a pilot study to determine the added percentage of POs-Ca to apple juice, the VAS average for the taste of 5% POs-Ca juice was very low. Given the unacceptable taste, 5% POs-Ca juice was excluded from the design of the main study. According to the results of study, 1% POs-Ca added to the apple juice had an acceptable taste and reduced erosivity, while 1.5% POs-Ca may be preferred in terms of erosive effects. In this regard, at the least erosive end of the spectrum (i.e. the highest possible POs-Ca concentration), the taste of product and therefore acceptability may be adversely affected. An optimal concentration of POs-Ca with both low erosivity and acceptable taste is thought to be between 1% and 1.5%; however, determination of an exact concentration with acceptable taste for the majority of consumers is beyond the scope of the current work.

Therefore, the null hypothesis of the study was rejected as addition of POs-Ca affected the erosive potential of the apple juice. Beneficial effects of higher concentrations of calcium from calcium salts used as food additives with respect to enamel erosion have been frequently reported. Recently, additives derived from natural resources have gained more popularity as safer alternatives (e.g. CPP-ACP, green tea extract, ovalbumin, etc). In this view, the use of POs-Ca, which is derived from potato, bears an advantage in comparison to chemical food additives, and appears to be a practical means of formulating beverages with reduced dental erosivity for consumption by the public.

CONCLUSIONS
In conclusion, addition of small amount (1% to 1.5%) of POs-Ca to apple juice could significantly reduce enamel erosion resulting from immersion in the drink, while maintaining an acceptable taste.

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