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Effects of soy and bovine milk beverages on enamel mineral content in a randomized, double-blind *in situ* clinical study

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ABSTRACT

Soy beverages are promoted as healthy alternatives to bovine milk even though they can contain added sugar.
Objectives: To compare enamel mineral content after consumption of bovine milk or a soy beverage in a double-blind, randomized, cross-over *in situ* clinical study.
Materials and Methods: Human enamel slabs with subsurface lesions were prepared and inserted into intra-oral appliances worn by volunteers who consumed 200 ml of either bovine milk or a soy beverage over a 60 s period once a day for 15 days. Enamel lesion depth and mineral content were measured using transverse microradiography. Saliva samples were collected immediately after consuming the beverages and calcium, inorganic phosphate and fluoride levels analysed. Data were statistically analysed using a linear mixed model.
Results: Depth of the enamel subsurface lesions increased by 7.1 ± 2.0 μm and mineral content decreased by 47 ± 22 vol% min.μm after consumption of the soy beverage indicating demineralization. However, after consumption of bovine milk the depth of the lesions decreased by 7.6 ± 3.5 μm and mineral content increased by 202 ± 43 vol% min.μm indicating remineralization. The changes were significantly different ($p < 0.001$) between the two beverages. Fluoride levels were similar in the saliva samples for both beverages, however the calcium and inorganic phosphate levels for the bovine milk group were significantly higher ($p < 0.02$) than those for the soy beverage group.
Conclusions: In this randomized, double-blind *in situ* clinical trial consumption of a soy beverage demineralized enamel whereas bovine milk produced remineralization.
Clinical Significance: Although soy beverages are promoted as healthy alternatives to bovine milk the added sugar and low calcium bioavailability of the soy drink makes frequent consumption a caries risk. (Trial registration no. ISRCTN19137849).

1. Introduction

The anticariogenic properties of bovine milk have been recognized for many years and have been attributed to its major protein casein and bioavailable calcium and inorganic phosphate ions [1–5]. Bovine milk has been shown to remineralize enamel subsurface lesions *in vitro* [6] and *in situ* [7–9] and increase the surface hardness of previously demineralized enamel *in situ* [10]. Milk casein contains phosphoseryl residues in cluster sequences (-Ser(P)-Ser(P)-Ser(P)-Glu-Glu) that stabilize amorphous calcium phosphate [6]. These complexes bind to the tooth surface providing a source of bio-available calcium and phosphate ions to help maintain saturation of the enamel mineral thereby inhibiting demineralization and promoting remineralization [11].

Soy beverages are a stable emulsion of water, oil, protein and mineral salts and are often promoted as healthy alternatives to bovine milk. They are a source of lecithin, isoflavones (non-steroidal oestrogens) and vitamin E. They do not contain lactose, have less saturated fat and have approximately the same protein content as milk. However, they usually have added sucrose and/or glucose to improve organoleptic (taste) properties [12]. Further, as bio-available calcium levels are low in soy beverages, calcium salts are usually added [13]. Soy products also contain phytate which chelates mineral ions such as calcium and, can therefore, reduce the bioavailability of these minerals [14].

Unlike bovine milk, soy beverages do not appear to protect enamel from acid erosion. A soy beverage with added calcium failed to protect enamel from acid erosion by chlorinated water, *in vitro* [15] and the surface hardness of enamel demineralized by exposure to orange juice

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was found to be increased by 30.5% after exposure to bovine milk but only by 8.6% following exposure to an unsweetened soy beverage [16].

It has been suggested that soy beverages may increase the risk of dental caries from the results of an *in vitro* study in which the acidogenic and buffering capacities of four brands of commercially-available Australian soy beverages and two bovine milk drinks were compared [12]. The rate of acid production by the cariogenic bacterium *Streptococcus mutans* was up to six times higher when incubated in the soy beverages than in the bovine milk [12]. Within 10 min after incubation with this bacterium, the soy beverages exhibited low pH values whereas the pH of the bovine milk remained unchanged. The soy beverage with the lowest bioavailable calcium level also exhibited the lowest buffering capacity and pH value after *S. mutans* fermentation [12]. These *in vitro* results suggest that frequent consumption of soy-based beverages may produce plaque acid and enamel demineralization *in situ* when compared with the remineralization usually observed with bovine milk [8,9]. Hence, the aim of this study was to compare enamel mineral content after consumption of a soy beverage or bovine milk in a randomized, double-blind, cross-over *in situ* study. The null hypothesis for the study was no significant difference in the change in enamel mineral content after consuming the soy beverage or bovine milk.

2. Materials and methods

2.1. Participants

This double-blind, randomized, cross-over study was approved by the University of Melbourne Human Research Ethics Committee (number 1,750,501). The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Eight healthy adults, living in Melbourne, Australia, with a fluoridated (0.9 ppm F), reticulated water supply, were recruited from staff and students of the University of Melbourne and provided informed, written consent to participate in the study. Study inclusion criteria were: age 18–60 years; at least 22 natural teeth; unstimulated whole salivary flow rate of ≥ 0.2 ml/min and chewing gum-stimulated whole salivary flow rate ≥ 1.0 ml/min. Exclusion criteria included: current use of antibiotics or medications which may affect saliva production; current dental caries; history of rampant dental caries; gingival disease; any other oral disease; allergy to milk proteins such as casein; allergy to soy products. The required sample size was calculated using G*Power software [17] using results from previous *in situ* studies on bovine milk [8,9]. Assuming a 90% probability of detecting a true significant difference between the effects of the two test products and, conversely, a 5% probability of falsely detecting a difference, a sample size of six participants was calculated. To allow for unexpected withdrawal of participants from the study, eight participants were recruited.

2.2. Intra-oral appliances and enamel subsurface lesions

Extracted third molars were collected from oral surgeons in private practice. The teeth were sterilized by storage for at least two weeks in 10% (v/v) neutral buffered formalin at room temperature. Enamel slabs were cut from the sterilized teeth and subsurface lesions created as described by Shen et al [18]. The enamel slabs containing subsurface lesions were cut into two half-slabs. One half-slab (test) was inserted into an intra-oral appliance (see below) and the other half-slab was used as a control [18,19]. A custom-made removable acrylic palatal appliance was produced for each participant. The appliances were retained intra-orally by four stainless steel clasps with troughs on each side of the palatal plate that each housed two demineralized enamel half-slabs each containing two demineralized subsurface lesions. The enamel half-slabs were inserted into the bilateral troughs of the appliance to create a plaque retention site over the enamel lesions as described by Cochrane et al. [19].

Table 1
Total carbohydrate, sugar, protein, fat and calcium levels of Vitasoy® Milky Regular and Woolworths Australian Full Cream Milk.

	Vitasoy® Milky Regular	Woolworths Australian Full Cream Milk
Carbohydrate (total)	3.1 g/100 ml	4.8 g/100 ml
Sugar	2.1 g per 100 ml (raw sugar)	4.8 g/100 ml (lactose)
Calcium	120 mg/100 ml	117 mg/100 ml
Protein	3.0 g/100 ml	3.3 g/100 ml
Fat (total)	3.0 g/100 ml	3.4 g/100 ml
Saturated	0.4 g/100 ml	2.2 g/100 ml
Trans	0 g/100 ml	< 1.0 g/100 ml
Polyunsaturated	1.3 g/100 ml	
Monounsaturated	1.3 g/100 ml	

2.3. Products tested

Two commercially-available products were tested in random order: 1) Vitasoy® Milky Regular; 2) Woolworths Australian Full Cream (bovine) milk. Ingredients on the label for the soy beverage included: filtered water; whole soybeans; raw sugar; sunflower oil; mineral (calcium phosphate); vegetable gums (460, 466, 407); flavour; sea salt; food acids (i) 340 - potassium phosphate, dibasic, monobasic, tribasic and (ii) 331 - sodium citrate/sodium dihydrogen citrate. Carbohydrate, sugar and calcium levels in the two products are summarized in Table 1.

The beverages were purchased from retail outlets and stored in a secure refrigerator at approximately 4 °C until just before the beverage was to be consumed. Each participant completed two 15-day treatment periods separated by a one week washout period during which they rested from the study. Participants were provided with Techno-Plas plain containers (St. Marys, South Australia) containing 200 ml of their allocated beverage.

2.4. Clinical trial protocol

The double-blind, randomized, cross-over *in situ* clinical trial was conducted at the Royal Dental Hospital of Melbourne in 2018. Participants were randomly assigned to one of the two different test products and crossed over to the other test products with one week washout in between. Each participant was assigned a number and randomization was effected using a standard randomization table for the coded test products. Each participant wore the custom-made palatal appliance containing four enamel half-slabs with subsurface lesions and one time per day for 15 days consumed 200 ml of test product. The product consumption involved 10–15 sips of the beverage over 60 s allowing the beverage during each sip to contact the enamel slabs in their appliance. Participants kept a diary of beverage consumption times and duration. Participants maintained their normal diet and oral hygiene procedures for the duration of the study, however the intra-oral appliances were removed during eating and drinking (except for the test beverage) and normal oral hygiene procedures. When out of the mouth the appliances were stored in sealed humid containers. All subjects brushed their teeth with standard 1450 ppm fluoride toothpaste for the duration of the study. The subjects returned to the clinical site with their appliances, diary and empty tubes at the conclusion of each 15-day treatment period. Researchers and participants were blind to the treatment code. An independent staff member held the treatment code which was only released after data collection and analysis. After each treatment period each test half-slab was paired with its control half-slab and embedded, sectioned and analysed by transverse microradiography to determine mineral content as described previously by Cochrane et al. [19].

2.5. Saliva collection during treatment periods

During the study participants provided a total of six saliva samples; one sample on each of three specified days during each treatment period. On each of these days, immediately after consuming the beverage, participants allowed all saliva that accumulated in their mouths over two minutes to flow into a pre-weighed sterile plastic tube (supplied) while keeping the appliance in their mouth. Immediately after collecting the saliva, the research team collected the pre-weighed tube containing saliva. The saliva samples were weighed and then stored in a secure freezer in a laboratory before their calcium, inorganic phosphate and fluoride levels were measured. Calcium and fluoride content were determined using a Dionex ICS-3000 ion chromatography system (Dionex Corporation, CA, USA) equipped with an IonPac CS12A cation column, an IonPac AS18 anion column, and ICS3000 conductivity detectors. Samples were diluted with deionized water and filtered through a 0.2 µm filter (Millex-FG, Millipore, MA, USA) before analysis. For inorganic phosphate analysis 100 µl of diluted sample was added to 500 µl of Malachite Green colour reagent containing ammonium molybdate in HCl followed by 20 µl of 1.5% Tween and then vigorously vortexed. These solutions were analysed after 30 min using a Varian 50 Bio ® UV–vis light spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at a wavelength of 660 nm.

2.6. Data analysis and statistical considerations

Initial lesion depth (LDb) and lesion depth after exposure to the test products (LDa) were measured (µm) and the change in lesion depth (LDb-LDa) calculated for each lesion. The mineral content profile of each enamel block's demineralized and treated lesions were compared with the mineral content profile for the median sound enamel of the same section. The difference between the areas under the densitometric profile of the demineralized lesion before exposure to the test products and the median sound enamel, calculated by trapezoidal integration, was represented by ΔZb (vol% min.µm). Hence ΔZb represents mineral loss before exposure to the test products. The difference between the areas under the densitometric profile of the lesions after exposure to the test products and the median sound enamel was represented by ΔZa. Hence ΔZa represents mineral loss after exposure to the test products. These parameters were then converted to mineral content change (ΔZb-ΔZa) where a negative value indicated demineralization and a positive value indicated remineralization of the pre-existing subsurface enamel lesions. A linear mixed model was used to measure differences between the two beverages for each lesion parameter with the beverage as the fixed factor and participants as the random and repeated factors. This model tested the effect of LDb as a covariate in the analysis of LDb-LDa and the effect of ΔZb as a covariate in the analysis of ΔZb-ΔZa. If the effect of the covariate was significant in the analysis it was retained in the model; otherwise they were removed from the model. The effects of bovine milk and soy beverage on enamel mineral content were compared using integrated mineral gain/loss (ΔZb-ΔZa) and lesion depth changes (LDb-LDa) as the primary and secondary outcome measures. Normality of residuals was checked using the Shapiro-Wilk test and normal probability plots. Homogeneity of variance was tested using Levene's test. The data were statistically analysed using a linear mixed model using a compound symmetry variance-covariance structure. Difference between each treatment was measured using a post hoc pairwise comparison. Significance was set at $p < 0.05$. Analyses were performed using SPSS Statistics for Windows, Version 24.0. (IBM Corp. Armonk, NY, USA).

3. Results

3.1. Participants and adverse events

The mean participant age was 43 years (range 29–60 years) with 5 females and 3 males. The mean unstimulated and stimulated flow rates of the participants were 1.2 ± 0.5 ml/min and 3.5 ± 0.7 ml/min, respectively. All participants completed the study with no reported adverse events.

Table 2
Effect of soy beverage and bovine milk consumption on enamel lesion parameters.

Treatment	LDb[a] (µm)	LDb-LDa (µm)	ΔZb[a] (vol% min.µm)	ΔZb-ΔZa (vol% min.µm)
Soy Beverage	97.2 ± 5.3	−7.1 ± 2.0[b]	2872 ± 454	−47 ± 22[b]
Bovine Milk	97.3 ± 3.6	7.6 ± 3.5[b]	2978 ± 437	202 ± 43[b]

All differences were analysed using a linear mixed model with a compound symmetry covariance structure.

[a] LDb and ΔZb describe the lesion depth and integrated mineral loss of the initial lesions before treatment (before intra-oral exposure to the test products: Soy Beverage and Bovine Milk).

[b] Values with same superscript letter in column significantly different ($p < 0.001$); all other comparisons not significantly different ($p > 0.05$).

3.2. Enamel mineral changes

The enamel subsurface lesion data are presented in Table 2. These data include means and standard deviations for: 1) initial depth of the demineralized lesions before exposure to the test products (LDb); 2) lesion depth change from before to after treatment (LDb-LDa); 3) initial mineral content of the demineralized lesions before exposure to the test products (ΔZb); and 4) mineral content change from before to after treatment (ΔZb-ΔZa). There was no significant difference in either the initial lesion depth or initial mineral content of the demineralized lesions prior to exposure to the two beverages. The lesion depth increased by 7.1 ± 2.0 µm following consumption of the soy beverage but decreased by 7.6 ± 3.5 µm following consumption of the bovine milk with the difference being significant ($p < 0.001$) (Table 2). Mineral content decreased by 47 ± 22 vol% min.µm following exposure to the soy beverage and increased by 202 ± 43 vol% min.µm following exposure to the bovine milk with the difference being significant ($p < 0.001$) (Table 2). These results showed that consumption of the bovine milk resulted in a significant increase in the mineral content of the enamel subsurface lesions (remineralization), whereas consumption of the soy beverage promoted enamel subsurface lesion progression (demineralization).

3.3. Saliva calcium and fluoride levels post-beverage consumption

The saliva samples collected immediately after beverage consumption were analysed and the results are presented in Table 3. The fluoride levels in the saliva samples for the bovine milk group and the soy beverage group were similar (0.9 ± 0.8 ppm and 0.8 ± 0.2 ppm, respectively). However, a significantly greater amount of calcium and inorganic phosphate were found in the samples from the bovine milk

Table 3
Bioavailable calcium, pH and fluoride levels in saliva samples collected after soy beverage and bovine milk consumption.

Treatment	pH	Calcium (mM)	Inorganic Phosphate (mM)	Fluoride (ppm)
Bovine Milk	6.9 ± 0.3	5.4 ± 5.3[a]	9.7 ± 3.2[a]	0.9 ± 0.8
Soy Beverage	6.9 ± 0.3	0.9 ± 0.6[a]	5.2 ± 2.1[a]	0.8 ± 0.2

Using a Wilcoxon Signed Rank test, the differences in Ca and Pi levels in post-rinse saliva between the milk and soy groups were significant ($p = 0.012$).

[a] Values with same superscript letter in column significantly different ($p < 0.02$) for both calcium and phosphate concentrations; all other comparisons not significantly different.

group (Table 3). In fact the calcium level in the saliva samples from the milk group (5.4 mM) was six times greater than that from the soy beverage group (0.9 mM). For the eight participants the calcium levels in saliva post-beverage consumption was significantly correlated with enamel lesion mineral content change [r = 0.709, P = 0.002].

4. Discussion

The results of this randomized, double-blind, cross-over *in situ* clinical study demonstrated that consumption of bovine milk resulted in significant remineralization of enamel subsurface lesions, whereas consumption of a soy beverage promoted further enamel demineralization. Hence the null hypothesis for the study was rejected. The remineralization by the bovine milk observed in this study corroborates the results of a previous *in situ* study by Walker et al [8] who demonstrated enamel subsurface lesion remineralization in participants consuming 200 ml of bovine milk per day for 15 days. The increase in lesion depth and decrease in mineral content produced by the soy beverage indicating further demineralization of the enamel in the current study is consistent with the *in vitro* findings of Dashper et al [12] who reported that soy beverages were acidogenic when incubated with a cariogenic bacterium (*Streptococcus mutans*).

Recently Lee et al [20] evaluated the cariogenic properties of soy beverage, almond beverage and whole bovine milk, and analysed their abilities to support *Streptococcus mutans* biofilm formation and acid production, and their capacity to buffer changes in pH. They reported that the soy beverage supported the most biofilm growth and exhibited the highest cariogenic potential. These *in vitro* study results support the findings of our present *in situ* clinical trial demonstrating that the consumption of a soy beverage resulted in enamel subsurface lesion progression. The reason that consumption of the soy beverage resulted in subsurface lesion progression in the current study is likely attributable to the added sucrose and the lower bioavailable calcium levels in the soy beverage (Table 3 and Dashper et al [12]). Mineral content of soy beverages vary with some having added calcium salts such as the soy beverage tested in this study. Calcium, inorganic phosphate and fluoride were measured in saliva samples collected immediately after consumption of the two beverages in this trial. A 532% higher bioavailable calcium level was observed in the saliva samples from the bovine milk group when compared with that from the soy beverage group. The low bioavailability of calcium added to soy beverages compared with bovine milk has been described previously by Heaney et al [13] and Dashper et al [12]. The results of this *in situ* clinical trial demonstrating the higher bioavailability of dairy calcium and the promotion of enamel subsurface lesion remineralization adds further support to the growing body of evidence on the oral health promoting effects of regular consumption of dairy products [21–25]. These anticariogenic properties of bovine milk have been attributed to the specific actions of the major phosphoprotein casein and its ability to inhibit enamel demineralization and to deliver bioavailable calcium to promote remineralization [6,11,26]. In conclusion, the current study suggests that frequent consumption of soy beverages with added sugar and low bioavailable calcium is likely to be a caries risk.

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