COMPARISON OF CHILDREN'S FOLLOW-ON INSTANT POWDERED COW'S MILK FORMULA, BUFFALO MILK FORMULA AND CHICKEN-BASED FORMULA ON ENAMEL MICROHARDNESS OF BOVINE TEETH *IN VITRO*

Kadkao Vongsavan[1] Praphasri Rirattanapong[1] and Rudee Surarit[2]

[1]Department of Pediatric Dentistry, [2]Department of Oral Biology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Abstract. Dental caries are a major public health problem worldwide. The aim of this study was to compare the effects of children's follow-on instant powdered cow's milk formula, buffalo milk formula and a chicken-based formula on microhardness of bovine enamel with artificial caries-like lesions. Forty bovine teeth were each placed in acrylic blocks and the enamel surfaces were polished to create flat 5 x 5 millimeter surfaces. The teeth surfaces were then demineralized using 0.1M lactic acid (pH 4.5) to achieve an enamel microhardness of 35-65 Vickers Hardness Numbers (VHN). All specimens were then randomly allocated into one of 4 groups (n=10/group). For remineralization, each group was soaked in a different kind of milk formula for 2 hours at 37°C except group 1 which was a negative control (artificial saliva) group. Group 2 was soaked in Murrah™ buffalo milk formula (a positive control), group 3 in S-26-Promil-Gold™ (cow's milk formula) and group 4 in a chicken-based formula (Siriraj Hospital, Mahidol University). The microhardness of the specimens was then measured again. Data were analyzed using a one-way ANOVA and paired t-test with a 95% confidence interval. After exposure to the formula, the mean VHN for each study group was significantly higher (paired t-test, $p<0.05$) except for group 1 ($p=0.345$). The mean VHN for the the Murrah™ buffalo milk formula, the chicken-based formula and the S-26-Promil-Gold™ formula group were not significantly different from each other (one-way ANOVA, $p>0.05$). In conclusion, S-26-Promil-Gold™ follow-on cow milk formula, Murrah™ buffalo milk formula and the chicken-based formula all increased bovine enamel microhardness after soaking for 2 hours.

Keywords: buffalo milk, chicken-based formula, enamel microhardness, follow-on formula, incipient caries-like lesions, remineralization

INTRODUCTION

Dental caries are major public health problem worldwide. In a study of 15–19 month old Thai children from low-income families with a low education level, the prevalence of early childhood caries (ECC) was 82.8% (Vachirarojpisan *et al*,

Correspondence: Kadkao Vongsavan, Department of Pediatric Dentistry, Faculty of Dentistry, Mahidol University, 6 Yothi Street, Bangkok 10400, Thailand.
Tel: 66 (0) 2200 7821 ext 30
E-mail: kutkao.von@mahidol.ac.th.

2004). ECC is a form of severe dental caries positively associated with the amount and duration of bottle feeding (Tinanoff and O'Sullivan, 1997). Dental caries are a multi-factorial infectious disease. Milk is one of many factors that have been identified as cariogenic for children. The cariogenic effect of cow's milk is controversial. Lipa (1988) found cow's milk was cariogenic and Peres *et al* (2002) found cow's milk infant formula to be more cariogenic than cow's milk. Infant formula is thought to be cariogenic due to the sugars found in it (Peres *et al*, 2009). Rugg-Gunn *et al* (1985) found cow's milk to be anticariogenic due to the high concentration of calcium and phosphate. The high concentration of calcium in cows milk in the form of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been found to remineralize erosions in teeth caused by chlorinated water (Vongsawan *et al,* 2010). Soy milk without calcium has been found to be less protective against dental erosions (Vongsawan *et al,* 2012).

Most studies in this area have focused on cow's milk; few have evaluated cow's milk and buffalo milk follow-on formulas. Follow-on formula is used for children under 2 years old. S-26 Promil Gold™ is a cow's milk follow-on formula. It is easily available in Thai supermarkets. Buffalo milk is used for all age groups because it is composed of whey protein that is easily absorbed (Mayuri *et al*, 2012). For children who are allergic to cow's milk-base formulas, protein hydrolysate formulas are an alternative. Recently a chicken-based formula has been produced by the Faculty of Medicine, Siriraj Hospital, Mahidol University. This formula is made from homogenizing chicken meat with vegetable oil, glucose polymer and vitamins and minerals as recommended by the Coordinated International Expert Group for Infant Formula (ESPGHAN) which can be used for all age groups.

The purpose of this *in vitro* study was to evaluate and compare the remineralization effects of follow-on cow's milk, buffalo milk and chicken-based formula and their anti-caries properties. The results of this study will clarify the relationship between formulas and initial caries lesions using tooth surface microhardness.

MATERIALS AND METHODS

Specimen preparation

Forty bovine teeth without wear or caries were used in this study. The roots and pulp were removed first. The enamel was then cut on the labial surface of the teeth and embedded in self-curing acrylic resin. The enamel surfaces were then ground and polished to create a flat plane using 150, 400, 600,1000, 2000 and 4000 grit silicon carbide sandpaper (Buehler, Lake Bluff, IL) with a rotating polishing machine (Grinder-Polisher, Metaserv 2000; Buehler, Lake Bluff, IL), followed by cleaning the enamel surface using an ultrasonic cleanser (TRU-SWEEP Model-275D Crest Ultrasonic Corp, Trenton, NJ). The majority of the enamel surface was concealed with nail varnish, except a 5 x 5 mm^2 left free to test for surface microhardness, determined using a Vicker indenter tester (FM-700e Type D; Future-Tech, Tokyo, Japan) using 100 grams of force for 15 seconds. Four tests for microhardness were performed on each specimen and the mean Vickers Hardness Number (VHN) value was recorded. All specimens used in this study had a baseline microhardness value between 300 and 350 VHN (Lippert *et al*, 2012).

Caries-like lesions formation

The specimens were soaked in demineralization solution (Lippert *et al*,

2012) at a pH of 4.5 at room temperature for 15 hours to created caries-like lesions and then immersed in artificial saliva for 30 minutes. The specimens were then rinsed with deionized water and blotted dry. The microhardness of each specimen was again tested in 4 places at least 50 μm apart and the mean VHN was again recorded prior to remineralization (Lippert *et al,* 2012).

Remineralization

The specimens were then divided randomly into 4 groups of 10 teeth each; Group 1 was a negative control group (artificial saliva). The 10 specimens were immersed in artificial saliva (Hosoya *et al,* 2013) for 2 hours and then rinsed with deionized water and blotted dry by wiping with 70% ethyl alcohol. Groups 2, 3 and 4 were treated in the same way as Group 1, except instead of artificial saliva they were respectively placed in Murrah™ buffalo milk formula (a positive control group), S-26 Promil Gold™ level 2 follow-on cow's milk formula and a chicken-based formula. The formulas were prepared according to the manufacturer's instruction. The water used for preparation was deionized. The teeth were again checked for microhardness after the above remineralization and the mean VHN value was recorded.

Fluoride, calcium, phosphate and protein content measurement

Fluoride and calcium ion concentrations were measured using an ion selective electrode connected to a potentiometer (Orion Expandable Ion Analyzer E940, Orion Research, Boston, MA). Measurements were taken 3 times and the average was recorded.

The phosphate ion concentration measured using arsenomolybdate solution and the total protein content was determined using a spectrophotometer (Genesys10S UV-Vis, Madison, WI) using an optical density of 680 nanometers (colorimetric assay) (Farnet *et al,* 2010) and 595 nanometers (Bio-Rad Protein Assay) (Life Science Group, Bio-Rad Protein Assay, Hercules, CA). The average of 3 measurements was recorded as the final value.

Statistical analysis

Paired *t*-tests (α=0.05) were used to compare the mean VHN values before and after remineralization for each group. The variables tested satisfied the assumptions of equality and normal distribution (Kolmogorov-Smirnov test, α=0.05 and Shapiro-Wilk test, α=0.05). The one-way ANOVA and Tukey's tests (α=0.05) were used to compare the VHN before and after remineralization among the groups.

RESULTS

Before remineralization the mean VHN values for each group ranged from 35-65 VHN; they were not statistically significantly different from each other (One-way ANOVA, p>0.05). After remineralization, the mean VHN values were significantly higher in all groups (paired-*t*-test, $p < 0.05$), except for the negative control group (Table 1).

S-26 Promil Gold™ cow's milk formula, the Murrah™ buffalo milk formula and the chicken-based formula had post-remineralization VHN values higher than the negative control group (one-way ANOVA, p<0.05, Tukey's test) but they were not significantly different from each other (p>0.05).

The concentrations of the calcium ions and phosphate ions in the Murrah™ buffalo milk formula, the S-26 Promil Gold™ cow's milk formula and the chicken-based formula were 26.67±5.77 and 3,394.75±162.67, 23.33±5.77

Table 1
Mean VHN values before and after the remineralization by studied formula.

Studied formula	VHN before remineralization ± SD	VHN after remineralization ± SD
Negative control group (artificial saliva)	50.61±3.80[b]	49.62±5.07[b]
Murrah™ buffalo milk formula	56.83±4.67[b]	68.32±4.37[a]
S-26 Promil Gold™ cow's milk formula	54.60±4.12[b]	61.24±5.74[a]
Chicken-based formula	56.10±2.48[b]	62.31±4.03[a]

VHN, Vickers hardness number; SD, standard deviation. Within columns, different superscript letters indicated significant differences among treatment groups (one-way ANOVA, Tukey's test, $p<0.05$). Within rows, different superscript letters indicated significant differences before and after the remineralization process (paired samples *t*-test, $p<0.05$).

Table 2
Concentration of various ions and proteins by type of studied formula.

Studied formula	Calcium ion concentraion (ppm)	Phosphate ion concentraion (ppm)	Fluoride ion concentraion (ppm)	Total protein concentraion (mg/ml)
Negative control group: Artificial saliva	20.00±0.00[b]	483.88±6.97[d]	0.006±0.00[d]	0±0.16[c]
Positive control group: Murrah™ buffalo milk formula	26.67±5.77[b]	3,394.75±162.67[a]	0.025±0.00[c]	22.69±0.50[b]
S-26 Promil Gold™: cow's milk formula	23.33±5.77[b]	2,113.59±95.99[b]	0.080±0.00[b]	41.57±8.69[a]
Chicken-based formula	40±0.00[a]	1,076.03±50.89[c]	0.123±0.01[a]	2.66±0.55[c]

Within columns, different superscript letters indicate significant differences among treatment groups (one-way ANOVA, Tukey's test, $p<0.05$).

and 2,113.59±95.99 and 40±0.00 and 1,076.03±50.89 ppm, respectively (Table 2). The fluoride ion concentrations and total protein levels were 0.025±0.00 ppm and 22.69±0.50 mg/ml, 0.080±0.00 ppm and 41.57±8.69 mg/ml and 0.123±0.01 ppm and 2.66±0.55 mg/ml, respectively. The chicken-based formula had the highest concentrations of calcium and fluoride ions ($p<0.05$). The Murrah™ buffalo milk formula had the highest concentrations of phosphate ions. The S-26 Promil Gold™ cow's milk formula had the highest protein content ($p<0.05$) (Table 2). The negative control group had the lowest concentrations of calcium ions, phosphate ions, fluoride ions and protein content ($p<0.05$).

DISCUSSION

In this study, S-26-Promil-Gold™ cow milk formula, Murrah™ buffalo milk formula and the chicken-based formula all increased bovine enamel microhardness of demineralized enamel, indicating remineralization. The finding of a cow's milk formula enhancing remineralization of emamel is supported previous studies (Rugg-Gunn *et al*, 1985; Bowen and Pearson, 1993; Grenbya *et al*, 2001). The cow and buffalo milk formulas enhancing remineralization was likely due to casein phosphopeptides and amorphous calcium phosphate complexes (CPP-ACP) (Cochrane *et al*, 2010). However, the process of remineralization of decalcified enamel is still unclear. It is likely to involve the promotion of ion deposition into crystal voids in the demineralized enamel causing a net mineral gain (Tung and Eichmiller, 2004). Our findings are supported by a previous study finding of the anticariogenic effect of caw's milk (Rugg-Gunn *et al*, 1985).

This is the first study demonstrating this the chicken-based formula increased bovine enamel microhardness. The chicken-based formula does not contain casein phosphopeptides (CPP), so the mechanism of remineralization produced by chicken-based formula may be different from that obtained with the cow and buffalo milk formulas. This might be because the chicken-based formula had significantly greater calcium and fluoride concentrations than the Murrah™ buffalo milk formula and the S-26-Promil-Gold™ cow's milk formula.

The concentrations of fluoride in the S-26-Promil-Gold™ cow's milk formula and Murrah™ buffalo milk formula in this present study were 0.025 and 0.080 ppm, respectively. Fomon and Ekstrand (1996) found fluoride concentrations in cows's milk were in the range of 0.03-0.06 ppm. In our study, the buffalo milk formula had a greater concentration of fluoride than the cow's milk formula. Higher concentrations of fluoride can enhance enamel remineralization more effectively (Kahama *et al*, 1998).

The Murrah™ buffalo milk formula was used for a positive control in this study because the results conducted in our laboratory (Songwatcharaporn A, personal communication) demonstrated enamel microhardness after soaking in it for 2 hours did not significantly differ from teeth treated with casein phosphopeptide and amorphous calcium phosphate complexes (CPP-ACP). The use of the Murrah™ buffalo milk formula as a positive control is beneficial for this experiment because the same soaking treatment method was used; whereas the CPP-ACP was applied topically.

In summary, all the studied formulas significantly increased VHN values of demineralized bovine teeth. *In vivo* studies needed to assess this effect.

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