UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

LUIZA DE PAULA SILVA CASSIANO

Effect of frequency of intake and amount of fluoride in milk on enamel and dentine caries: *in situ* study

Efeito da frequência de ingestão e quantidade de fluoreto no leite sobre cárie de esmalte e dentina: estudo *in situ*

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Dissertation presented to the Bauru School of Dentistry of the University of São Paulo to fulfillment of the requirements for the degree of Master in Dentistry in the area Stomatology and Oral Biology.

Supervisor: Prof. Drª Marília Afonso Rabelo Buzalaf

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ABSTRACT

Effect of frequency of intake and amount of fluoride in milk on enamel and dentine caries: *in situ* study

This study analysed the effect of frequency of intake and amount of fluoride in milk on the remineralisation of artificial enamel and dentine caries lesions *in situ*. Pre-demineralised bovine enamel and dentine slabs were randomly allocated to 5 *in situ* phases. Twenty-three subjects wore removable appliances with 2 enamel and 2 dentine slabs for 7 days each phase (separated by a 7-day washout period), following a crossover double-blind protocol. In each phase, treatment was performed with milk containing 2.5 ppm fluoride (F) everyday (T1), 2.5 ppm F every other day (T2), 5.0 ppm F every day (T3), 5.0 ppm F every other day (T4) or no treatment (control; T5). The subjects were instructed to immerse the appliance in 100 ml of milk for 5 minutes and then drank 200 ml of the respective milk. The enamel alterations were quantified by surface hardness (%SHR) and transversal microradiography (TMR, ΔΔZ) and dentine by TMR only. Data were analysed by repeated-measures ANOVA/Tukey´s tests ($p<0.05$). For enamel, the highest %SHR was found for groups treated with fluoridated milk every day compared to control, without significant differences between T1 and T3. All groups showed positive values of ΔΔZ, except for T4; significant differences were seen between T1/T3 and T4. For dentine, the only group that presented remineralisation was T2. Fluoridated milk every day seems to have better remineralising effect on enamel than its use every other day, but no dose-response effect was seen. Dentine, however, does not seem to benefit from every day use of fluoridated milk.

Keywords: Enamel. Dentine. Fluoride. Milk. Remineralisation. Caries.

RESUMO

O presente estudo teve como objetivo analisar o efeito do leite fluoretado com concentrações e frequências diferentes na remineralização de lesões de cárie de esmalte e dentina produzidas artificialmente *in situ.* Blocos de esmalte e dentina bovino previamente desmineralizados foram distribuídos aleatoriamente em cinco grupos. Vinte e três indivíduos usaram aparelhos removíveis contendo 2 blocos de esmalte e 2 blocos de dentina por 7 dias em cada fase (separadas por um período de washout de 7 dias), seguindo um protocolo duplo-cego, cruzado. Em cada fase, o tratamento realizado foi com leite contendo 2,5 ppm de flúor (F), todos os dias (T1), 2,5 ppm de F em dias alternados (T2), 5,0 ppm F todos os dias (T3), 5,0 ppm F em dias alternados (T4) ou sem tratamento (T5). Os sujeitos foram instruídos a mergulhar o aparelho em 100 ml de leite por 5 minutos e, em seguida, beberam 200 ml do mesmo leite. As alterações no esmalte foram quantificadas por dureza superficial e microradiografia transversal (TMR), e dentina apenas por microradiografia transversal. Os dados foram analisados por medidas repetidas ANOVA / Tukey ($p < 0,05$). Para o esmalte, o mais alto valor de porcentagem de recuperação de dureza superficial foi encontrado para os grupos tratados com leite fluoretado todos os dias em relação ao controle, sem diferenças significativas entre T1 e T3. Todos os grupos apresentaram valores positivos de $\Delta\Delta Z$, com exceção de T4; foram observadas diferenças significativas entre T1/T3 e T4. Para a dentina, o único grupo que apresentou remineralização foi T2. O leite fluoretado todos os dias parece ter melhor efeito remineralizante sobre o esmalte do que seu uso em dias intercalados, mas nenhum efeito dose-resposta foi visto. A dentina, no entanto, não parece se beneficiar do uso diário de leite fluoretado.

Palavras-chave: Esmalte. Dentina. Flúor. Leite. Remineralização. Cárie

SUMÁRIO

1 INTRODUÇÃO

1 INTRODUCTION

Dental caries is a multifactorial disease; it depends on the interaction between the time that the biofilm is present on the tooth surface, the type of microorganism which comprises the oral environment and the diet of each individual. Thus, dental caries happens when the bacteria present in the dental plaque metabolize carbohydrates from the diet of the host, resulting in the production of acids that dissolve the hard tooth tissues (YEUNG et al., 2005).

From the understanding of disease and public awareness, dental caries showed a decline in recent decades, due to the control of etiologic factors, administration of fluoride in the water and use of fluoridated toothpaste as the main primary preventive measures for disease control (EINARSDOTTIR; BRATTHALL, 1996). Fluoride plays the role of inhibiting demineralization, accelerating remineralization and reducing bacterial metabolism. Fluoride exerts these functions through its topical action when used in vehicles such as toothpastes, gels, varnishes and restorative materials. Despite there are also several sources of systemic fluoride, such as water, supplements, salt and milk, its action is essentially topical since fluoride comes into contact with the teeth while passing through the oral cavity or when it returns to the oral cavity through saliva (BUZALAF et al., 2011).

Although there was a decline in the prevalence of caries in the past 20 years, populations in less developed countries and of lower socio-economic classes still have a significant incidence of the disease (CAMPUS et al., 2007; MASEREJIAN et al., 2009). This is due to the fact that water fluoridation as the main primary preventive measure in caries control is not possible in many areas because of political, geographical or technical reasons. Therefore, attention was focused towards the possibility of including fluoride into other products (KUNZEL, 1993), such as milk. This beverage has an important nutritional function. It is an important part of the human diet in the first years of life, containing essential nutrients like proteins, fats, carbohydrates, vitamins and minerals.

The use of milk as a source of nutrients has been studied and used in public health programs because of the benefits that this type of drink brings to the

population (YEUNG et al., 2005). Randomized clinical trials were conducted and showed that the increase in milk consumption in adolescent girls contributed significantly to the increase in bone mass (CADOGAN et al., 1997). In another study, supplying milk in schools for 2 years for needy children, contributed little but significantly for the growth of these children (BAKER et al., 1980).

In addition to the systemic benefits, milk has become an important vehicle for the prevention of dental caries. Studies done in the mid 40's revealed that milk improved oral health (SPRAWSON,1932; SPRAWSON,1947), probably for having components that are able to protect the tooth structure from carious lesions. Among these components are listed lactose, a carbohydrate present in high concentration in milk (80%)that is the least cariogenic sugar in the diet. In addition there are casein, other proteins and lipids (RUGG-GUNN, 1993).

Casein is a phosphoprotein found in milk in larger percentage and is considered as one of the main components able to protect the tooth structure from the appearance of carious lesions (JOHANSSON, 2002). Studies show that casein inhibits the activity of glucosyltransferase, an enzyme produced by *Streptococcus mutans*, which transforms sucrose into glucose monomers. These monomers are rearranged promoting adherence of other bacteria and increasing the thickness of the dental biofilm (VACCA-SMITH; BOWEN, 1995; VACCA-SMITH et al., 1994; VACCA SMITH; BOWEN, 2000). Thus, the adhesion of bacteria and some salivary components present in the acquired pellicle on the enamel surface is compromised, preventing then the occurrence of carious lesions.

The presence of calcium and phosphate also helps to prevent the dissolution of enamel against acid challenges. The results of an in vitro study comparing the protective effect of human milk, cow's milk, lactose and sucrose solution on enamel, showed that the two types of milk protected the enamel from dissolution, but cow's milk had a greater protective effect, probably due to higher amounts of calcium and phosphate (RUGG-GUNN; ROBERTS; WRIGHT, 1985). Another in vitro study showed that milk had a significant inhibitory effect of demineralization of enamel when compared to a saline solution and a remineralizing solution (ARNOLD et al., 2003).

Given the benefits to oral health that milk promotes, the process of milk fluoridation started in Japan, United States and Switzerland around the 1950's (RUSOFF et al., 1962; ZIEGLER, 1956, 1964). Milk was then regarded as another vehicle for the prevention of dental caries, and mostly able to reach the population that did not have access to fluoridated water. As in water, the addition of fluoride in milk does not promote change in flavor or other characteristics of the beverage and its use allow for a more accurate control of the amount of fluoride that is ingested daily (BANOCZY; RUGG-GUNN; WOODWARD, 2013).

Several studies *in vitro*, *in situ* and clinical trials have demonstrated a very satisfactory effect of fluoridated milk in relation to caries prevention (BANOCZY; RUGG-GUNN; WOODWARD, 2013), but in some systematic reviews the results are still sketchy regarding prevention of dental caries through the consumption of fluoridated milk and more studies need to be done in order to establish more solid evidences (CAGETTI et al., 2013; YEUNG et al., 2005).

Currently there are over one million children receiving fluoridated milk in several countries such as Chile, Thailand, UK, Russia, Bulgaria and Republic of Macedonia as part of the public health program. The fluoridated milk is offered in schools. Depending on each place and on the background exposure to fluoride, the concentration varies between 0.5 and 0.85 mg per day, while the frequency of intake varies between 200 and 365 days per year (BANOCZY; RUGG-GUNN; WOODWARD, 2013).

In order to evaluate the advantages and disadvantages of ingestion of fluoridated milk in different concentrations and frequencies, some studies *in vitro* and *in situ* have been performed. Malinowski et al. (2012) evaluated *in situ* fluoridated milk containing 2.5 and 5.0 ppm for preventing enamel demineralization. The results showed that for both concentrations milk had a protective effect on the demineralization process when compared with the milk without fluoride, but no difference was found between milk with different concentrations of fluoride. Recently, an *in vitro* study assessed the remineralization of initial enamel carious lesions using fluoride milk at concentrations of 2.5, 5.0 and 10.0 ppm, and different frequencies of intake (once a day, twice a day or on alternate days). The results showed that milk with 2.5 ppm of fluoride used twice daily had a better effect on enamel

remineralization (ONGTENCO et al., 2014). To date, only one study evaluated the influence of different fluoride amounts added in milk on enamel remineralization *in situ* (LIPPERT; MARTINEZ-MIER; ZERO, 2014). It was observed that milk containing 1.5 or 3.0 mg fluoride had positive effects. However, these amounts of fluoride added to milk are well above those used in school-based milk fluoridation programs. In addition, the depth of the lesions was not assessed, since the authors evaluated surface rehardening. In addition, there are no studies in the literature that evaluated the effect of different fluoride concentrations in milk on dentin remineralization *in situ*. The interplay between different fluoride concentrations and frequencies of intake of milk on enamel and dentin caries remineralization was never evaluated *in situ*. The present study aimed to add more evidence on the current recommendations regarding the fluoride concentration and frequency of intake of milk on enamel and dentin caries remineralization. Using an in situ crossover design, the impact of different fluoride concentrations (2.5 or 5.0 ppm) and frequencies of milk intake (every day or every other day), resembling the schemes of school-based milk fluoridation programs, on enamel and dentin caries remineralization was evaluated.

2 ARTIGO

2 ARTIGO

Frequency of intake and amount of fluoride in milk on enamel and dentine artificial caries: *in situ* study

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Short title: Effect of fluoridated milk on enamel and dentine caries
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Abstract

This study analysed the effect of frequency of intake and amount of fluoride in milk on the remineralisation of artificial enamel and dentine caries lesions *in situ*. Pre-demineralised bovine enamel and dentine slabs were randomly allocated to 5 *in situ* phases. Twenty-three subjects wore removable appliances with 2 enamel and 2 dentine slabs for 7 days each phase (separated by a 7-day washout period), following a crossover double-blind protocol. In each phase, treatment was performed with milk containing 2.5 ppm fluoride (F) everyday (T1), 2.5 ppm F every other day (T2), 5.0 ppm F every day (T3), 5.0 ppm F every other day (T4) or no treatment (control; T5). The subjects were instructed to immerse the appliance in 100 ml of milk for 5 minutes and then drank 200 ml of the respective milk. The enamel alterations were quantified by surface hardness (%SHR) and transversal microradiography (TMR, ΔΔZ) and dentine by TMR only. Data were analysed by repeated-measures ANOVA/Tukey´s tests ($p<0.05$). For enamel, the highest %SHR was found for groups treated with fluoridated milk every day compared to control, without significant differences between T1 and T3. All groups showed positive values of ΔΔZ, except for T4; significant differences were seen between T1/T3 and T4. For dentine, the only group that presented remineralisation was T2. Fluoridated milk every day seems to have better remineralising effect on enamel than its use every other day, but no dose-response effect was seen. Dentine, however, does not seem to benefit from every day use of fluoridated milk.

Introduction

Fluoride is considered the main responsible for the dramatic decrease in caries incidence and prevalence observed worldwide over the last decades [Bratthall et al., 1996]. The current scientific evidence on the mechanisms of action of fluoride in caries control indicates that the ion reduces the acid solubility of enamel and promotes enamel remineralisation. *In vitro*, fluoride is also able to inhibit glucose uptake and utilisation by acidogenic bacteria, and seems to have bacteriostatic or bactericidal effects, but the *in vivo* implications of this are still not clear [Buzalaf et al., 2011]. It has been recently reported that fluoride inhibits matrix metalloproteinases 2 and 9, which might impact the progression of dentine caries [Kato et al., 2014].

Fluoride can be administered by community, self-applied and professional methods, which are often used in association [Pessan et al., 2011]. Among the community methods, water fluoridation has been regarded as the main primary preventive and public health measure for caries control, as it reaches most of the population, including socially deprived groups. The benefits of water fluoridation, however, are unavailable to a large proportion of the world's population, mainly due to political, geographical and technical reasons [Sampaio and Levy, 2011]. In order to overcome this problem, other methods of community fluoridation have been suggested. As milk is an important part of children's diet, fluoridated milk has been used in school-based preventive programmes for many decades, in different parts of the world. One of the main advantages of this method is that it allows the delivery of a precise amount of fluoride under controlled conditions [Banoczy et al., 2013].

The effectiveness of fluoridated milk in caries control has been assessed by *in vitro*, *in situ* and clinical studies, which show a positive effect of its regular consumption on caries prevention [Banoczy et al., 2013]. However, while the evidence of water fluoridation in caries control has been firmly established over the last decades [McDonagh et al., 2000], systematic reviews have concluded that the number of studies with good quality that evaluated the effects of fluoridated milk in preventing caries is insufficient. Overall, the included studies suggested that fluoridated milk was beneficial to school children, to prevent caries in the permanent [Yeung et al., 2005] or primary [Cagetti et al., 2013] dentitions. However, high-quality studies are still necessary in order that unequivocal evidence can be established. In addition, many aspects involving the milk fluoridation programmes still need to be addressed.

There are distinct ongoing school based milk fluoridation programmes worldwide in countries like Chile, Thailand, UK, Russia, Bulgaria and Republic of Macedonia, involving over a million children. These fluoridated milk schemes comprise a single drink of cow´s milk at school during a morning break. However, the amount of fluoride delivered through milk in these programmes ranges between 0.5 and 0.85 mg per day [Banoczy et al., 2013]. Also the frequency of fluoridated milk consumption varies in the different milk fluoridation schemes. In the UK, the amount of fluoride delivered from milk is 0.5 mg per day on school days (200 days/year), while in Chile amounts ranging between 0.25 and 0.75 mg per day (depending on the age of the children) are delivered 365 days each year [Banoczy et al., 2013].

The impact of these differences in the amounts of fluoride delivered and frequencies of intake on the caries preventive potential of milk fluoridation schemes is not completely known. The influence of two different fluoride concentrations in milk (2.5 and 5.0 ppm, corresponding to amounts of 0.5 and 1.0 mg fluoride delivered in 200 mL of milk, respectively) on the prevention of demineralisation of sound enamel was recently evaluated *in situ*. Both fluoride concentrations significantly protected enamel from demineralisation when compared with non-fluoridated milk, but did not significantly differ from each other [Malinowski et al., 2012a]. A recent *in vitro* study evaluated the effect of different fluoride concentrations in milk (2.5, 5.0 or 10.0 ppm) and also of distinct frequencies of use (once daily, twice daily or on alternate days) for remineralising initial enamel carious lesions. The best remineralising effect was observed for 2.5 ppm fluoride milk used twice daily [Ongtenco et al., 2014]. However, only one study evaluated the influence of different fluoride concentrations in milk on enamel remineralisation *in situ*, but the amounts of fluoride used added in milk (1.5 or 3.0 mg) were higher than those typically employed in milk fluoridation schemes and the authors evaluated only surface rehardening [Lippert et al., 2014].

In addition, to date no studies evaluated the effect of distinct fluoride concentrations in milk on dentine remineralisation *in situ* neither the effect of frequencies of use of milk on the remineralisation of dentine and enamel caries *in situ*. Thus, the present study evaluated if there is any additional benefit of increasing the amount of fluoride in milk from 2.5 ppm to 5 ppm per day on *in situ* enamel and root dentine remineralisation. The effect of different frequencies of drinking fluoridated milk (every day or every other day) was also investigated. It was hypothesized that higher fluoride concentration and frequency of milk intake would lead to enhanced remineralisation of enamel and dentine.

Materials and Methods

Ethical Aspects and Subjects

The study followed a double-blind, randomised, crossover protocol, comprising 5 phases of 7 days each, with an interval of 7 days among them. Twenty-three young adult subjects (2 male, 21 female) took part in the study, after approval by the IRB of Bauru Dental School, University of São Paulo, Brazil (No.179/2011). Sample size was based on an *in situ* study recently conducted with similar research protocol

[Malinowski et al., 2012a]. The inclusion criteria were: stimulated salivary flow > 1 mL/min, unstimulated salivary flow > 0.25 mL/min; salivary pH > 6, good oral conditions (presenting full permanent dentition, no open cavities or deficient restorations, absence of gingivitis and periodontal disease). Other factors taken into consideration were related to the overall health of the volunteer. Pregnant women, patients with systemic diseases and using chronic medication were not eligible to participate. Subjects signed an informed consent document prior to the beginning of the study.

Specimen Preparation

Bovine lower incisors were freshly extracted and stored in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. Teeth were submitted to careful visual inspection in order to detect stains and cracks. If these were detected, the teeth were excluded. Selected teeth were carefully cleaned from gingival tissue using a periodontal curette (Duflex, Duflex do Brasil, Brazil) before being cut. Initially the roots were separated from the crowns using a diamond disk (Diaflex-F, Wilcos do Brasil, Petrópolis, Brazil) by sectioning the cervical portion of the tooth. The crown was used for obtaining enamel slabs and, from the root, dentine slabs were prepared. Then the crowns or roots were fixed in acrylic plaques (40 X 40 X 5 mm) that were placed in the ISOMET Low Speed Saw cutting machine (Buehler Ltda., Lake Bluff, IL, USA). Two diamond disks (XL 12205, 102 X 0.3 X 12.7 mm, Extec Corp., Enfield, CT, USA), which were separated by a 4-mm diameter spacer (7 cm diameter, 4 mm thickness and central role of 1.3 cm), were used to cut the slabs (300 rpm, under refrigeration). One enamel and one dentine slabs (4 X 4 mm) were obtained from the flattest portion of each crown or root, respectively, through double sections in the longitudinal and transversal directions. The surface of the slabs was ground flat with water-cooled carborundum discs (320, 600 and 1200 grades of Al_2O_3 papers; Buehler, Lake Bluff, IL, USA) and polished with felt paper wet by diamond spray (1 µm; Buehler), resulting in removal of about 100 µm depth of the enamel. This was controlled with a micrometer. After polishing, the specimens were cleaned in an ultrasonic device with deionised water for 10 minutes.

Baseline surface hardness (BSH) determination was performed on all the enamel slabs (*n*=230) for selection purposes (five indentations; Knoop diamond, 25 g, 10 seconds; HMV-2; Shimadzu Corporation, Tokyo, Japan) (Mean KHN

335.3±2.4). Dentine slabs (*n*=230) were not submitted to surface hardness analysis since previous studies have revealed that this type of analysis is not accurate for this substrate since hardness even in sound dentine is not evenly distributed [Moron et al., 2013]. After that, one third of the (enamel/dentine) surface was covered with nail varnish to create a sound control area. The slabs were subjected to the formation of artificial caries lesions by immersion in a solution containing 50 mM lactate, 3 mM $CaCl_2$, 3 mM KH_2PO_4, 6 µM tetraetil methyl diphophanate and traces of thymol, pH 5.0 (10 M KOH to adjust pH) (30 mL per block), for 7 days (dentine) and 6 days (enamel), at 37 ºC [Buskes et al., 1985]. The mean (±) lesion depth was 64.0 ± 20.1 µm and 82.9 ± 18.9 µm for enamel and dentine, respectively, as evaluated by TMR. After demineralisation, surface hardness (SHD) determination was done in enamel slabs and the other outer one third of the surface (enamel/dentine) was protected with nail varnish (demineralised control area). For the in situ experiment, 230 pre-demineralised enamel and 230 pre-demineralised dentine were randomly allocated to 23 subjects (n=2/subject/phase) according to SHD means.

In situ experiment

Acrylic palatal appliances were constructed individually for each volunteer and for each phase. They were made with four cavities (5 x 5 mm), distributed into 2 rows of 2 spaces each. Enamel and dentine slabs were randomly accommodated into the cavities and fixed in place with wax. The study comprised 5 crossover phases of 7 days each, separated among each other by a washout period of 7 days (no treatment) [Afonso et al., 2013]. In each phase, four-five volunteers were assigned to one of the five treatments, as follows: T1: fluoridated milk (0.5 mg F), administered every day (200 mL of milk containing 2.5 ppm F as NaF); T2: fluoridated milk (0.5 mg F), administered every other day; T3: fluoridated milk (1.0 mg F), administered every day (200 mL of milk containing 5.0 ppm F as NaF); T4: fluoridated milk (1.0 mg F), administered every other day; T5: No treatment. Fluoridated pasteurised whole milk (Batavo Total, BRF SA, Teutônia, RS, Brazil) was prepared by the addition of powdered sodium fluoride (Merck, Darmstadt, Germany). Preparation was done every day and fluoride concentrations were checked using an ion specific electrode, after hexamethyldisiloxane-facilitated diffusion [Taves, 1968].

One week before the *in situ* phase started, volunteers received dental prophylaxis in order to remove dental biofilm. Dental prophylaxis was also done

between the *in situ* periods. The devices were worn by the volunteers 12 h before starting the treatments and challenges, allowing the salivary pellicle to form. To perform the experiment, in the days that milk should be used (every day for T1 and T3 or every other day for T2 and T4), the volunteers were instructed as follows: in the morning, during breakfast, they were asked to remove the device from the mouth and to immerse it in a cup containing 100 mL of milk for 5 minutes. Then they were asked to drink 200 mL of milk and reinsert the device in the mouth immediately after. Fluoridated milk was freshly prepared each day. Palatal devices were used during the entire day, except when drinking or eating (in total 4h/day). The devices were kept in wet gauze inside a plastic container during the time they were not being used by the volunteers. It is important to highlight that the study was conducted in a fluoridated area (0.6-0.8 ppm) [Buzalaf et al., 2013], but the volunteers drank bottled water with low fluoride concentration (<0.2 ppm) provided by the researchers throughout the experimental period. The same water was used for cooking and preparing beverages. The volunteers were instructed to keep their usual eating habits and to perform oral hygiene using toothbrush, dental floss and non-fluoridated toothpaste provided by the researchers during all stages of the study. Only the surface of the device that was in contact with the palate could be brushed. The volunteers were also instructed not to use any type of fluoride or antibacterial product during the *in situ* period. At the end of each phase, the devices of all volunteers were collected and washed with deionised water and the slabs were again set on their respective acrylic disks to be prepared for hardness and transverse microradiography analysis.

Hardness measurement

After the *in situ* phase, final surface hardness (FSH) analysis was performed on all the enamel slabs as described above. The % surface hardness recovery (%SHR) was calculated, as follows: %SHR = [(FSH – SHD) / (BSH – SHD)]*100.

Transverse microradiography

Two longitudinal sections were made at the center of the block. Each piece had a thickness of approximately 1.3 mm. The cut was perpendicular to the orientation of the protective nail polish. Thus, 1/3 of the surface corresponded to

sound enamel (or dentine), 1/3 to enamel (or dentine) artificially demineralized and 1/3 to enamel (or dentine) after *in situ* remineralisation. After cutting, 1/3 of the block was used for TMR analysis, while the remainder was stored in eppendorf tubes filled with deionised water for future analyses, if necessary. Fragments were then hand polished to obtain a specimen of an approximate thickness of 100 µm using water-cooled silicon-carbide discs (600-and 1,200-grade papers ANSI grit; Buehler, Enfield, CT, USA). The final thickness of each specimen was checked with a micrometer. The dentine specimens were immersed in ethylene glycol (Sigma-Aldrich, Steinheim, Germany) for 24 h before exposure to avoid shrinkage during X-ray exposure due to desiccation [Buchalla et al., 2003]. After that, the specimens were bonded with adhesive tape on the sample holder (around 30-40 samples/specimen holder, containing the aluminium calibration step wedge with different thicknesses, which generates different shades of grey. Then the sample holder was inserted into the cassette with the glass plate in a dark room. The cassette was placed inside a black bag and taken to the X-ray generator (Softex, Japan). The glass plate was sensitized by x-ray (20 kV and 20 mA for enamel and 20 kV and 15 mA for dentine) for 20 min. After each exposure, the glass plate was developed for 6 min, fixed for 3 min in a dark room at 20°C and washed with running water for 10 minutes. The developed plate was analysed using a transmitted light microscope fitted with a 20x objective (Zeiss, Germany), a CCD camera (Canon, Japan) and a computer. The images were taken using data-acquisition (version 2012) and interpreted using calculation (version 2006) software from Inspektor Research System (Amsterdam, The Netherlands). From the analysis of 11 slices of aluminium (step wedge for the construction of "standard curve") and for each microradiogram, it was possible to calculate the integrated mineral loss (ΔZ) and lesion depth (µm) using the formula by Angmar et al. [1963]. The ΔZ (vol.µm%) is the product of the difference between the percentage of mineral volume of sound enamel or dentine and the percentage of mineral volume of demineralised enamel or dentine in relation to the depth of the lesion (µm). The depth of the lesion (LD) is defined by the distance from the surface (0 vol% min) to the depth at which the enamel/dentine again has a mineral content equal or greater than 95% of the mineral content of sound enamel/dentine [Arends and ten Bosch, 1992]. The mineral volume of the sound enamel and dentine corresponds to 87% and 50%, respectively. To calculate the value obtained at the end of the treatments, the

ΔΔZ/ΔL was calculated as the difference between ΔZ/LD lesion and ΔZ/LD effect (after treatments T1/T2/T3/T4/T5).

Statistical analysis

The software GraphPad InStat (version 3.0) was used. Initially, data were tested for normality and homoscedasticity, using Kolmogorov-Smirnov and Bartlett's tests, respectively. Data regarding %SHR for enamel, and ΔΔZ for enamel and dentine were analysed by repeated-measures ANOVA and Tukey's post-hoc test. Data regarding lesion depth were analysed by Friedman's test (non-parametric repeated-measures ANOVA) and Dunn's multiple comparison test. In all conditions, the significance level was set at 5%.

Results

Mean %SHR of enamel slabs with artificial caries lesions after *in situ* remineralisation is presented in Table 1. Repeated-measures ANOVA found a significant difference among the treatments (F = 3,691, p = 0.0081). No dose-response was observed for the two fluoride concentrations in milk evaluated, regardless the frequency of intake. The highest %SHR was found for the groups treated with fluoridated milk every day, regardless the amount of F (T1 and T3) that did not significantly differ from each other but performed significantly better than T5 (no treatment). Treatment with fluoridated milk every other day (T2 and T4) presented an intermediate effect and did not significantly differ from T1, T3 and T5.

For enamel, significant differences were found among the groups for ΔΔZ (Fr = 18.647, p = 0.0009). All groups showed positive values of ΔΔZ, which means remineralisation, except for T4 that showed negative values (demineralisation). The two groups with the highest remineralisation (T1 and T3) significantly differed from T4. The differences among the other groups were not significant. The same pattern was found for the lesion depth, but in this case the differences among the groups were not significant (Fr = 8.988, p=0.061). Separate statistical analysis was performed for T1, T3 and T5 only. In this case, treatment with fluoridated milk every day, regardless the F amount, significantly increased the ΔΔZ (F = 4.898, p = 0.014). The difference for lesion depth again was not significant among the groups (F = 1.534, p = 0.231) (Table 2; Figure 1).

For dentine, no significant differences among the groups were found for lesion depth (Fr = 4.985, p = 0.289). The results found for ΔΔZ were quite different from those obtained for enamel. A significant difference was found among the groups (repeated-measures ANOVA, F = 3.75, p = 0.010). The only group that presented remineralisation was T2 that significantly differed from all the others that suffered demineralisation (Table 3; Figure 2).

Discussion

Despite fluoridated milk has been recommended as an alternative vehicle to deliver fluoride since 1953 and some clinical studies have attested the preventive effect of this measure to prevent caries, evidence on this regard has not been firmly established so far [Cagetti et al., 2013; Yeung et al., 2005]. Recently, various mechanistic studies have attempted to contribute to a better understanding of the effect of fluoridated milk against caries, using *in vitro* and *in situ* models. These studies have evaluated different parameters, such as fluoride concentration of milk [Giacaman et al., 2012; Itthagarun et al., 2011; Lippert et al., 2012; Lippert et al., 2014; Malinowski et al., 2012b, a], temperature of milk [Lippert et al., 2012], volume of ingested milk [Lippert et al., 2014] and frequency of milk ingestion [Ongtenco et al., 2014] on the anticariogenic properties of fluoridated milk. In general, *in vitro* studies have reported that milk containing 2.5 ppm fluoride significantly increases enamel remineralisation and that increasing fluoride concentrations do not have an additive effect [Itthagarun et al., 2011; Malinowski et al., 2012b]. Additionally, the use of fluoridated milk twice per day remineralised enamel lesions *in vitro* to a greater extent when compared with the use once per day or every other day [Ongtenco et al., 2014]. Despite *in vitro* models are broadly employed to evaluate the anticariogenic effect of different treatments [Buzalaf et al., 2010], some very important variables such as the presence of saliva can be more precisely reproduced using *in situ* designs [Cochrane et al., 2012]. This is the first *in situ* study to evaluate the remineralising potential of milk with different fluoride concentrations and frequencies of use on both enamel and dentine.

The fluoride concentrations (2.5 and 5.0 ppm) were chosen because they are the most used in milk fluoridation schemes worldwide, where 200 mL of milk containing 0.5 or 1.0 mg fluoride are typically offered to children in school based

programmes, depending on the age and background exposure to fluoride [Banoczy et al., 2013]. The frequencies of milk intake (every day or every other day) were chosen to mimic the provision of milk to school children every day, as happens in some schemes [Marino et al., 2001] or on school days only (around 200 days per year), which is the case for most of the milk fluoridation programmes. Our control group received no treatment instead of milk, since there is no programme offering non-fluoridated milk in order to prevent dental caries. Additionally, plain milk (without fluoride) presented rehardening effect similar to milk containing 1.5 mg fluoride [Lippert et al., 2014]. In addition, fluoridated milk (5.0 ppm fluoride) was not significantly different from non-fluoridated milk regarding dentine demineralisation [Giacaman et al., 2012].

Since the aim of the present study was to assess the remineralising potential of fluoridated milk, enamel and dentine slabs with artificially produced caries lesions were used. The protocol of artificial demineralisation used was shown to produce satisfactory lesions both for enamel [Buskes et al., 1985; Magalhaes et al., 2009] and dentine [Moron et al., 2013]. The *in situ* remineralisation period chosen (7 days) was based on the study by Afonso et al. [2013]. In that study, the authors tested remineralisation periods of 3 and 7 days when fluoridated dentifrices (0-1100 ppm) were used, using surface and cross-sectional hardness as response variables. They observed more pronounced dose-response relationship for the lesions remineralised for 3 days than for those remineralised for 7 days. However, the fluoride concentrations used ranged between 0 and 1100 ppm delivered through dentifrices. In the present study, since the fluoride concentrations in milk were much lower, volunteers drank non-fluoridated water and used a fluoride-free dentifrice throughout the experimental period, we decided to use 7 days of experimental period, in order to increase the extent of remineralisation. The degree of remineralisation, however, was low.

For enamel specimens, %SHR ranged between 5 and 10%. However, it was possible to see a significant increase in %SHR for the groups treated with fluoridated milk every day (T1 and T3), regardless the fluoride concentration in milk, without significant differences between these groups. These results were reflected in the subsurface of the lesions, where the highest degrees of remineralisation were seen for T1 and T3 (Table 2). These results confirm previous *in vitro* studies that revealed that milk containing 2.5 ppm fluoride significantly increases enamel remineralisation

and that increasing fluoride concentrations do not have an additive effect [Itthagarun et al., 2011; Malinowski et al., 2012b]. It is important to highlight that only when milk was used every day it was possible to see significant enamel remineralisation in comparison to control (no treatment). This is in agreement with recent *in vitro* findings [Ongtenco et al., 2014] and is also in-line with the current knowledge regarding the mechanism of action of fluoride for caries control that emphasises the need of constant low-fluoride levels of fluoride in the oral fluids [Buzalaf et al., 2011]. It should be noted again that the degree of remineralisation, both at the surface and in deeper layers of enamel, was low. This might have been due to the low fluoride concentration in milk (2.5-5.0 ppm, compared to 1000 ppm when fluoride is included in dentifrice formulations) and low exposure to fluoride from other sources, since volunteers used fluoride-free dentifrice and drank bottled water with low fluoride concentration throughout the experimental period.

Current milk fluoridation programmes are directed to school children and, due to this, nearly all studies in the literature focus on the potential of fluoridated milk to control enamel caries. In the present study we decided to evaluate also the protective effect of fluoridated milk against dentine caries because milk fluoridation schemes could be implemented to older people that might suffer both from dentine caries (due to gingival recession) and osteoporosis and could benefit from fluoridated milk. The results found for dentine, however, were remarkably different from that observed for enamel. Notably, remineralisation was only observed for the group that was treated with the lowest fluoride concentration (2.5 ppm) every other day (T2). These results are difficult to explain and information available in the literature on the effect of fluoridated milk on dentine caries is quite scarce, which limits data interpretation. The only study available in the literature was conducted *in vitro* and employed an *S. mutans* biofilm model with pH cycles. The authors observed that fluoridated milk (5 ppm fluoride) was not significantly different from milk alone regarding dentine demineralisation, while it was for enamel demineralisation. It was also noted that while fluoridated milk did not significantly differ from the positive control (0.05% NaF) regarding enamel demineralisation, for dentine fluoridated milk had a worse performance when compared with the positive control. The authors attributed this to the less mineralised nature of the dentinal tissue [Giacaman et al., 2012]. In the present study, however, we employed dentine slabs with artificial caries lesions that are expected to contain a superficial layer of demineralised organic matrix [Buzalaf et

al., 2012; Tjaderhane et al., 1998]. It is possible that the distinct components of milk, such as fat or proteins, have interacted with this organic layer and interfered with the fluoride , since the only group that experience remineralisation was T2, with the lowest fluoride concentration and frequency. However, this does not explain why T4 (5.0 ppm fluoride milk applied every other day) did not suffer remineralisation. Interestingly, for enamel T4 was the only group that had additional demineralisation during the *in situ* period. Since the present study is the only one that dealt with dentine remineralisation by fluoridated milk so far, additional studies are necessary to confirm and explain our findings.

Our data suggest that use of fluoridated milk every day seems to have better remineralising effect on enamel than its use every other day (situation that mimics use of fluoridated milk on school days only). Additional clinical studies should confirm the findings. If the results are similar, then current milk fluoridation programmes should consider the need to provide fluoridated milk to children for every day use, instead of use on school days only. Dentine, however, does not seem to benefit from every day use of fluoridated milk, which needs confirmation by further studies.

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Table 1. Mean percentage of surface hardness recovery (%SHR) of enamel slabs with artificial caries lesions remineralised *in* situ, as function of treatment with fluoridated milk containing 2.5 or 5.0 ppm fluoride every day or every other day

	Treatments				
	T1	**T2**	**T3**	**T4**	**T5**
Mean	9.4^{a}	8.6^{ab}	9.7^{a}	8.8^{ab}	5.1^{b}
SD	*5.3*	*5.2*	*5.5*	*3.5*	*4.8*

Different superscript letters indicate significant differences among the treatments (*n*=22). Repeated-measures ANOVA and Tukey´s test ($p<0.05$). T1: fluoridated milk (2.5 ppm F) every day; T2: fluoridated milk (2.5 ppm F) every other day; T3: fluoridated milk (5.0 ppm F) every day; T4: fluoridated milk (5.0 ppm F) every other day; T5: no treatment.

Table 2. Average lesion depth (µm, ±SD; ∆L= L lesion – L treatment) and integrated mineral loss (ΔΔZ; ΔZ lesion – ΔZ treatment) for the enamel slabs with artificial caries lesions submitted to *in situ* remineralisation, as function of treatment with fluoridated milk containing 2.5 or 5.0 ppm fluoride every day or every other day

Treatment	**ΔL - depth (µm)**	**ΔΔZ (vol.µm%)**
T1	6.5 ± 9.2	247.3 ± 198.5^{aA}
T2	2.3 ± 8.7	110.9 ± 303.2^{ab}
T3	0.9 ± 10.2	226.0 ± 299.2^{aA}
T4	-5.2 ± 9.0	-274.5 ± 407.3^{b}
T5	3.5 ± 6.6	5.0 ± 288.0^{abB}

No significant differences were detected among the treatments for ∆L (Friedman´s test, $p>0.05$). For ΔΔZ, distinct lower case letters denote significant differences among the five groups, while distinct upper case letters show significant differences for analysis conducted for T1, T3 and T5 separately (repeated-measures ANOVA and Tukey's test, $p<0.05$). T1: fluoridated milk (2.5 ppm F) every day; T2: fluoridated milk (2.5 ppm F) every other day; T3: fluoridated milk (5.0 ppm F) every day; T4: fluoridated milk (5.0 ppm F) every other day; T5: no treatment. *n* = 17.

Table 3. Average lesion depth (µm, ±SD; ∆L= L lesion – L treatment) and integrated mineral loss (ΔΔZ; ΔZ lesion – ΔZ treatment) for the dentine slabs with artificial caries lesions submitted to *in situ* remineralisation, as function of treatment with fluoridated milk containing 2.5 or 5.0 ppm fluoride every day or every other day

Treatment	ΔL - depth (µm)	ΔΔZ (vol.µm%)
T1	-3.22 ± 6.85	-117.9 ± 344.8[a]
T2	3.15 ± 16.0	350.0 ± 657.5[b]
T3	1.3 ±11.1	-182.7 ± 269.5[a]
T4	1.8 ± 10.0	-185.0 ± 360.3[a]
T5	-0.6 ± 7.5	-127.0 ± 332.39[a]

No significant differences were detected among the treatments for ∆L (Friedman's test, $p>0.05$). For ΔΔZ, distinct letters denote significant differences among the groups (repeated-measures ANOVA and Tukey's test, $p<0.05$). T1: F-milk (2.5 ppm F) every day; T2: F-milk (2.5 ppm F) every other day; T3: F-milk (5.0 ppm F) every day; T4: F-milk (5.0 ppm F) every other day; T5: no treatment. *n* = 13.

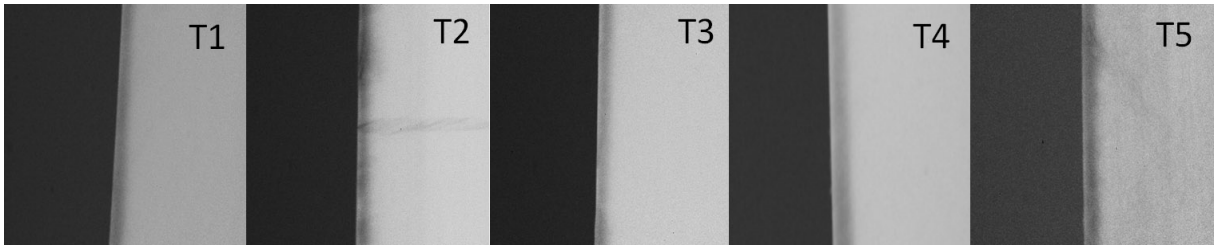

Figure 1 – Typical TMR images of enamel remineralised *in situ* for 7 days. T1 (2.5 ppm F milk every day); T2 (2.5 ppm F milk every other day); T3 (5.0 ppm F milk every day); T4 (5.0 ppm F milk every other day); T5 (no treatment)

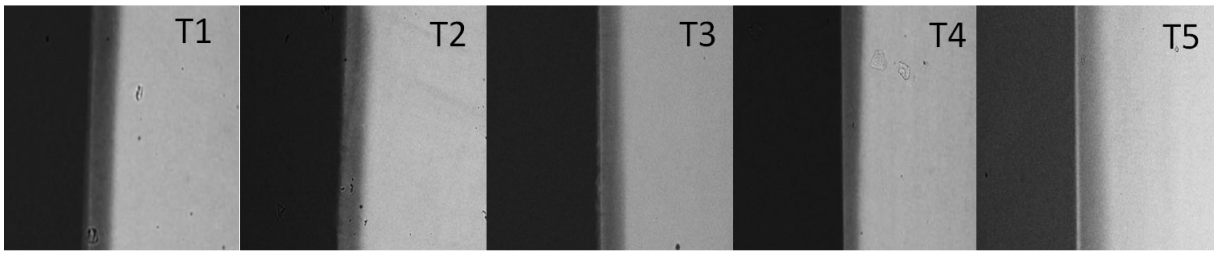

Figure 2 - Typical TMR images of dentine remineralised in situ for 7 days. T1 (2.5 ppm F milk every day); T2 (2.5 ppm F milk every other day); T3 (5.0 ppm F milk every day); T4 (5.0 ppm F milk every other day) ; T5 (no treatment)

3 DISCUSSION

3 DISCUSSION

The widespread use of fluoride through different vehicles has been recognized as the main responsible for the sharp decline in caries prevalence that has been observed worldwide in the last half of the past century (EINARSDOTTIR; BRATTHALL, 1996). Despite the mechanism of action of fluoride to fight caries is essentially topical, systemic vehicles of fluoride delivery, such as water, milk and salt have been also shown to be effective when implemented through community methods for caries prevention (SAMPAIO; LEVY, 2011). However, despite these methods are described as systemic, their caries preventive effect is essentially topical and happens while fluoride from these agents comes into contact with the tooth structure after being dissolved in the oral fluids (BUZALAF et al., 2011).

Water fluoridation has been regarded as the main primary preventive and public health measure for caries control, as it reaches most of the population, including socially deprived groups (MCDONAGH et al., 2000). The benefits of water fluoridation, however, are unavailable to a large proportion of the world's population, mainly due to political, geographical and technical reasons (YEUNG et al., 2005). In order to overcome this problem, other methods of community fluoridation have been suggested. As milk is an important part of children's diet, fluoridated milk has been used in school-based preventive programmes for many decades, in different parts of the world since the 1950's (BANOCZY; RUGG-GUNN; WOODWARD, 2013). Despite fluoridated milk has been recommended as an alternative vehicle to deliver fluoride since the 1950's and some clinical studies have attested the preventive effect of this measure to prevent caries, evidence on this regard has not been firmly established so far (CAGETTI et al., 2013; YEUNG et al., 2005).

Many mechanistic studies have been conducted in an attempt to understand the processes that occur for this protection to be truly effective, in attempt to optimize the caries preventive effect and give support to the design of prevention schemes. The most frequently studied questions are the fluoride concentration of milk (GIACAMAN et al., 2012; ITTHAGARUN et al., 2011; LIPPERT; MARTINEZ-MIER; SOTO-ROJAS, 2012; LIPPERT; MARTINEZ-MIER; ZERO, 2014; MALINOWSKI et al., 2012a, 2012b), temperature of milk (LIPPERT; MARTINEZ-MIER; SOTO-ROJAS,

2012), volume of ingested milk (LIPPERT; MARTINEZ-MIER; ZERO, 2014) and frequency of milk ingestion (ONGTENCO et al., 2014). Results from *in vitro* studies have shown that milk containing 2.5 ppm fluoride significantly increases enamel remineralisation and that increasing fluoride concentrations do not have an additive effect (ITTHAGARUN et al., 2011; MALINOWSKI et al., 2012a). Regarding the frequency of milk ingestion, the use of fluoridated milk twice per day was shown to remineralized enamel lesions to a greater extent when compared with the use once per day or every other day (ONGTENCO et al., 2014). However, *in vitro* models do not reproduce important variables such as the presence of saliva (BUZALAF et al., 2010) that can be mimicked using *in situ* models (COCHRANE; ZERO; REYNOLDS, 2012). This is the first *in situ* study to evaluate the interplay of two important variables (fluoride concentration in milk and frequency of milk intake) on the remineralizing potential of milk on pre-demineralized enamel and dentin slabs.

The protocol of the study was defined to mimic the schemes of school-based milk fluoridation programs worldwide. In these schemes, typically 200 mL of milk containing 0.5 or 1.0 mg fluoride are offered to children, depending on the age and background exposure to fluoride (BANOCZY; RUGG-GUNN; WOODWARD, 2013). This implies that the fluoride concentration in the milk that is offered to the children ranges between 2.5 and 5.0 ppm, which were the concentrations tested in the present study, contrarily to another *in situ* remineralization study that employed milk with much higher fluoride concentrations (7.5 and 30.0 ppm) (LIPPERT; MARTINEZ-MIER; ZERO, 2014). The same rationale was applied to define the frequencies of milk intake. In most of the school-based milk fluoridation schemes, fluoridated milk is provided to children on school days only (around 200 days per year) (BANOCZY; RUGG-GUNN; WOODWARD, 2013), while in Chile milk is provided every day (MARINO; VILLA; GUERRERO, 2001). To reproduce these situations, in the present study fluoridated milk was administered every day or every other day. Another important methological issue involved the choice of the control group. In the present study, the control group received no treatment instead of milk, since there is no programme offering non-fluoridated milk in order to prevent dental caries. Additionally, plain milk (without fluoride) presented rehardening effect similar to milk containing 1.5 mg fluoride (LIPPERT; MARTINEZ-MIER; ZERO, 2014). Furthermore,

fluoridated milk (5.0 ppm fluoride) was not significantly different from non-fluoridated milk regarding dentin demineralization (GIACAMAN et al., 2012).

In order that the remineralizing potential of fluoridated milk could be evaluated, enamel and dentin slabs with artificially produced caries lesions were employed. The protocol used to produce artificial caries lesions was used in previous studies and shown to produce satisfactory lesions both in bovine enamel (BUSKES; CHRISTOFFERSEN; ARENDS, 1985; MAGALHAES et al., 2009) and dentine (MORON et al., 2013). The period of *in situ* remineralization chosen (7 days) was based on the study by Afonso et al. (2013) that tested remineralization periods of 3 and 7 days when fluoridated dentifrices (0-1100 ppm) were used. The response variables used were surface and cross-sectional hardness. The authors observed more pronounced dose-response relationship for the lesions submitted to remineralization during 3 days than for those remineralized for 7 days. It should be pointed out, however, that the fluoride concentrations used in that study ranged between 0 and 1100 ppm delivered through dentifrices, while in the present study the fluoride concentrations in milk were much lower (2.5 or 5.0 ppm). In addition, the volunteers drank non-fluoridated water and used a fluoride-free dentifrice throughout the experimental period. Due to this low exposure to fluoride, we decided to use 7 days of experimental period, in order to increase the extent of remineralization. Even so the degree of remineralization was very low.

The %SHR (surface hardness recovery) for enamel specimens ranged between 5 and 10%. This might have been due to the low fluoride concentration in milk (2.5-5.0 ppm, compared to 1000 ppm when fluoride is included in dentifrice formulations) and low exposure to fluoride from other sources, since volunteers used fluoride-free dentifrice and drank bottled water with low fluoride concentration throughout the experimental period. Despite the low rate of remineralization, it was possible to see a significant increase in the %SHR for the groups treated with fluoridated milk every day (T1 and T3), regardless the fluoride concentration in milk, without significant differences between these groups. These results reflected in the subsurface of the lesions. The highest degrees of remineralization were seen for T1 and T3, confirming previous *in vitro* studies that revealed that milk containing 2.5 ppm fluoride significantly increases enamel remineralization and increasing fluoride concentrations do not have an additive effect (ITTHAGARUN et al., 2011;

MALINOWSKI et al., 2012a). It is important to emphasize that significant enamel remineralization in comparison to control (no treatment) was only achieved when milk was used every day. This is in agreement with recent *in vitro* findings (ONGTENCO et al., 2014) and is also in-line with the current knowledge regarding the mechanism of action of fluoride for caries control. This mechanism highlights the need of constant low-fluoride levels of fluoride in the oral fluids (BUZALAF et al., 2011).

Currently, milk fluoridation programmes are directed to school children. For this reason, nearly all studies in the literature focus on the potential of fluoridated milk to control enamel caries, since dentin caries is not as prevalent in children as is enamel caries. However, milk fluoridation schemes could be implemented to older people that might suffer both from dentin caries (due to gingival recession) (AMER; KOLKER, 2013) and osteoporosis (CAROLI et al., 2011) and could benefit from fluoridated milk. For this reason, in the present study we decided to evaluate also the protective effect of fluoridated milk against dentin caries. Interestingly, the results found for dentin were remarkably different from that observed for enamel. Only the group treated with the lowest fluoride concentration (2.5 ppm) every other day (T2) suffered remineralization. These results are difficult to explain, especially when compared with those obtained for enamel. In addition, the paucity of information available in the literature on the effect of fluoridated milk on dentin caries limits data interpretation. So far only one *in vitro* study was conducted, employing an *S. mutans* biofilm model with pH cycles. It was observed that fluoridated milk (5 ppm fluoride) was not significantly different from milk alone regarding dentine demineralization, contrarily to which was seen for enamel. In addition, while fluoridated milk did not significantly differ from the positive control (0.05% NaF) regarding enamel demineralization, for dentin fluoridated milk had a worse performance when compared with the positive control. This was attributed to the less mineralized nature of the dentinal tissue (GIACAMAN et al., 2012). In the present study, however, the dentin slabs employed had artificial caries lesions. In this situation, there is a superficial layer of demineralized organic matrix (BUZALAF; KATO; HANNAS, 2012; TJADERHANE et al., 1998). It cannot be excluded the possibility that the distinct components of milk, such as fat or proteins, have interacted with this organic layer and interfered with the fluoride, since the only group that experienced remineralization was T2 (lowest fluoride concentration and frequency). Anyway, this

does not explain why T4 (5.0 ppm fluoride milk applied every other day) did not experience remineralization. In fact, for enamel T4 was the only group that had additional demineralization during the *in situ* period. Considering that the present study is the only one that dealt with dentin remineralization by fluoridated milk so far, additional studies are necessary to confirm and explain the present findings.

In conclusion, our data suggest that use of fluoridated milk every day seems to have better remineralizing effect on enamel than its use every other day (situation that mimics use of fluoridated milk on school days only), which should be confirmed by clinical studies. If the results are similar, then current milk fluoridation programmes should consider the need to provide fluoridated milk to children for every day use, instead of use on school days only. Dentin, however, does not seem to benefit from every day use of fluoridated milk, which required confirmation by further studies.

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ANEXOS

ANEXOS

ANEXO 1

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comitê de Ética em Pesquisa

50 ANOS 1962 - 2012 FOB USP

Processo nº 179/2011

Bauru, 1 de dezembro de 2011.

Senhor Professor,

O projeto de pesquisa encaminhado a este Comitê de Ética em Pesquisa em Seres Humanos, denominado "**Efeito da frequência de ingestão e quantidade de fluoreto no leite sobre cárie e erosão de esmalte e dentina: estudo in situ**", de sua autoria e co-autoria do Prof. Dr. Juliano Pelim Pessan e Profª Drª Ana Carolina Magalhães, que será desenvolvido sob sua orientação, foi enviado ao relator para avaliação e apreciado em reunião realizada no dia **30 de novembro de 2011.**

O CEP-FOB/USP considerou o projeto APROVADO lembrando que a condição de aprovação da pesquisa propriamente dita exige o que segue:

- que sejam encaminhados ao CEP-FOB/USP relatórios anuais sobre o andamento da pesquisa (parciais e finais), conforme o cronograma apresentado;
- que sejam notificados ao CEP-FOB/USP, com a devida justificativa, qualquer modificação na metodologia e/ou título e a inclusão ou exclusão de autores;
- na apresentação do relatório final, incluir todos os TCLEs e/ou termos de doação de dentes devidamente assinados e rubricados.

Atenciosamente,

Prof. Dr. Flávio Augusto Cardoso de Faria
Coordenador

Profª Drª Marília Afonso Rabelo Buzalaf
Docente do Departamento de Ciências Biológicas

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ANEXO 2

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comitê de Ética em Pesquisa

Proc. CEP nº 179/2011

Bauru, 14 de julho de 2014.

Senhora Professora,

Em atenção à solicitação de Vossa Senhoria para alterações na pesquisa de sua autoria juntamente com o Prof. Dr. Juliano Pelim Pessan e Profª Drª Ana Carolina Magalhães, quais sejam:

- ✓ alteração no título, anteriormente denominado "*Efeito da frequência de ingestão e quantidade de fluoreto no leite sobre cárie e erosão de esmalte e dentina: estudo in situ*", de autoria de Suelen Cristina da Costa Pereira, para "*EFEITO DA FREQUÊNCIA DE INGESTÃO E QUANTIDADE DE FLUORETO NO LEITE SOBRE CÁRIE DE ESMALTE E DENTINA: ESTUDO IN SITU*";
- ✓ inclusão de pesquisadora responsável, Luiza de Paula Silva Cassiano;
- ✓ alteração no cronograma para finalização em agosto/2014.

informamos que foram analisadas por um relator e consideradas APROVADAS em reunião deste Colegiado realizada no dia 14 de julho de 2014.

Lembramos que ao final da pesquisa, os autores deverão enviar um relatório com os resultados obtidos, conforme orientações deste CEP, para análise e parecer.

Atenciosamente,

Profª Drª Izabel Regina Fischer Rubira de Bullen
Coordenadora

Profª Drª Marília Afonso Rabelo Buzalaf
Docente do Departamento de Ciências Biológicas

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ANEXO 3

INSTRUÇÕES AOS VOLUNTÁRIOS

1. Todos os materiais utilizados na pesquisa não acarretam em custo.
2. Durante o experimento, vocês deverão utilizar apenas a escova, fio dental e dentifrício não fluoretado, fornecidos pela autora do trabalho.
3. A pesquisa será composta por cinco fases de sete dias cada uma, com 7 dias de descanso entre elas. Vocês participarão das 5 fases experimentais (não necessariamente nesta ordem)

Fase A	leite deverá ser administrado TODOS os dias consecutivamente (1x/dia)
Fase B	leite deverá ser administrado de forma intercalada (um dia sim outro não)
Fase C	leite deverá ser administrado TODOS os dias consecutivamente (1x/dia)
Fase D	leite deverá ser administrado de forma intercalada (um dia sim outro não)
Fase E	NÃO haverá exposição ao leite

4. Vocês deverão utilizar um dispositivo bucal palatino e só o removerão para as principais refeições diárias (café da manhã, almoço, lanche e jantar, 1h cada) e para os procedimentos de aplicação de sacarose/leite.
5. Enquanto o dispositivo permanecer fora da boca durante as refeições, este deverá ficar envolvido em gaze umedecida em água deionizada fornecida pela autora do trabalho.
6. Durante o uso do dispositivo, nenhum tipo de alimento ou bebida poderá ser ingerido, exceto água (retirar o dispositivo para a ingestão de água e recolocá-lo imediatamente após a ingestão).
7. Evite que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para a refeição (máximo de 1 hora por refeição) e um intervalo de pelo menos 2-3 h entre elas.
8. Realize sua higiene bucal normalmente, utilizando o dentifrício fornecido.
9. Durante as fases, às 8 horas da manhã, TODOS os dias (com exceção das fases específicas, quando a exposição ao leite será intercalada – fases B e D, e quando o leite não será empregado-fase E), vocês deverão imergir o aparelho em 100 mL de leite (garrafinha marrom) por 5 minutos, e na sequência recolocar o aparelho na boca. Em seguida, deverão ingerir 200 mL de leite (garrafinha branca). NÃO lavar a boca por pelo menos 1 hora após esse procedimento.
10. Dentro das atividades, vocês deverão aplicar 1 gota de solução de sacarose (fornecida pela autora) em cada bloco da fileira azul apenas, 8 vezes ao dia, com um intervalo de 2 horas entre as aplicações. A primeira aplicação deve ser sempre às 8:30 horas (30 minutos após a ingestão do leite). A segunda deverá ser às 10:00 horas e as demais a cada 2 horas. A sacarose NÃO deverá ser gotejada nos blocos da fileira verde.

11. Aplicar **1 gota de solução de sacarose** em cada bloco da fileira **azul** (4 blocos), com o aparelho fora da boca. Aguardar **5 minutos** e recolocar o aparelho na boca sem lavá-lo.
12. Muito cuidado deverá ser tomado para que a solução de sacarose aplicada na fileira vermelha não escorra para a fileira verde. Esse cuidado é de extrema importância.
13. Uma vez ao dia, os voluntários poderão realizar a escovação do dispositivo, somente da face voltada para o palato (céu da boca) e com o dentifrício fornecido pela autora.
14. A água com baixa concentração de flúor será fornecida pela autora e esta deverá ser utilizada tanto para ingestão como para escovar os dentes e o dispositivo.
15. Quando qualquer material estiver acabando, entrar em contato com a autora, para que este seja reposto.
16. Favor verificar todos os dias se os fragmentos estão em suas lojas e se a tela plástica e sua proteção em cera permanecem intactas. Caso não estejam, entrar em contato imediatamente com a autora.
17. Qualquer dúvida, entrar em contato com a autora do trabalho pelos telefones abaixo: Luiza Cassiano

Celular: (14) 96675554
Residência Bauru: (14) 32451408
Laboratório de Bioquímica FOB/USP: (14) 32358246

RESUMO DAS INSTRUÇÕES AOS VOLUNTÁRIOS

Durante o período de utilização do aparelho, você deverá utilizar apenas o dentifrício, a escova e o fio dentário fornecidos pela autora para a realização da pesquisa. Não poderá realizar bochechos com enxaguatórios bucais, como por exemplo, Cepacol, Listerine, Periogard. Também não poderá ser submetido à aplicação tópica de flúor profissional.

1. Primeira noite: apenas dormir com o aparelho.
2. Durante 7 dias: retirar o aparelho **4 vezes ao dia** (café da manhã, almoço, lanche e jantar), por um período de no máximo **1 hora** por vez. Enquanto isso o aparelho deverá permanecer envolvido em gaze umedecida com água deionizada. Intervalo entre as refeições deve ser de pelo menos 2-3h.
3. Após cada período, realizar a higiene bucal com dentifrício e escova dental fornecidos pela autora, antes do aparelho ser recolocado na boca.
4. Às 8 horas da manhã, TODOS os dias (com exceção das fases específicas, quando a exposição ao leite será intercalada – fases B e D, e quando o leite não será empregado- fase E), retirar o aparelho da boca e imergí-lo em 100 mL de leite (garrafinha marrom) por 5 minutos. Em seguida, recolocar o aparelho na boca e ingerir 200 mL de leite (garrafinha branca) .
5. Aplicar **1 gota de solução de sacarose** em cada bloco da fileira **azul** (4 blocos), com o aparelho fora da boca. Aguardar **5 minutos** e recolocar o aparelho na boca, sem lavá-lo. Ao gotejar a sacarose, posicionar o aparelho sobre uma superfície plana, de modo que a solução não escorra, evitando contaminar os blocos da fileira verde Este procedimento deverá ser realizado **8 vezes ao dia**, de preferência com um intervalo de 2h entre as aplicações (8:30h/10h/12h/14h/16h/18h/20h/22h)
6. **Nada deverá ser realizado** na fileira **verde**

Obs: Ao fazer a aplicação de sacarose na fileira azul tomar o máximo de cuidado para que esta não escorra para a fileira verde.

No último dia, dormir com o dispositivo. No dia da entrega do aparelho você pode escovar seus dentes normalmente (sem o aparelho na boca), mas não tomar café da manhã. Em seguida, você deve ir ao laboratório de Bioquímica com o aparelho posicionado na boca, no horário previamente agendado pela pesquisadora. Quando chegar no laboratório, será feita a última exposição ao leite, e 1 hora depois será coletado o biofilme. Após a coleta, um lanche será oferecido no laboratório.

Muito grata.

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