*Acta Odontologica Scandinavica.* 2014; 72: 737–744

informa
healthcare

**ORIGINAL ARTICLE**

# Remineralization of initial enamel carious lesions using fluoridated milk *in vitro*

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**Abstract**

***Objectives.*** Milk is a universal dietary component and it is now recognized as an effective medium for the delivery of fluoride (F). This study sought (i) to evaluate fluoridated milk (2.5 ppm, 5 ppm, 10 ppm) for remineralizing carious lesions and (ii) to determine the optimum frequency for treating carious lesions with fluoridated milk. ***Materials and methods.*** Artificial carious lesions, 90–180 μm deep, were created on extracted third molar teeth that were sectioned to produce specimens of 100–120 μm thickness. Specimens were randomly divided into 13 groups ($n = 20$) for treatment with deionized water, plain milk or fluoridated milk (2.5 ppm, 5 ppm, 10 ppm); once daily, twice daily or on alternate days as part of a 20-day pH cycling model. Lesion depth (LD) and mineral content were evaluated before and after pH cycling. Paired *t*-test, ANOVA and Student-Newman-Keuls tests were employed to make comparisons within and between the different groups. ***Results.*** Fluoridated milk significantly reduced LD and increased the mineral content of the lesions compared to plain milk and deionized water ($p < 0.05$). The greatest reduction in LD was with 2.5 ppm F milk used twice daily ($p < 0.05$). ***Conclusions.*** Milk with 2.5 ppm F used twice daily demonstrated the greatest remineralization of artificial enamel carious lesions *in vitro.*

**Key Words:** *fluoride, milk, caries, demineralization, remineralization*

## Introduction

Fluoride (F) is a major protective factor that can outweigh the pathologic factors and tip the 'caries balance' in favour of remineralization [1], hence it has been the basis of many dental caries prevention programmes. Furthermore, the use of F has been attributed to the reduction in the prevalence of dental caries. Studies that have investigated the effects of F on demineralization and remineralization have demonstrated that the presence of low levels of F ion in the enamel/plaque/saliva interface speeds up the process of remineralization [2].

Milk is a universal dietary component that is readily available in most regions of the world. Milk and water have much in common as vehicles for F, in that the concentration of fluoride is low and they are both drunk as part of the diet. It is recognized as an effective medium for the delivery of F. In 1994, the WHO [3] classified milk, together with water and salt, as a cost efficient vehicle for F delivery. The efficacy of fluoridated milk to reduce carious lesions has been demonstrated by several *in vitro* [4,5] and clinical studies [6,7]. It has been successfully used for community fluoridation schemes in many parts of Europe, the UK, Thailand, Russia, China and Chile.

Recently, a Cochrane review [8] on the efficacy of fluoridated milk in preventing dental caries has identified only two randomized clinical trials [6,9] with an intervention or follow-up period of at least 3 years. They concluded that there were insufficient studies with good quality evidence evaluating the effects of fluoridated milk in dental caries prevention [8]. While several *in vitro* and clinical trials support the remineralizing potential of fluoridated milk, they have failed to establish a consensus on the appropriate concentration of F to be added to the milk to remineralize carious lesions. To date, there are no published studies evaluating the optimum concentration and frequency of fluoridated milk in remineralizing

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*(Received 2 July 2013; accepted 24 February 2014)*

ISSN 0001-6357 print/ISSN 1502-3850 online 
DOI: 10.3109/00016357.2014.903515

carious lesions. Therefore, the objectives of this *in vitro* study were to (i) evaluate the efficacy of fluoridated milk (2.5 ppm, 5 ppm and 10 ppm) in remineralizing artificial carious lesions compared with plain milk and deionized water and (ii) determine if the frequency of treating artificial carious lesions with these fluoridated milks (a) twice daily and (b) on alternate days would alter its remineralization potential in comparison with the artificial carious lesions treated once daily with the same F concentration.

## Materials and methods

### *Lesion formation*

Extracted third molars (erupted), stored in normal saline, with intact buccal and lingual surfaces were cleansed of soft tissue and debris and inspected for any hypoplasia, cracks or caries under a stereomicroscope. Sticky wax (Model Cement®, Hong Kong, Dentsply) was used to envelope the roots up to the cemento-enamel junction, so as to seal the apical foramina and the furcation area completely. The teeth were painted with an acid resistant nail varnish (Revlon®, Newyork, USA), leaving a 1 mm wide rectangular window, to enable several sections, on the buccal surfaces and were allowed to dry overnight. The following day an additional coating of the nail varnish was applied, avoiding the rectangular window, to ensure complete coverage. The teeth were immersed in demineralizing solution (10 ml/tooth) in an airtight container and stirred, for 96 h to produce homogenous artificial carious lesions of 90–180 μm deep. They were then sectioned longitudinally through the carious lesions using a hard tissue saw microtome (Leica® 1600 saw microtome, Wetzlar, Germany) to produce specimens that were ~100–120 μm thick. The thickness of each specimen was measured using a micrometer. The specimens were then painted completely, except for the superior margin of the lesion, under a stereomicroscope using an acid resistant nail varnish (Revlon®). The sections were stored in 100% humidity until the start of the pH cycle.

### *Preparation of demineralizing and remineralizing solutions*

The de-/remineralizing buffered solutions were prepared from certified chemicals and de-ionized water. The demineralizing solution contained 2.2 mM $CaCl_2$, 2.2 mM $NaH_2PO_4$ and 0.05 M acetic acid; the pH was adjusted to 4.4 using 1M KOH. The remineralizing solution contained 1.5 mM $CaCl_2$, 0.9 mM $NaH_2PO_4$, 0.15 M KCl at pH 7.0. These solutions were similar to those utilized by ten Cate and Duijsters [10].

All of the solutions were freshly prepared before each phase of the pH cycle. The pH of the demineralizing, remineralizing and treatment solutions was checked prior to inserting the specimens during each phase of the pH cycle. The different concentrations of fluoridated milk were prepared using homogenized and pasteurized fresh bovine (cow's) milk manufactured by Nestle® Hong Kong Limited which contained the following ingredients: calcium 110 mg/100 ml; protein 3.1 g/100 ml; carbohydrate 4.6 g/100 ml; fat 3.5 g/100 ml. F stock solutions were added to the plain milk in a container with a magnetic stirrer to produce fluoridated milk solutions, 2.5 ppm, 5 ppm or 10 ppm, in a fixed volume ratio. During the pH cycling, all groups were placed on an orbital shaker to ensure all specimens were immersed in the treatment solutions.

### *pH cycling model and test groups*

The pH cycling model used in this study was a modified version of the one proposed for investigating root caries by Ivancakova et al. [4]. The protocol was modified, the difference being the duration of the treatment, in which the originally proposed 14 days of treatment was extended to 20 days. Two hundred and sixty specimens (sections) were randomly assigned to 13 treatment groups; 20 specimens per group (Figure 1). Three individual cycles, with different treatment protocols, were conducted simultaneously, see Figure 2. In cycle 1, specimens in Groups 1, 4, 7, 10 and 13 were subjected to F treatment once daily. This involved 4 h of demineralization, 6 h of F treatment and 14 h of remineralization each day. In cycle 2, specimens in Groups 3, 6, 9 and 12 were subjected to F treatment on alternate days, i.e. 4 h of demineralization, 6 h of F treatment and 38 h of remineralization. Furthermore, in cycle 3, specimens in Groups 2, 5, 8 and 11 were subjected to F treatment twice daily to allow 6 h of F treatment, 4 h of demineralization, 6 h of F treatment and 8 h of remineralization.

### *Test groups*

- Group 1: 2.5 ppm F milk treatment once daily after demineralization cycle.
- Group 2: 2.5 ppm F milk treatment twice daily, before and after demineralization cycle.
- Group 3: 2.5 ppm F milk treatment on alternate days, after demineralization cycle.
- Group 4: 5 ppm F milk treatment once daily after demineralization cycle.
- Group 5: 5 ppm F milk treatment twice daily, before and after demineralization cycle.
- Group 6: 5 ppm F milk treatment on alternate days, after demineralization cycle.
- Group 7: 10 ppm F milk treatment once daily after demineralization cycle.

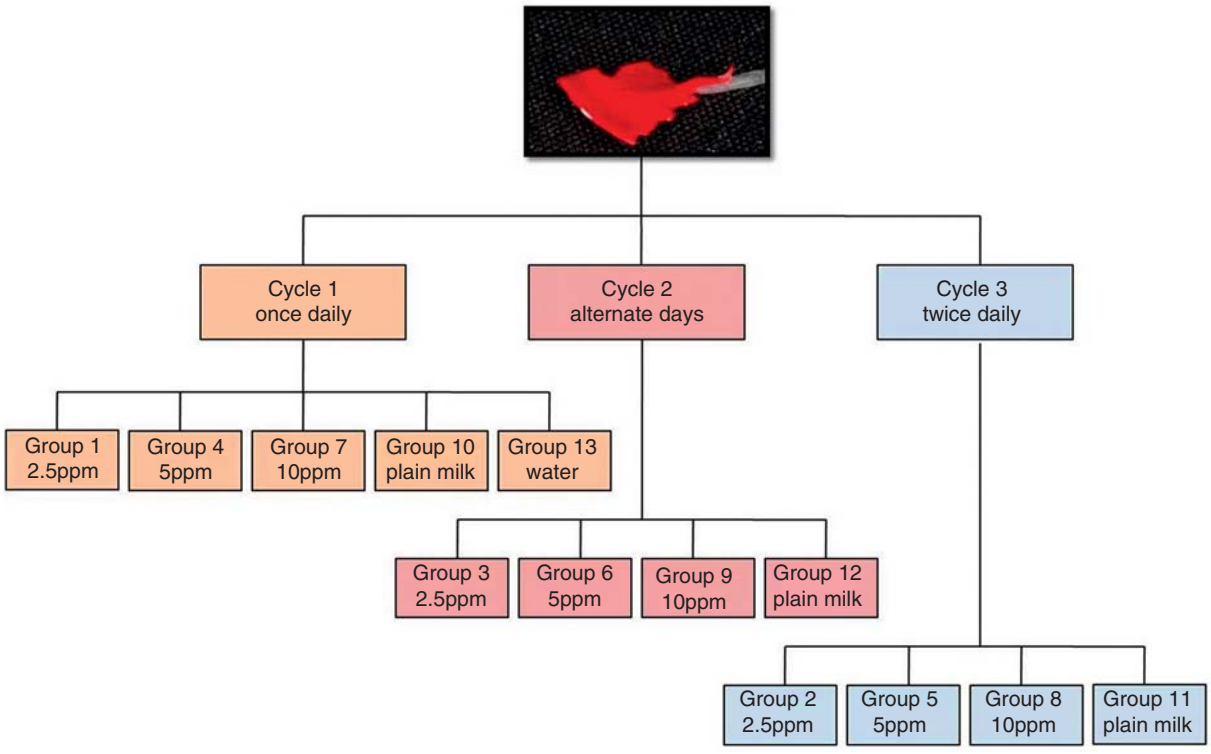


Figure 1. Flow chart illustrating the different groups and their treatment protocols.

- Group 8: 10 ppm F milk treatment twice daily, before and after demineralization cycle.
- Group 9: 10 ppm F milk treatment on alternate days, after demineralization cycle.
- Group 10: milk without fluoride, treatment once daily after demineralization cycle.
- Group 11: milk without fluoride, treatment twice daily, before and after demineralization cycle.
- Group 12: milk without fluoride, treatment on alternate days, after demineralization cycle.
- Group 13: deionized water, treatment once daily after demineralization cycle.

*Evaluation techniques*

The lesions were evaluated qualitatively under a polarized light microscope (PLM; Orthoplan®, Leitz, Germany), before and after the pH cycling. Each specimen was imbibed in deionized water and subsequently viewed under a polarized light microscope to exhibit a clear demarcation between sound enamel and the lesion area [11]. The photomicrographs (Kodak® ektachrome transparency film E100GX) were taken at a fixed standard magnification (6.3× objective, 8× camera lens) for all specimens before

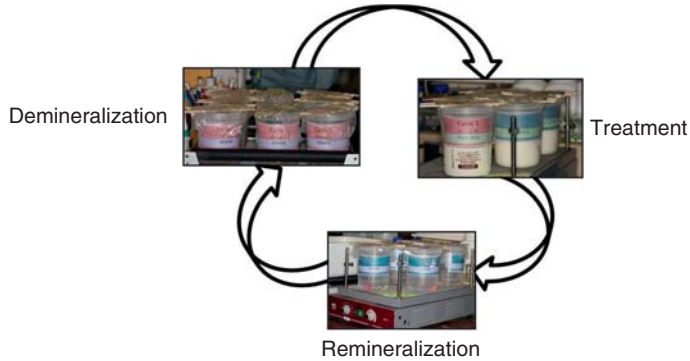


Figure 2. Different protocols for each treatment cycle.

and after the pH cycle to allow visual comparisons between pre- and post-treatment.

Quantitative evaluation of the lesions was performed using microradiography (MRG). Specimens were mounted on an electron microscopy film (Kodak® electron microscopy film 4489) with a step wedge and exposed to Cu (K$\alpha$) X-rays (Softex IRS-20, JIRA, Japan) at a voltage of 7 kV and 3 mA current for 30 s. Subsequently each film was immersed in the developing solution for 8 s, rinsed for 60 s in running water, after which they were placed in the fixing solution for 60 s. Each microradiograph was washed, air dried and then mounted onto a glass slide to be observed under a microscope (Zeiss®, Germany). The images of the microradiographs were scanned at a resolution of 12 800 dots per inch and saved as high quality tiff files. The scanned images were then used with Image J® software to quantify the changes in the lesion depth (LD) and the mineral content of all the specimens before and after the pH cycling procedure. The software calculated the relative mineral density based on the data from sound enamel [12].

### *Statistical analysis*

Within the context of correlation, as this study encompassed a positive directional hypothesis the statistician consented that at $n = 20$ the observed value would be significant at the 5% level. Paired *t*-test was used to compare differences in lesion depths within each group before and after the pH cycle. One-way analysis-of-variance (ANOVA) and Student-Newman-Keuls (SNK) tests were employed to determine the differences between the different treatment groups with $p < 0.05$ considered statistically significant. Mean and standard deviation was used for the descriptive data.

## Results

### *Lesion depth changes in the artificial carious lesions*

There was no statistical difference between the pre-treatment LD among the different groups within each cycle and between the three cycles prior to the experimental phase of the study ($p = 0.87$, $p = 0.29$ and $p = 0.37$ for cycles 1, 2 and 3, respectively, ANOVA).

All the specimens treated with F milk, either once daily, twice daily or on alternate days, exhibited a statistically significant difference in LD between pre- and post-treatment ($p < 0.01$ paired *t*-test), see Table I. All three of the milks containing fluoride (2.5 ppm, 5 ppm and 10 ppm) exhibited significant reductions in LD compared with plain milk or water, while plain milk exhibited a significantly lower rise in LD compared with water ($p < 0.05$, ANOVA, SNK). When comparisons were made between the different fluoridated milk groups, no statistically significant differences were evident in specimens treated once daily or on alternate days. Nevertheless, specimens

Table I. Lesion depth changes after treatment with different concentrations of fluoridated milk.

| | | Mean lesion depth (μm) ± SD | | |
|---|---|---|---|---|
| Treatment (50 ml/section) | Sample Size | Pre-treatment | Post-treatment | % Change |
| *Once daily* | | | | |
| 2.5 ppm fluoride milk | 20 | 151.65 ± 26.6 | 146.95 ± 25.5* | −3.04 ± 2.1*a*1 |
| 5 ppm fluoride milk | 20 | 151.80 ± 29.9 | 148.10 ± 28.1* | −2.23 ± 3.7*a*1 |
| 10 ppm fluoride milk | 20 | 149.40 ± 33.7 | 146.55 ± 31.9* | −1.62 ± 3.6*a*1 |
| Plain milk | 20 | 146.70 ± 30.3 | 153.85 ± 27.8* | 5.76 ± 8.1*b*1 |
| Deionized water | 20 | 157.40 ± 28.4 | 173.85 ± 27.7* | 11.24 ± 10.2*c*1 |
| *Alternate days* | | | | |
| 2.5 ppm fluoride milk | 20 | 148.90 ± 33.4 | 146.85 ± 32.5* | −1.25 ± 3.1*a*2 |
| 5 ppm fluoride milk | 20 | 150.00 ± 24.53 | 147.55 ± 23.4* | −1.45 ± 5.0*a*2 |
| 10 ppm fluoride milk | 20 | 159.00 ± 32.76 | 157.00 ± 32.3* | −1.22 ± 3.5*a*2 |
| Plain milk | 20 | 155.55 ± 28.5 | 160.35 ± 28.7* | 3.22 ± 3.5*b*2 |
| *Twice daily* | | | | |
| 2.5 ppm fluoride milk | 20 | 153.85 ± 32.4 | 143.80 ± 26.0* | −5.94 ± 5.0*a*3 |
| 5 ppm fluoride milk | 20 | 148.10 ± 27.2 | 144.55 ± 28.4* | −2.57 ± 5.3*b*3 |
| 10 ppm fluoride milk | 20 | 148.55 ± 26.7 | 145.45 ± 26.9* | −2.12 ± 3.5*b*3 |
| Plain milk | 20 | 152.70 ± 24.6 | 156.20 ± 21.3* | 2.81 ± 4.9*c*3 |

*Significant change ($p < 0.01$ paired *t*-test) in lesion depth post-treatment.
Different superscript letters and numbers indicate statistically significant differences between the groups ($p < 0.05$, ANOVA, SNK).

Table II. Comparison of the lesion depth changes between the different treatment groups.

| | | % Change (μm) ± SD | | |
|---|---|---|---|---|
| Treatment (50 ml/section) | Sample size | Alternate days | Once daily | Twice daily |
| 2.5ppm fluoride milk | 20 | $-1.25 \pm 3.1^{b}$ | $-3.04 \pm 2.1^{b}$ | $-5.94 \pm 5.0^{a}$ |
| 5ppm fluoride milk | 20 | $-1.45 \pm 5.0^{b}$ | $-2.23 \pm 3.7^{b}$ | $-2.57 \pm 5.3^{b}$ |
| 10ppm fluoride milk | 20 | $-1.22 \pm 3.5^{b}$ | $-1.62 \pm 3.6^{b}$ | $-2.12 \pm 3.5^{b}$ |
| Plain milk | 20 | $3.22 \pm 3.5^{c}$ | $5.76 \pm 8.1^{c}$ | $2.81 \pm 4.9^{c}$ |

Different superscript letters indicate statistically significant differences between the groups ($p < 0.05$, ANOVA, SNK).

treated with 2.5 ppm F milk twice daily exhibited a statistically significant reduction in the LD compared to those treated with 5 ppm and 10 ppm F milk twice daily ($p < 0.05$, ANOVA, SNK).

When multiple comparisons were made between the different groups among the three cycles (Table II), specimens treated with 2.5 ppm F milk twice daily exhibited the greatest reduction in the LD compared to those treated once daily or on alternate days with the same or higher F concentration ($p < 0.05$, ANOVA, SNK). The specimens treated with 2.5 ppm F milk once daily also exhibited an increased reduction in their LD compared to those specimens treated on alternate days with the same F concentration.

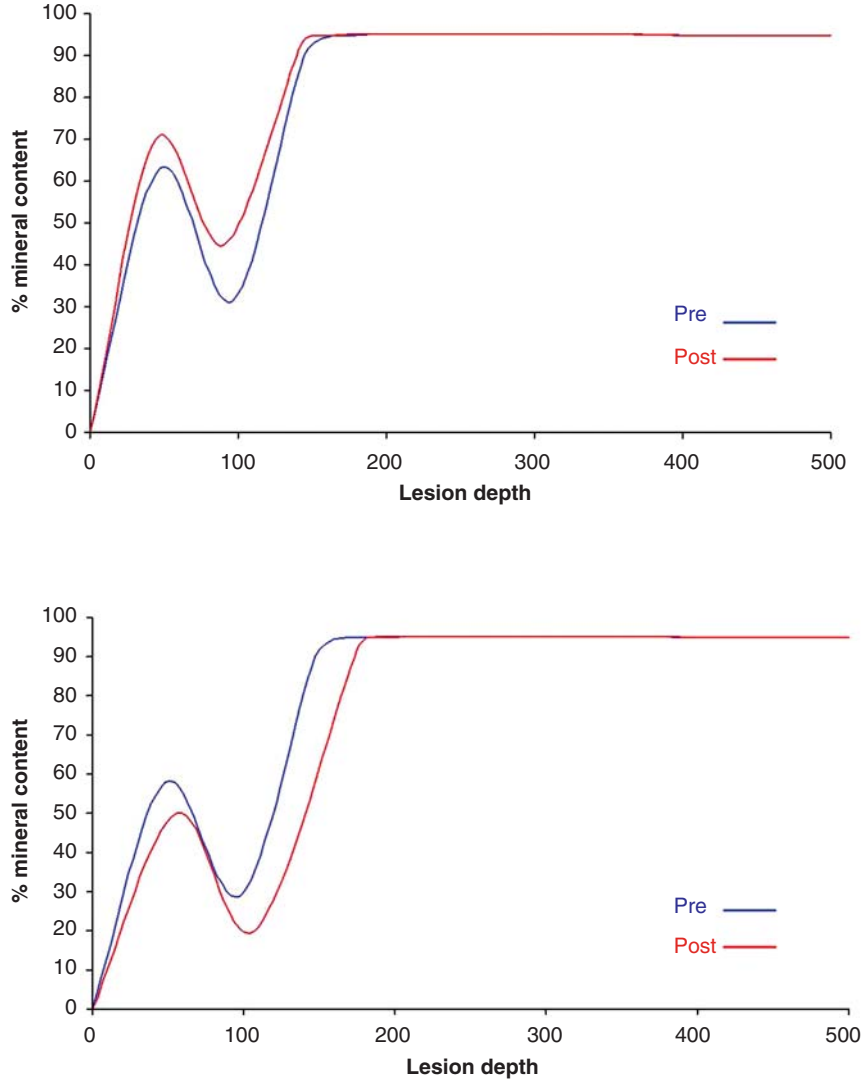


Figure 3. Representative graphs (Group 2: 2.5 ppm F twice daily) showing the relationship between the lesion depth and the relative percentage of mineral content, pre- and post-treatment, for the fluoride groups (top) and control groups (bottom).

However, this did not reach statistical significance. There were no significant differences between the 5 ppm and 10 ppm F milk groups among the three cycles. Nevertheless, there was a trend indicating that treatment with 5 ppm and 10 ppm F milk twice daily exhibited a higher remineralizing potential compared to treatment once daily and that treatment once daily exhibited a higher remineralizing potential than treatment on alternate days with the same F concentration.

The PLM photomicrographs illustrate that the groups subjected to treatment with different F concentrations had decreased the LD, while groups treated with plain milk and deionized water exhibited an increase in the LD. Similarly, the mineral density profiles demonstrate that only groups treated with different F concentrations exhibited a higher mineral content after pH cycling. Examples of the microradiographs illustrating the increase and decrease in LD and the changes in the mineral content after pH cycling are evident in Figure 3.

## Discussion

Artificial enamel carious lesions are of a more reproducible nature than natural carious lesions [13], as it ensures the possibility of testing multiple variables at any given time. The *in vitro* pH cycling model is designed to simulate the dynamic variations in both mineral saturation and pH associated with the natural carious process [14]. Recently, Ivancacova et al. [4] used a 14 day pH cycling model to evaluate the remineralizing potential of fluoridated milk and concluded that 14 days of treatment duration was insufficient to elicit marked changes in the lesions; leading them to recommend longer treatment duration. Therefore, a 20-day pH cycling model was used in this present study so as to allow sufficient time to create marked changes in the lesions and, simultaneously, not to make the process too lengthy so as to produce damage to the thin enamel specimens.

In the present study, the solutions for the pH cycling model were freshly prepared for each phase of the cycle. The aim was to prevent saturation or exhaustion of the solution, due to accumulation of enamel dissolution products [10]. All specimens were placed on an orbital shaker during the pH cycle to avoid deeper lesions and greater mineral loss in comparison to unstirred systems [15]. The pH of deionized water, plain milk and fluoridated milk ranged between 6.6–6.8, which was well above 5.5; the critical pH. The milk used in this study was from Nestle® Hong Kong Limited and was declared melamine-free by the Centre for Food Safety of Hong Kong (CSF). Fresh milk was used every day to avoid storage problems and the addition of F solution to the milk did not alter its taste, appearance or odour. Separate containers were used for all treatment solutions in each phase of the pH cycle to minimize cross-contamination.

All the specimens in our study were randomly divided into the different groups. No statistically significant differences were evident between the specimens within each group, among the different groups, within each cycle and between the different cycles prior to the experimental phase of the study. This represents the absence of sampling bias at baseline and implies that, although the specimens were sectioned from different teeth, the variations among these teeth would not have influenced the demineralization progression before the pH cycling. Therefore, it was logical to disregard these variations when de-/remineralization efficacy of different concentrations of F milk were evaluated and compared after the pH cycle.

Milk is an effective medium for F delivery [3] and it has been demonstrated that the availability of F in milk is unaffected by the presence of calcium. Although milk is calcium rich, most of it is bound to casein and citrate within the milk matrix and only minute amounts are freely available to interact with higher concentrations of F, to produce calcium fluoride. It has also been shown that this interaction does not occur at lower levels of F concentrations [16]. Therefore, the relatively low F concentrations used in this present study would have had minimum interactions with the calcium in milk.

Several reviews have emphasized the role of plain milk and dairy products in caries prevention [17–19] and some studies have successfully used bovine milk to remineralize sub-surface enamel lesions [20]. In the present study, although treatment with plain milk increased the LD, this increase in the LD was significantly lower than that noted in the specimens treated with deionized water; which highlights the beneficial effects of plain milk in reducing the progression of a carious lesion.

The efficacy of topical F is dependent on factors such as: its concentration, pH of the topical medium, solubility of the tooth, exposure time and the availability of free calcium and phosphate ions. F in milk exhibits both topical and systemic effects. When present in the oral cavity it binds with free calcium and phosphate ions to form fluorapatite [$Ca_{10}(PO_4)_6F_2$], a complex that is more resistant to an acidic challenge. F also gets incorporated into the plaque and remains in the saliva to form a F reservoir. When consumed systemically it will be subsequently excreted through the salivary glands [21], especially during the first 60 min; thus, elevating the available F level both in the saliva and supragingival plaque to exert its topical effects [22].

All the specimens treated with fluoridated milk either once daily, twice daily or on alternate days exhibited lesions with significantly reduced LD ($p < 0.05$, ANOVA, SNK) compared with those

treated with plain milk and deionized water. Among the fluoridated milk groups, specimens treated with 2.5 ppm fluoridated milk twice daily exhibited the greatest decrease in LD. Similarly, when comparing the percentage changes in LD between the different groups treated with the same concentration, only those groups treated with 2.5 ppm fluoridated milk demonstrated significant changes.

No statistically significant differences were evident between groups that received 5 ppm and 10 ppm fluoridated milk either once daily, twice daily or on alternate days. Although a trend was evident which indicated that increased frequency of treatment produced greater remineralization, it failed to reach statistical significance. This could be due to the length of the pH cycling used in this study. Perhaps increasing the length of the pH cycle might have produced more noticeable changes.

Recently, Malinowski et al. [23] using an *in situ* model and reported that 2.5 ppm and 5 ppm F in milk significantly protected enamel from demineralization. Subsequently, an *in vitro* pH cycling study by the same authors [24] demonstrated that F concentration in milk exhibited a clear dose dependency and that the presence of F even at low concentrations (below 2.5 ppm F) promoted remineralization in their pH-cycling model. However, the pH cycling method used in their study was very different to ours in that they exposed the slabs alternatively to demineralization and remineralization with exposure twice daily to the test milk and milk/saliva slurry. Nevertheless, the present study did not study concentrations lower than 2.5 ppm in milk, hence eliminating comparisons between the two studies.

In the present study 2.5 ppm F appeared to be more effective than 5 ppm F and 10 ppm F; which might be due to the amount of available F in the fluoridated milk. When F is added to milk, it may interact partially with the intrinsic ions, including calcium, or be incorporated into the milk proteins. Calcium present in high concentrations in milk is believed to interact with F resulting in the precipitation of calcium fluoride, but this interaction does not occur when the concentration of F in milk is low (2–5 ppm). This is because only a very small fraction of the calcium, ~80 mg/l, is free for interaction; the rest of the calcium in milk is already complexed within the milk matrix with citrate and casein. Therefore, calcium fluoride does not precipitate out at low levels of F. When the volume is increased the total amount of fluoride available for re-mineralization is increased, whereas at higher concentrations of F, the calcium in milk may interact with F, resulting in the precipitation of calcium fluoride.

The findings of the present study demonstrate that fluoridated milk (2.5 ppm, 5 ppm and 10 ppm) is effective in reducing the progression of carious lesions. Furthermore, it appears that 2.5 ppm fluoridated milk has the potential to be effective in remineralizing carious lesions and increasing its frequency (twice daily) would result in a higher remineralization. Although this result might be exciting, one must be cautious while extrapolating these results to a clinical situation due to the limitations of the present *in vitro* model which does not account for saliva, plaque/pellicle and the carbohydrate challenge. Therefore, prior to making clinical recommendations the findings of the present study should be validated using an *in situ* model.

Therefore, based on the findings of this *in vitro* study, it can be concluded that (i) artificial carious lesions treated with 2.5 ppm, 5 ppm and 10 ppm F milk exhibited greater remineralization when compared to those treated with plain milk or deionized water, (ii) increasing the frequency of treating artificial carious lesions with the same concentration of F milk showed a 'trend' to improve their remineralization efficacy, (iii) specimens treated with 2.5 ppm F milk exhibited the greatest remineralizing potential of artificial carious lesions and (iv) artificial carious lesions treated twice daily with 2.5 ppm F milk demonstrated the highest remineralizing potential compared to those treated once daily or on alternate days.

## Acknowledgements

The technical assistance of Mr Yip and Mr Shadow is gratefully acknowledged. This study was funded by a grant from the Borrow Foundation, UK.

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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