The Effect of Varying Concentrations of Fluoridated Milk on Enamel Remineralisation in vitro

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Abstract
The aim of this in vitro single blind study was to investigate the dose response of fluoride in milk on enamel demineralisation and remineralisation under pH cycling using transverse microradiography (TMR). Enamel slabs (n = 11) with caries-like lesions were exposed to milk containing 6 different fluoride concentrations (0, 0.25, 0.5, 1.0, 5.0 and 10.0 ppm F). On each of the 14 days of the cycling period the lesions were exposed to five 2-minute periods of cariogenic challenge (1.5 mM CaCl₂, 0.9 mM KH₂PO₄ and 50 mM acetic acid at pH 5.0) and two 5-min periods in milk plus 10 min in a milk/saliva (1:3) slurry. The slabs were stored at 37 °C in artificial saliva throughout the cycling period and demineralisation and remineralisation was assessed by TMR using dedicated image software. Remineralisation (ΔZ) was observed in all fluoride groups in contrast to demineralisation in the non-fluoride control. Remineralisation was significant (p < 0.05) for all concentrations above 1.0 ppm F. The results showed that fluoride concentration in milk exhibited a clear dose dependency and that the presence of fluoride even at low concentrations promoted remineralisation in this pH-cycling model.

Key Words
Demineralisation · Enamel · Fluoride · Milk · Remineralisation

The Swiss paediatrician Ziegler [1953] recommended that milk could be used as an alternative vehicle to deliver fluoride (F). Since that time, a number of studies have established the caries-preventive efficacy of fluoride in milk [Stephen et al., 1984; Bánóczy et al., 1990; Bánóczy et al., 2009]. The first studies were conducted in the USA [Russoff et al., 1962] and Switzerland [Wirz, 1964]. These showed DMF reductions of between 16 and 82% for children after 3–6 years of fluoridated milk (1.0 ppm F) consumption. In a 5-year double-blind trial conducted in Scotland [Stephen et al., 1984], caries reductions were found to be 35.8 and 48% for DMFT and DMFS, respectively. In general, studies have shown a reduction of caries between 18 and 65% [Kunzel, 1993].

Values of 1.0 and 2.5 ppm F and even higher in milk have been recommended as optimal concentrations, using sodium fluoride [FDI, 1984]. An animal study [Stösser et al., 1995] showed that a reduction in the number of carious lesions in rats (36–40%) was observed at 3 concentrations of fluoridated milk (5.0, 10.0 and 15.0 ppm F) which was independent of the compound or the fluoride concentration used.

A large scale community-based milk fluoridation project which included 3,000 children aged 3–10 years old in Bulgaria [Pakhomov et al., 1995] claimed that after 5 years of fluoridated milk consumption (200 ml milk containing 1 mg of fluoride) reductions in the DMFT and
DMFS values were 44 and 77%, respectively. Riley et al. [2005] found a difference of 31% in DMFT and a difference of 37% in DMFS between those children attending schools where fluoridated milk (0.5 mg F in 189 ml of fresh milk) was available and those where it was not. These findings are comparable to the studies conducted in Scotland by Stephen et al. [1981, 1984], where the fluoride dosage was substantially higher at 1.5 mg in 200 ml. However, a number of unanswered questions remain and one of them is to establish the concentration of fluoride in milk which should be used for such school-based programmes.

A number of studies have investigated the influence of fluoride on enamel demineralisation and remineralisation [Takagi et al., 2000; Lippert et al., 2012]. It is now widely accepted that the main mechanism of action of fluoride for caries control is due to the presence of low levels of fluoride ion in the enamel/plaque/saliva interface [Buzalaf et al., 2011]. Lynch and ten Cate [2005] found that fluoride at concentrations normally found in plaque could promote remineralisation at pH values often associated with demineralisation. There have not been many studies carried out using in vitro demineralisation models to investigate the effect of fluoridated milk on human dental enamel. Toth et al. [1997] showed that 10 mg/l of fluoride in milk produced a significant decrease in acid solubility. A pH-cycling experiment [Herkströter et al., 1991] showed that 2 ppm fluoride was needed to stop the demineralisation of enamel almost completely, but not that of dentine. A recent study [Itthagarun et al., 2011] suggested that 2.5 ppm fluoride milk provided similar remineralisation potential to higher fluoride concentrations. However, they did not study concentrations lower than 2.5 ppm in milk and the cycling regime involved prolonged periods of demineralisation and remineralisation, which do not reflect the actual time this would happen in a real life scenario.

From the review of studies on fluoridated milk it seems that there is a need to investigate and determine the optimum fluoride concentration in milk that produces the greatest reduction in dental caries. The aim of our study was to use a pH-cycling model to investigate the demineralisation and remineralisation of subsurface caries-like lesions exposed to different concentrations of fluoride in milk.

**Materials and Methods**

**Tooth Selection, Cleaning and Preparation**
The enamel slabs used in the study were from human premolar teeth extracted for orthodontic purposes. The teeth were cleaned with a pumice powder and stone to remove any soft tissue and screened by transillumination and transmitted light using low-power microscopy for the detection of cracks (Leitz, Wetzlar®, Germany), caries or any malformations. Suitable teeth were lightly abraded with fine abrasive paper (P1000 Wet & Dry paper, 3M) in order to remove the outer enamel surface (100–200 µm). This helps to achieve flatness and removes the pellicle while also achieving a degree of standardisation, as the fluoride history of the teeth is unknown and teeth may have accumulated fluoride in the surface layer. The teeth were painted with two coats of an acid-resistant, coloured nail varnish (Max Factor, UK) – with a 24-hour interval in between – except for a window of exposed enamel of approximately 6 × 2 mm on the buccal surface. Each tooth was attached to the plastic spoon of a ‘Sterilin’-type universal tube with dental wax to hold it in the demineralising gel.

**Lesion Preparation**
Artificial white spot lesions were created with an acidified gel as described by Edgar [1983]. The teeth were submerged in universal tubes containing demineralising gel of 6% w/v hydroxyethyl cellulose (pH 4.5). They were left in the gel for 7 days at room temperature until a white spot was clearly visible. The exposed window on each tooth was then carefully sectioned with a diamond saw (Well 3242, Switzerland) to give 4 equally sized enamel portions of approximately 1.5 × 2 × 1.5 mm, which were used for the cycling regime; 1 section was kept as the baseline/control slab.

**Transverse Microradiography**
A thin slice was cut from each enamel section and hand-ground. This was verified using a micrometer (Mitutoyo Digmatic, USA) to a thickness of 80–100 µm, using a diamond disc, which was impregnated with 15-micrometer diamond particles (Buehler, Ill., USA). The sections were then placed into pockets made from acetate sheets, and into specially fabricated radiographic plate-holding cassettes, incorporating an aluminium step-wedge (steps of 20-µm thickness). The cassette was loaded with type 1A high-resolution glass X-ray plates (IMTECH, Calif., USA) and exposed Cu-Kx X-ray source for 8 min at an anode voltage of 20 kV and tube current of 10 mA at a focal distance of 30 cm.

The microradiographs were examined under the optical microscope (Leica Leitz DMR, Germany). The mineral loss (ΔZ) and lesion depth (µm) were measured using an image-analysis computer program TMRW, 2000 (version 20.1.5.1) (Inspector Research Systems, Amsterdam, The Netherlands).

**Experimental Protocol/Regime**
It was estimated that 10 slabs per group were required. Eleven enamel slabs with artificial caries-like lesions were used per group in case a slab was damaged during the study. Each slab was allocated randomly to one of the study groups, with one slab from each tooth allocated to each group using a table of random numbers. The following concentrations of F in milk were used: 0 ppm F (control), 0.25, 0.5, 1.0, 5.0 and 10.0 ppm F. Slabs were cycled for 14 days. At the start of each day, the slabs were exposed for 5 min to one of the concentrations of F milk, followed by 10 min in slurry of 1 part milk and 3 parts artificial saliva. The slabs were then subjected to demineralising solution (pH 5.0)
5 times daily for 2 min, whilst immersing them in the supersaturated saliva in between dippings for 1 h. They were then finally exposed to milk and milk/saliva slurry and stored overnight (fig. 1) in saturated night-time artificial saliva.

Two types of artificial saliva of pH 6.8 were used for this study, one (supersaturated with respect to hydroxyapatite) for use in between treatments during the day (CaCO3 0.7 mM, MgCO3 0.2 mM, KH2PO4 4 mM, HEPES 20 mM and KCl 31.4 mM) and one (saturated with respect to hydroxyapatite for overnight) as a storage solution (CaCO3 0.5 mM, MgCO3 2 mM, KH2PO4 0.5 mM, HEPES 20 mM and KCl 31.4 mM). The enamel slabs were immersed overnight in artificial saliva saturated with respect to hydroxyapatite in order to ensure that no further precipitation occurred on the slab surface; when the cycling was resumed the next morning, the slabs were in the same state of demineralisation and remineralisation. The experiment was carried out at 37 °C by keeping the slabs in an incubator throughout the cycling period.

**Statistical Analysis**

SPSS for Windows (Apache Software Foundation, 2004, USA) was used to statistically analyse the data. The Shapiro-Wilk test showed that the data was not normally distributed. A Wilcoxon test was used to compare the mineral loss (ΔZ) among and within groups with different fluoride concentrations. In addition, a paired Student t test was used to measure the 95% confidence intervals within the group. The significance level for all tests was set at 5%.

**Results**

Table 1 shows the mineral loss for each group at baseline and after treatment and the mean difference with standard deviations. Mineral loss in the lesion between baseline and after treatment when compared within each group was statistically significant for the 1.0-, 5.0- and 10.0-ppm F groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline SD</th>
<th>After pH cycling SD</th>
<th>Mean difference SD</th>
<th>Significance</th>
<th>95% confidence interval of the difference of the means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk (0 ppm F)</td>
<td>725.88</td>
<td>308.72</td>
<td>-200.90</td>
<td>0.074</td>
<td>-469.21 67.41</td>
</tr>
<tr>
<td>Milk (0.25 ppm F)</td>
<td>830.76</td>
<td>225.93</td>
<td>121.11</td>
<td>0.314</td>
<td>-88.77 330.98</td>
</tr>
<tr>
<td>Milk (0.5 ppm F)</td>
<td>979.43</td>
<td>307.97</td>
<td>139.42</td>
<td>0.386</td>
<td>-124.87 403.71</td>
</tr>
<tr>
<td>Milk (1.0 ppm F)</td>
<td>847.08</td>
<td>203.63</td>
<td>268.44</td>
<td>0.041*</td>
<td>5.60 531.28</td>
</tr>
<tr>
<td>Milk (5.0 ppm F)</td>
<td>908.91</td>
<td>426.37</td>
<td>482.55</td>
<td>0.003*</td>
<td>332.01 633.08</td>
</tr>
<tr>
<td>Milk (10.0 ppm F)</td>
<td>942.96</td>
<td>176.10</td>
<td>516.96</td>
<td>0.008*</td>
<td>143.73 890.18</td>
</tr>
</tbody>
</table>

* p < 0.05 was significant, Wilcoxon test.

Table 2 shows the comparison of mean difference (ΔZ) of mineral loss between the groups. The data showed that changes in mineral loss between the following groups were statistically significant: 0 and 5.0 ppm F (p < 0.01); 0 and 10.0 ppm F (p < 0.05); 0.25 and 5.0 ppm F (p < 0.01); 0.5 and 5.0 ppm F (p < 0.05).

The 0-ppm F control demonstrated net demineralisation compared to all fluoridated groups which showed remineralisation, with a trend of increasing remineralisation with increasing fluoride concentration in milk (fig. 2).

**Fig. 1.** The daily cycling regime.
Lesions which are produced in vitro are ‘caries-like’ lesions and their use in experiments has widened our understanding of the processes of demineralisation and remineralisation. Furthermore, in vitro experiments have been useful in indicating what features of the natural situation can be dispensed when attempting to produce lesions with features similar to those of natural caries [Robinson et al., 1995].

In our study, fluoridated milk was used following the demineralisation period of the pH-cycling regime; it is clear from the results that this had a dose dependent effect, but this was not linear as it was not so evident beyond 5.0 ppm. The statistical analysis of the results showed significant differences in mineral loss between baseline and after treatment for the higher fluoride concentrations (1.0, 5.0 and 10.0 ppm F) in milk, but not for the lower ones (0.25 and 0.5 ppm F). This may be due to the amount of available fluoride in fluoridated milk.

**Table 2.** Comparison between different groups of the effect of concentrations of fluoride in milk on the mineral loss of enamel

<table>
<thead>
<tr>
<th>Group comparison</th>
<th>Mean difference</th>
<th>SD Difference</th>
<th>95% confidence interval of the difference</th>
<th>Significance (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk + 0 ppm F vs. milk + 0.25 ppm F</td>
<td>113.06</td>
<td>623.79</td>
<td>-408.45 634.56</td>
<td>0.575</td>
</tr>
<tr>
<td>Milk + 0 ppm F vs. milk + 0.5 ppm F</td>
<td>87.23</td>
<td>524.18</td>
<td>-315.69 490.15</td>
<td>0.767</td>
</tr>
<tr>
<td>Milk + 0 ppm F vs. milk + 1.0 ppm F</td>
<td>235.59</td>
<td>608.81</td>
<td>-199.93 671.11</td>
<td>0.241</td>
</tr>
<tr>
<td>Milk + 0 ppm F vs. milk + 5.0 ppm F</td>
<td>524.65</td>
<td>530.68</td>
<td>145.03 904.27</td>
<td>0.009*</td>
</tr>
<tr>
<td>Milk + 0 ppm F vs. milk + 10.0 ppm F</td>
<td>634.53</td>
<td>597.43</td>
<td>135.07 1,133.99</td>
<td>0.017*</td>
</tr>
<tr>
<td>Milk + 0.25 ppm F vs. milk + 0.5 ppm F</td>
<td>85.02</td>
<td>469.89</td>
<td>-307.82 477.85</td>
<td>0.674</td>
</tr>
<tr>
<td>Milk + 0.25 ppm F vs. milk + 1.00 ppm F</td>
<td>289.30</td>
<td>472.10</td>
<td>-73.59 652.19</td>
<td>0.110</td>
</tr>
<tr>
<td>Milk + 0.25 ppm F vs. milk + 5.00 ppm F</td>
<td>476.64</td>
<td>220.42</td>
<td>307.21 646.07</td>
<td>0.008*</td>
</tr>
<tr>
<td>Milk + 0.25 ppm F vs. milk + 10.00 ppm F</td>
<td>387.73</td>
<td>648.21</td>
<td>-211.76 987.23</td>
<td>0.063</td>
</tr>
<tr>
<td>Milk + 0.5 ppm F vs. milk + 1.00 ppm F</td>
<td>135.83</td>
<td>568.74</td>
<td>-271.03 542.68</td>
<td>0.575</td>
</tr>
<tr>
<td>Milk + 0.5 ppm F vs. milk + 5.00 ppm F</td>
<td>370.92</td>
<td>444.07</td>
<td>53.25 688.59</td>
<td>0.037*</td>
</tr>
<tr>
<td>Milk + 0.5 ppm F vs. milk + 10.00 ppm F</td>
<td>287.95</td>
<td>410.18</td>
<td>-54.97 630.87</td>
<td>0.069</td>
</tr>
<tr>
<td>Milk + 1.0 ppm F vs. milk + 5.00 ppm F</td>
<td>214.10</td>
<td>498.89</td>
<td>-121.06 549.26</td>
<td>0.155</td>
</tr>
<tr>
<td>Milk + 1.0 ppm F vs. milk + 10.00 ppm F</td>
<td>185.92</td>
<td>654.64</td>
<td>-317.28 689.12</td>
<td>0.515</td>
</tr>
<tr>
<td>Milk + 5.0 ppm F vs. milk + 10.00 ppm F</td>
<td>21.55</td>
<td>655.23</td>
<td>21.55 655.23</td>
<td>0.859</td>
</tr>
</tbody>
</table>

* p < 0.05 was significant.

**Discussion**

Lesions which are produced in vitro are ‘caries-like’ lesions and their use in experiments has widened our understanding of the processes of demineralisation and remineralisation. Furthermore, in vitro experiments have been useful in indicating what features of the natural situation can be dispensed when attempting to produce lesions with features similar to those of natural caries [Robinson et al., 1995].

*Fig. 2.* Bar chart showing the difference in mineral loss (vol% × μm; ΔZ) of enamel between groups with different concentrations of fluoride in milk after cariogenic challenge. Error bars indicate standard deviation.
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pasteurised milk stored at 4°C remained at 5 mg/l for 3 days. It needs to be taken into consideration that when fluoride is added to milk, it may interact partially with the intrinsic ions, including calcium, or be incorporated into milk proteins. Although 35% of calcium has been shown to exist in the ionic form in milk [Ling et al., 1961], the small proportion of free calcium ions (80 mg/l) available in milk, compared to the total calcium content (1,200 mg/l) is unlikely to cause calcium fluoride precipitation at low fluoride concentrations [Holt et al., 1981].

Transverse microradiography (TMR) is widely used in the study of enamel demineralisation and remineralisation, and although relatively complex, gives a more direct measurement of mineral loss and an insight into the lesion progression/regression. TMR also requires the presence of a lesion before the experiment. This means that TMR can measure overall remineralisation and provide information on lesion progression. Using this technique, a statistically significant difference was observed between both 5.0 and 10.0 ppm F in milk and in the control (0-ppm F milk) in terms of a change in mean difference in the mineral loss. The mean difference was not statistically significant for the group with low fluoride concentrations in milk (0.25, 0.5 and 1.0 ppm F). The results also showed that there was no significant difference when lower concentrations (0.25 and 0.5 ppm), were compared with 1.0 ppm. Significant differences were only noted on comparison with 5.0 ppm. It was also interesting to note that there did not seem to be a further difference between 5.0 and 10.0 ppm, only a slight trend which was not statistically significant. A recent study by Itthagarun et al. [2011] also showed that when the concentration of fluoride in milk was increased from 2.5 to 250 ppm, there was no statistical difference between the percentage decrease in lesion depth using an in vitro cycling method. The cycling method used in that study was very different to ours in that the enamel slabs were subjected to prolonged periods of continuous demineralisation, milk treatment and then remineralisation. The method we used is a true cycling model which exposed the slabs alternatively to demineralisation and remineralisation with exposure twice daily to the test milk and milk/saliva slurry. However, when comparing our results with this study, it would be interesting to speculate that the maximum benefit in our model might have been somewhere between 1.0 and 5.0 ppm, with little added preventive effect to be gained by further increases in the concentration of fluoride in milk. This needs to be investigated further.

The net demineralisation or remineralisation observed in the cycling model was due to the balance of remineralisation, caused by the artificial saliva, versus the demineralisation produced by the acidic challenges and the ability of fluoride in milk to influence the degree of each process. In our study, the time that the enamel slabs were in contact with the demineralising solution was 2 min 5 times daily, i.e. a total of 10 min. In comparison, the total contact time with the milk-fluoride solution was 15 min twice daily, i.e. a total of 30 min.

The current in vitro study does not include an organic component such as plaque or pellicle, which is present in vivo. Silverstone [1972] found that natural saliva remineralised the artificial lesions more rapidly than artificial saliva. However, artificial saliva remineralised the lesions in the partially demineralised tissue and not only in the superficial layers of the lesion. Plaque may prevent the diffusion of ions away from the enamel so that the mineral deposits and fluoride in the plaque are released when the plaque pH is low. In our study, as we could not use plaque or pellicle due to the in vitro design, the cariogenic challenge was acetic acid rather than a carbohydrate challenge as would be used in an in situ model with plaque overlying the enamel slabs. Our study was designed to examine the dose-dependent response of fluoride in milk in a carefully controlled experimental situation in preparation for an in situ study. An investigation of this nature can contribute to our understanding of the optimal concentration of fluoride in milk that would carry a wide margin of safety in children and provide maximum caries-preventive efficacy.

As far as we are aware, this is the first study that has demonstrated a clear dose-response benefit of fluoride in milk for the prevention of demineralisation of enamel under a cariogenic challenge in vitro using a continuous pH-cycling regime whilst including very low concentrations of fluoride of below 1.0 ppm. The benefit was obvious from 1.0 ppm F and a clear increase in benefit was observed with the concentration increasing up to 5.0 ppm F, but possibly not beyond.

Acknowledgement

This study was supported by a PhD project grant from the Borr-ow Foundation.
References


