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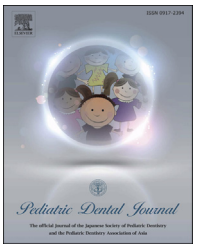

Research Paper

Warmed acidulated phosphate fluoride enhances release of fluoride from human enamel surfaces, promoting lesion remineralization *in vitro* and *in situ*

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ABSTRACT

Background: The calcium fluoride-like material deposited on the enamel surface is important as a fluoride reservoir. Elevated temperatures significantly increase the acquisition of KOH-soluble fluoride on sound tooth surfaces *in vitro*.

Methods: We investigated the efficacy of warmed APF solution on remineralization of subsurface enamel lesions, both *in vitro* and *in situ*. Hardness recovery was measured every week for 4 weeks to assess remineralization efficacy, and the behavior of fluoride release from sound and demineralized enamel with APF solution applied at 25 °C and 50°C was compared.

Results: Application of APF to enamel at 50 °C showed a significantly greater degree of F^- release up to 18 h than the 25 °C group in sound specimens and up to 48 h in demineralized specimens. Moreover, longer-lasting and greater amounts of F^- release were observed in demineralized specimens versus sound specimens. The profiles of changes in hardness over time *in vitro* and *in situ* showed that the hardness was significantly greater in the 50 °C group than in the 25 °C and control groups at all measurement points and the mean value of the hardness recovery in the 25 °C and 50 °C groups was observed by 2 weeks, although a significant increase was only noted from baseline to 1 week in the 25 °C group. Overall, the recovery of hardness was inadequate, compared to the original enamel.

Conclusion: In conclusion, application of the warmed APF solution to the demineralized enamel lesion showed potential to increase the fluoride release and enhance the rate of remineralization than that at 25 °C.

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1. Introduction

The caries-preventing benefits of in-home-use fluoride products, such as dentifrice [1] and mouth rinse [2], and professional topical fluoride products such as acidulated phosphate fluoride (APF) [3] and fluoride varnish [4] have been well established through many studies. Fluoride incorporated into enamel [5] and plaque [6] enhances resistance to acid demineralization. Particularly regarding professional products, previous studies have emphasized the importance of the calcium fluoride-like material (CFM) deposited on enamel surfaces as a fluoride reservoir over time [7,8]. The more the CFM precipitates on enamel surfaces, the more fluoride is expected to be supplied to the oral environment, leading to greater inhibition of demineralization.

Incipient enamel caries (subsurface lesions), referred to clinically as "white spot lesions," can be observed under *in situ* conditions [9] and clinically [10] to be remineralized or arrested. White spot lesions are observed more frequently in the cervical area and around orthodontic brackets [11] and are a symptom pediatric dentists often encounter in the clinic. Although remineralization, using fluoride dentifrice [1], fluoride-releasing sealant [11], and calcium/phosphate-containing gum [12], is well documented, there are very few reports on the effects of APF application in remineralization [13].

To increase fluoride acquisition during APF application to enamel, Okuno et al. [14] investigated the influence of the temperature of the APF solution and found that an elevated temperature significantly increased the acquisition of KOH-soluble and KOH-insoluble fluoride on sound tooth surfaces *in vitro*. Similarly, other groups have studied the effects of temperature on fluoride uptake from a mouth rinse [15], of milk on caries lesion rehardening [16], and of SnF_2 solution on tin and fluoride uptake by bovine enamel [17]. These studies have all revealed positive effects of elevated temperatures.

In a previous study, it was found that natural salivary macromolecules containing phosphoproteins greatly affected the preferential remineralization at the demineralized enamel surface layer when fluoride ions (F^-) were present [18]. Hence, in our *in vitro* analyzes, we included a homolog of such salivary phosphoproteins, casein. Artificial saliva (AS) containing casein and 1 ppm F^- has been reported to inhibit the precipitation of minerals such as fluoridated apatite on sound enamel and dentin surfaces but to allow remineralization in the lesion body [19,20].

Recently, we developed a new device that can circulate warmed APF solution to the dental arch without dilution by saliva contamination for a given period of time [21]. We expected that this device would promote F^- incorporation into enamel better than the conventional application method. We investigated the efficacy of warmed APF solution (50 °C) on remineralization of incipient subsurface enamel lesions, both *in vitro* and *in situ*.

2. Materials and methods

2.1. *Experiment for fluoride release*

2.1.1. *Preparation of sound and demineralized enamel specimens*

The enamel surface of extracted human premolars was polished with waterproof sandpaper (Astra waterproof paper #1000, Noritake Co., Ltd., Aichi, Japan) to remove the superficial enamel surface and polished premolars were cut into ~3 × 3 × 3 mm block specimens using a diamond disk attached to a dental engine. The specimens were embedded in self-curing resin with the enamel surface exposed, and then the margin between enamel and resin was covered with nail varnish (Maquillage, Shiseido Cosmetics, Tokyo, Japan) to prevent reactions on the side surfaces of the enamel specimens.

A demineralized subsurface lesion was prepared using a demineralizing solution (2.2 mmol/L $CaCl_2$, 2.2 mmol/L NaH_2PO, 50 mmol/L acetic acid, 100 mmol/L NaCl, pH 4.5, adjusted with NaOH) [22]. The resin-cured and nail-varnished enamel specimens were immersed in 10 mL demineralizing solution at 37 °C for 1 week. The lesions were confirmed to be typical subsurface lesions by observing the demineralized surface and cut surface by microscopy.

2.1.2. *Fluoride release after application of APF solution*

Nine specimens each were used for APF application at 25 °C (the 25 °C group) and 50 °C (the 50 °C group) and another seven with no APF application served as a control group. APF solution (2.0% NaF, 0.15 mol/L H_3PO_4) was prepared according to Brudevold et al. [23]. Each specimen was immersed for 5 min in 10 mL APF solution at 25 °C or that warmed at 50 °C. The temperature of 50 °C was selected as a safe temperature that would not affect dental pulp tissue histologically [24]. The temperature of 25 °C was used as the normal temperature of APF used in the clinic.

After APF application, each specimen was rinsed briefly with distilled water (DW) and then immersed in 10 mL DW for 1 h. Next, each immersed specimen was wiped briefly and transferred to more fresh 10 mL DW for 1 h. This procedure was repeated at 3, 4, 6, 12, 18, and 24 h for sound enamel. For the demineralized specimens, they were further immersed in DW for 48 and 72 h. Concentrations of released fluoride were measured using a standard method for each specimen with a fluoride-specific ion electrode (9609BNWP, Thermo Fisher Scientific, Waltham, MA, USA). The amount of released fluoride was calculated, taking into consideration the surface area of each enamel specimen measured with Vernier calipers, the concentrations of fluoride and the volume of DW. Specimens with no fluoride application served as a control group.

2.2. In vitro *and* in situ *remineralization experiments*

2.2.1. *Preparation of demineralized bovine specimens*

To minimize potential differences in demineralized specimen preparation, bovine enamel was used. A carborundum point

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attached to a dental engine was used to remove the surface cementum layer of extracted bovine lower incisors. In total, 45 enamel block specimens (window area: ~3 × 3 mm) were cut out using a low-speed saw (Isomet, Buehler Company, Lake Bluff, IL, US) and embedded in self-curing resin. Eight to nine specimens could be cut out from one bovine tooth. The enamel surface was polished with waterproof sandpaper (#1000). After washing with DW, the margin between the enamel and resin was sealed using nail varnish in the same fashion as in the fluoride release experiment.

The embedded bovine enamel specimens were demineralized for 19 days in a demineralizing solution at 37 °C (5.0 mmol/L $CaCl_2$, 4.0 mmol/L NaH_2PO, 50 mmol/L acetic acid, 0.02% NaN_3, pH adjusted to 4.8) [25]. The demineralized specimens were observed to confirm typical subsurface lesions, as described above.

2.2.2. In vitro *remineralization of the lesion applied by APF*

After APF application at 25 °C and 50 °C, each demineralized specimen was immersed in 1 mL remineralizing solution (1.0 mmol/L $CaCl_2$, 3.0 mmol/L NaH_2PO, 100 mmol/L acetic acid, 100 mmol/L NaCl, 0.02% NaN_3, 100 ppm casein, pH adjusted to 6.3) [20] for 1 week and the Vickers microhardness (HV) of the demineralized surface was measured using a microhardness tester with a load of 2.94 N and 15 s duration time (HMV-G21, Shimadzu, Kyoto, Japan). Five points on the enamel surface per specimen were measured and the average value was used as the representative hardness. The remineralizing solution was renewed every week to evaluate the rate of remineralization over time. The measurement was conducted on the same enamel surface before demineralization, after demineralization, and after 1, 2, 3, and 4 weeks of remineralization.

2.2.3. In situ *remineralization of the lesion applied by APF*

An oral appliance was prepared with self-curing resin for each of five subjects (five males, aged 29–32) and three shallow dents were made on the palatal surface where the enamel specimens were mounted using sticky wax (Fig. 1). Three specimens, APF applied at 50 °C and 25 °C and control, were systemically mounted in each of the dents to randomize the potential influence of F^- contamination released from the neighboring specimens (Fig. 1). All appliances were sterilized with ethylene oxide prior to intra-oral application. The subjects wore the appliance except during meals and sleep, during which the appliance was stored in a sealed container with 100% moisture. The subjects refrained from using fluoride toothpaste and chewing gum for the 4-week experiment. The specimens were removed every week for microhardness measurements and remounted on the appliance with sticky wax.

This *in situ* experiment was approved by the ethical committee of Iwate Medical University (approval no. #01247). Written informed consent was obtained from the five volunteers after explanation on the project using pamphlet that describes the detailed ethical concerns of the study.

2.3. *Statistical analyses*

Data for F^- release and microhardness are expressed as means ± SDs. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Scheffe's multiple-comparison test. Statistical significance was set at $p < 0.05$.

3. Results

As shown in Figs. 2 and 3 and Table 1, the 50 °C group showed significantly greater amounts of F^- release up to 18 h than the 25 °C group in sound specimens and up to 48 h in the demineralized specimens. Moreover, longer lasting and greater amounts of F^- release were observed in the demineralized specimens than in the sound specimens. The cumulative amount of F^- released was 2.4 times and 3.0 times greater in the demineralized group than in the sound groups at 25 °C and 50 °C, respectively. A sharp decrease in F^- release was also observed over several hours, followed by smaller amounts of F^- release in both sound and demineralized specimens at both temperatures.

Fig. 4 and Table 2 provide data on changes in hardness over time in the *in vitro* experiment. Although the hardness recovery from baseline was greatest in the 50 °C group at all time points, a significant increase in hardness was only noted from the baseline to 1 week in the 25 °C group and to 2 weeks in the 50 °C group. Subsequently, the recovery leveled off. No significant increase in hardness was seen in the control group. The mean hardness values after 4 weeks were 69 and 115 in the 25 °C and 50 °C groups, respectively, indicating that the overall recovery was insufficient.

The trends in hardness recovery in the *in situ* experiments were similar (Fig. 5, Table 3). Although the 50 °C group showed the highest hardness among the three groups at all time points, a significant increase in hardness was noted only from the baseline to 1 week in the 25 °C group and to 2 weeks in the 50 °C group. The mean increase in hardness was not remarkable. Indeed, the mean values after 4 weeks were 32 and 53 in the 25 °C and 50 °C groups, respectively, lower than in the *in vitro* experiments.

4. Discussion

Generally, the rate of any chemical reaction, including APF application to enamel in this case, should be increased by elevating the temperature, which increases the rate of collision between particles such as ions and molecules. In addition, it was anticipated that a higher temperature would decrease the viscosity of the APF solution. According to a handbook of physics, viscosity coefficients of water at 20 °C and 50 °C are 1002×10^6 and 546×10^6 Pa s, respectively. Moreover, the volume expansion coefficients of water at 20 °C and 50 °C are 0.206×10^3 and 0.457×10^3 K^{-1}, suggesting that a higher temperature may expand micro-porous spaces between crystallites or enamel rods. As a result, the viscosity of the APF would be lower at the higher temperature and expanded micro-porous spaces in the enamel would favor deeper penetration of F^- into enamel tissue. Thus, a greater amount of CFM formation on the surface and inside enamel micro-porous spaces as well as greater amounts of adsorbed F^- ion on the enamel crystal surface would be expected. These assumptions may explain the increased amount of fluoride

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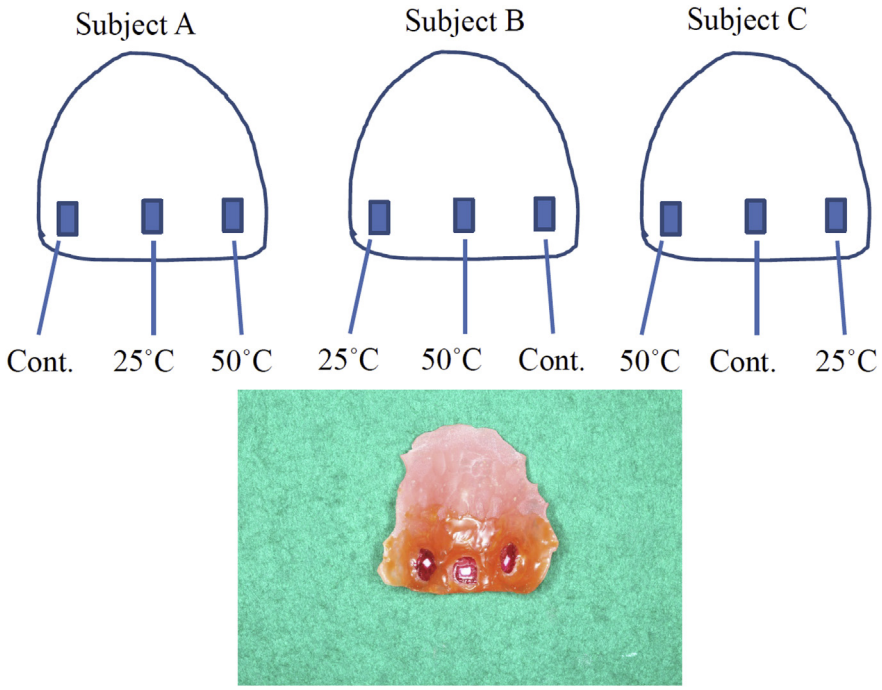

Fig. 1 – Photograph and schematic diagram of the intraoral device for *in situ* experiments. Three shallow dents were made on the palatal surface and a demineralized specimen in each group was mounted with sticky wax. They were mounted systematically by shifting the specimen position to randomize the potential influence by F^- contamination released from the neighboring specimens.

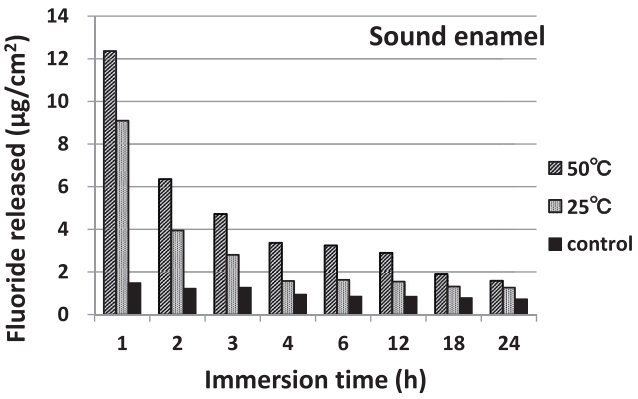

Fig. 2 – Amount of F^- released from sound enamel surface with APF applied. The 50 °C group showed significantly greater amounts of F^- release up to 24 h than control but the 25 °C group showed significantly greater amounts of F^- release than control only up to 3 h. When the 50 °C group is compared with the 25 °C group, the 50 °C group showed significantly greater amounts of F^- release up to 18 h than the 25 °C group.

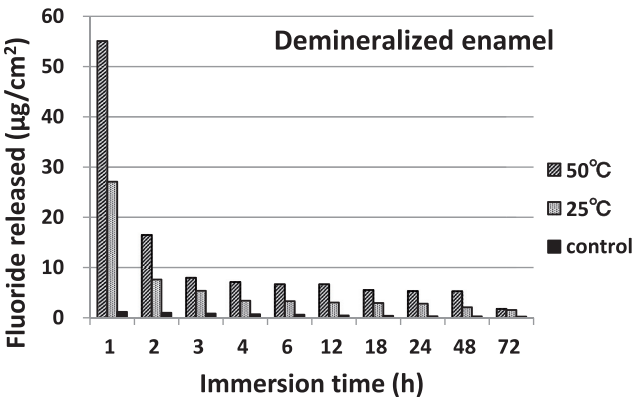

Fig. 3 – Amount of F^- released from demineralized enamel surface with APF applied. Longer lasting and greater amounts of F^- release were observed in the demineralized specimens than in the sound specimens. Both the 50 °C group and the 25 °C group showed significantly greater amounts of F^- release than control up to 72 h. Also the significant differences were observed between the 50 °C group and the 25 °C group up to 48 h.

released from sound and demineralized enamel at 50 °C than at 25 °C, followed by a higher rate of hardness recovery in demineralized lesions. As shown in Figs. 2 and 3, demineralized enamel specimens released greater amounts of F^- than sound specimens. This could also be explained by increased porosity in the demineralized surface layer, indicating an increased surface area for F^- acquisition.

APF application to enamel results in several types of fluoride, including CFM that precipitates onto the apatite crystal surface, fluoridated apatite (FA) as a lattice ion, and F^- that is loosely adsorbed to the enamel crystal surface and can be removed by brief washes in water [26]. To the best of our knowledge, however, no studies have examined how applied APF reacts with enamel at 50 °C. CFM is the main product and very little FA formation occurs, compared to CFM at room or

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Table 1 – Statistical significance of the amount of F^- released by paired comparisons among the 25 °C, 50 °C, and control groups, in both sound and demineralized specimens, as well as by neighboring comparisons of immersion time.

	1 h	2 h	3 h	4 h	6 h	12 h	18 h	24 h
Sound enamel								
50 °C VS Cont	**	**	**	**	**	**	**	**
25 °C VS Cont	**	**	*	–	–	–	–	–
50 °C VS 25 °C	*	**	**	*	*	**	*	–

	1 h	2 h	3 h	4 h	6 h	12 h	18 h	24 h	48 h	72 h
Demineralized enamel										
50 °C VS Cont	**	**	**	**	**	**	**	**	**	**
25 °C VS Cont	**	**	**	**	**	**	**	**	*	**
50 °C VS 25 °C	**	**	**	**	**	**	**	**	**	–

–: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$.

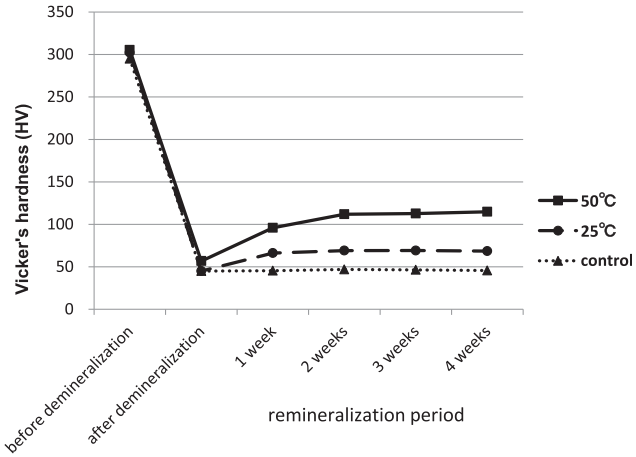

Fig. 4 – Profiles of changes in Vickers hardness over time in the *in vitro* experiment. Solid and dotted lines indicate 50 °C and 25 °C groups, respectively. Both the 50 °C group and the 25 °C group showed significantly greater hardness recovery than control throughout the four weeks of remineralization period. The differences between the 50 °C group and the 25 °C group were also significant throughout the four weeks. However, a significant increase in hardness was only noted from the baseline to 1 week in the 25 °C group and to 2 weeks in the 50 °C group.

Table 2 – Statistical significance of differences in hardness by paired comparisons among the 25 °C, 50 °C, and control groups in the *in vitro* experiments, as well as by neighboring comparisons of remineralization period.

	1 week	2 weeks	3 weeks	4 weeks
in vitro				
50 °C VS Control	**	**	**	**
25 °C VS Control	**	**	**	**
50 °C VS 25 °C	*	**	**	**
Control				
Baseline	–	–	–	–
1 week		–	–	–
2 weeks			–	–
3 weeks				–
25 °C				
Baseline	**	**	**	**
1 week		–	–	–
2 weeks			–	–
3 weeks				–
50 °C				
Baseline	**	**	**	**
1 week		*	**	**
2 weeks			–	–
3 weeks				–

–: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$.

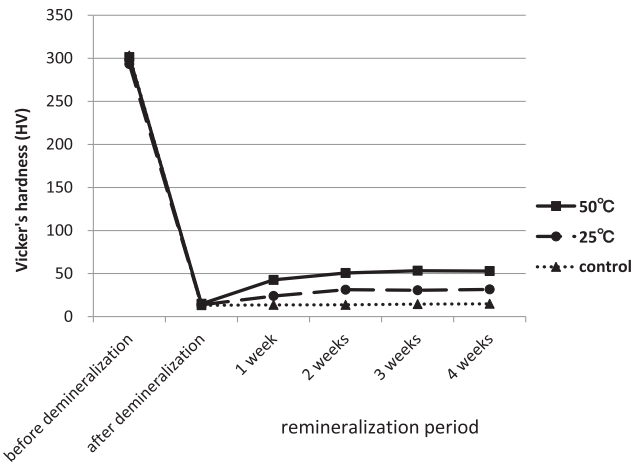

Fig. 5 – Profiles of changes in Vickers hardness over time in the *in situ* experiment. Solid and dotted lines indicate 50 °C and 25 °C groups, respectively.

physiological temperatures [26], whereas FA will not significantly release F^- because of its very low solubility [27]. The initial burst of F^- release during 1–2 h was assumed to be from F^- loosely adsorbed to enamel crystallites (Figs. 2 and 3). After this initial burst, the slow release over time might be from CFM.

Regarding the F^- release behavior, there may seem to be a discrepancy between two findings in this study: fluoride release behavior in the demineralized enamel specimens (Fig. 3) and the duration of *in vitro* and *in vivo* remineralization potential. In the 50 °C group, ~94% of cumulated fluoride was released in 24 h and the F^- concentration decreased markedly from 2.2 ± 0.4 ppm at the first 1 h incubation to a very low concentration (0.07 ± 0.03 ppm) after 72 h incubation (data not shown). However, the hardness continued to increase in the *in vitro* and *in situ* experiments for at least 2 weeks. This discrepancy between incubation time and the term of remineralization progression may be explained as follows. One reason is probably due to differences in the medium volume and ingredients. In the former, 10 mL DW water was used and was renewed nine times over 72 h, enhancing removal of F^- from the demineralized enamel (Fig. 3). However, in the latter case, a smaller volume of AS (1.0 mL) was used and was renewed every week for *in vitro* remineralization. The smaller volume of AS should have less capacity to contain released F^- than the DW, leading to a longer period for remineralization, at least 2 weeks. A second reason may have been the presence of phosphate ions (3 mmol/L) in AS. Rølla reported that the

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Table 3 – Statistical significance of differences in hardness by paired comparisons among the 25 °C, 50 °C, and control groups in the *in situ* experiments, as well as by neighboring comparisons of remineralization period.

	1 week	2 weeks	3 weeks	4 weeks
in situ				
50 °C VS Control	**	**	**	**
25 °C VS Control	–	**	*	*
50 °C VS 25 °C	**	**	**	**
Control				
Baseline	–	–	–	–
1 week		–	–	–
2 weeks			–	–
3 weeks				–
25 °C				
Baseline	**	**	**	**
1 week		–	–	–
2 weeks			–	–
3 weeks				–
50 °C				
Baseline	**	**	**	**
1 week		*	**	*
2 weeks			–	–
3 weeks				–

–: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$.

presence of phosphate ions in DW inhibited F^- release from pure CaF_2 [28]. In addition, we used casein as a homolog of salivary phosphoproteins [19,20], which have strong adsorption affinities to apatite surfaces [29]. Saxegaard reported that calcium fluoride dissolved more readily in water than in saliva and that CFM was more stable in saliva than in water after a 3-week incubation [30]. Considering the similarity between casein and salivary phosphoproteins, we assumed that casein might adsorb to the surface of CFM, resulting in the inhibition of rapid dissolution of CFM and more prolonged F^- release.

We used casein as a homolog of salivary phosphoproteins, which play important roles in mineral regulation [31] by sustaining supersaturation in saliva in terms of apatite minerals and also via pellicle formation on enamel surfaces [32], inhibiting apatite deposition on the surface. Fujikawa et al. reported that such salivary macromolecules inhibited the deposition of fluoridated apatite onto surface enamel when 1 ppm F^- was present in AS, by which porous channels at the surface layer were preserved, allowing mineral ions including F^- to penetrate deeper into the lesion body, whereas macromolecules themselves inhibited lesion remineralization [18]. They concluded that salivary macromolecules enhanced remineralization when F^- was present in saliva. We assumed that casein in this study played the same role as the salivary macromolecules.

Comparisons between *in vitro* and *in situ* remineralization experiments indicated similar patterns of hardness increase by 2 weeks. In both datasets, the increase was greater in the first week, followed by a lower increase in the second week. This was likely due to less F^- release in the second week in both 25 °C and 50 °C groups. Moreover, the mean hardness recovery from baseline was greater in the *in vitro* experiment than in the *in situ* experiment in both groups by the second week. Several explanations are possible: one is that the baseline hardness before remineralization was significantly lower (t-test, $p < 0.01$) *in situ* (13.8 and 14.9 for 25 °C and 50 °C, respectively) than *in vitro* (45.0 and 52.2, respectively) although the same demineralization conditions were used. Regarding the difference in baseline mineral loss (ΔZ: vol%·μm), Strang et al. reported that when the mineral loss at baseline was higher, the mineral gain (rate of remineralization) was lower [33]. A second possibility is that the mineral-regulating power is stronger in natural saliva (*in situ*) than under *in vitro* conditions. In saliva, there may be other factors that affect remineralization such as lower concentrations of Ca^{2+} or phosphate ions and a higher viscosity due to organic components (mucin, bacteria, other proteinaceous materials), leading to less ion penetration into the lesion body. A third possibility may be that the exposure time to saliva was shorter in the *in situ* experiment for patient convenience (sleeping, meal times) than in the *in vitro* experiment. Finally, it could be due to potential differences in mineral regulatory activity between casein and the macromolecules in natural, whole saliva.

Our results demonstrate the efficacy of warmed APF application in the progression of remineralization. However, a significant increase from the baseline in the hardness was noted only at the first and second weeks; subsequently, the trend leveled off. These findings indicate that the recovered surface hardness at 4 weeks was not sufficiently resistant to protect the surface from mechanical damage. This suggests that the fluoride acquired in the demineralized specimens might be lost within a short time period. Thus, more frequent APF application may be necessary to achieve clinically meaningful remineralization. Further study is needed to examine the effects of application frequency and initial status of white spot lesions on APF efficacy.

In the previous study [14] we found that APF warmed to 50 °C significantly increased acquisition of fluoride on the tooth surface and also the pulp temperature increased *in vitro* to 40 °C when the tooth was dipped in a water bath at 50 °C. Baldissara et al. [34] reported that an intra-pulpal temperature increase of 8.9–14.7 °C in human teeth, which includes the temperature change of pulp in this study, did not produce any pathological change in the pulp. Although preliminary trial conducted in authors' mouth indicated that 50 °C is actually acceptable temperature for oral application, further investigations on its effects to the oral organs like mucosa and pulp are required in detail in order to apply this temperature to the clinical use.

5. Conclusions

Application of warmed APF solution (50 °C) to sound enamel and artificial white spot lesions increased the amount of released fluoride compared to application at 25 °C. Furthermore, the rate of remineralization, evaluated by microhardness, was greater at 50 °C than at 25 °C.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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