# Enhancing the Remineralization Potential of Child Formula Dentifrices: An *In Vitro* Study

Mohammed Nadeem Ahmed Bijle*/ Lai Pui Tung **/ Jasmine Wong ***/Manikandan Ekambaram ****/ Edward CM Lo *****/Cynthia Kar Yung Yiu******



***Objective(s):*** *The incorporation of Arginine (Arg) in NaF-containing child dentifrice might enhance its remineralizing potential, reducing fluorosis risk with significant anti-caries benefit. The study objective was to examine the remineralizing potential of arginine in child formula dentifrice (600-ppm NaF).* ***Study Design:*** *Primary teeth enamel specimens (n = 10) with artificial caries-like lesion were randomly divided to 4 treatment groups: A: 2% Arg-(600-ppm) NaF; B: 600-ppm NaF; C: 1100-ppm NaF; and D: deionized water subjected to 7-day pH-cycling. The mineral density (MD) of the treated specimens was assessed using micro-CT. The pre-/post-treated artificial caries-like lesion were acid-etched for enamel fluoride uptake (EFU) evaluation, Ca and P element analysis using ICP-OES, and the inorganic phosphate ($PO_4^{3-}$) determination using colorimetric assay.* ***Results:*** *The percentage remineralization of the 2% Arg-NaF and 1100-ppm NaF groups was significantly higher than the 600-ppm NaF group ($p<0.001$). However, no significant difference in remineralization was observed between the two groups ($p>0.05$). The EFU, Ca/P ratio, $PO_4^{3-}$ content of the 2% Arg-NaF group were significantly higher than the 600-ppm NaF group ($p<0.01$); while no significant difference was found between the 2% Arg-NaF and 1100-ppm NaF groups.* ***Conclusion:*** *Within the limitations of the present study, incorporation of 2% arginine in 600-ppm NaF child formula dentifrice enhanced the remineralization potential of artificial enamel caries, to a level comparable to 1100-ppm NaF adult formula dentifrice.*

***Keywords:*** *arginine, child, dentifrices, enamel, remineralization.*

## INTRODUCTION

Dental caries, a biofilm-mediated disease, is a major oral health problem affecting 60-90% children.[1–3] Prevention or delimiting the progress of dental caries at an incipient stage help to control its severity globally. Until now, fluoride remains the standard for caries prevention.[4] Fluoride promotes remineralization–a natural process that deposits calcium (Ca) and phosphate ($PO_4^{3-}$) ions in the demineralized enamel. Daily use of fluoride dentifrices is the most common form of topical fluoride application.

Although fluoride dentifrices have substantially contributed to worldwide caries reduction, the use of high fluoride concentration dentifrice in very young children (< 6 years) is not preferred, due to the risk of fluorosis.[5,6] Hence, low fluoride dentifrices (< 1000 ppm F) have been recommended to pre-school children.[7,8] However, the reduced fluoride concentration has been shown to have a negative impact on the anti-caries effect of the dentrifice.[9] Hence, alternative strategies to enhance the anti-caries efficacy of these low fluoride dentifrices for use in children is needed.

Arginine, a semi-essential α-amino acid, is naturally available in saliva and plaque through dietary protein metabolism. Ammonia synthesized by oral arginine metabolism increases the pH of biofilm.[10–12] The arginolytic potential of the oral micro-environment increases with arginine supplementation.[13] Thus, biofilm

*Mohammed Nadeem Ahmed Bijle, BDS, MDS, Paediatric Dentistry, Faculty of Dentistry, The University of Hong Kong
**Lai Pui Tung, BDS, Faculty of Dentistry, The University of Hong Kong
***Jasmine Wong, BDS, Faculty of Dentistry, The University of Hong Kong
****Manikandan Ekambaram, BDS, MDS, PhD, Paediatric Dentistry, Faculty of Dentistry, University of Otago, Dunedin, New Zealand.
*****Edward CM Lo, BDS, MDS, PhD, Dental Public Health, Faculty of Dentistry, The University of Hong Kong, Hong Kong
******Cynthia Kar Yung Yiu, BDS, MDS, PhD, Paediatric Dentistry, Faculty of Dentistry, The University of Hong Kong,

**Send all correspondence to:**
Cynthia Kar Yung Yiu
Paediatric Dentistry, Faculty of Dentistry, The University of Hong Kong, Prince Philips Dental Hospital, 34 Hospital Road, Sai Ying Pun, Hong Kong Island, Hong Kong.
E-mail: ckyyiu@hkucc.hku.hk

alkalinization could be stimulated by external arginine, which inhibits the cariogenic biofilm formation, leading to ecological homeostasis.[11] Several studies have demonstrated the enhanced remineralization effect of fluorides with arginine that primarily affects the metabolic activity of biofilms.[14,15] Therefore, the incorporation of arginine into topical fluoride agents appears a promising anti-caries therapy that largely affects the biofilms.

Recently, arginine, in combination with 500-ppm sodium fluoride (NaF) solution, has been shown to promote enamel fluoride uptake, thereby increasing the resistance of enamel to carious demineralization.[16] It has been proposed that the protonated positively charged guanidinium group of arginine interacts with negatively charged F ions, forming arginine-fluoride complexes in the enamel during remineralization. Also, arginine is reported to have a synergistic effect with NaF on cariogenic biofilm, suppressing the acidogenic *S. mutans*.[17] The commercially available 1.5% arginine and fluoride dentifrice (1450 ppm F) with sodium monofluorophophate (MFP) in an insoluble calcium base has been shown to effectively reverse the early enamel carious lesions compared to dentifrices containing fluoride alone.[18–22]

It has been shown that the incorporation of 2% arginine in 1100-ppm NaF toothpaste significantly enhanced the remineralization potential of NaF toothpaste.[23] However, the synergistic effect of arginine and fluoride in child formula dentifrices on the remineralization of primary teeth enamel caries remains unexplored. Therefore, the aim of this study was to examine the remineralizing potential of arginine in NaF (600-ppm)-containing child formula dentifrice and compare it to 1100 ppm NaF adult formula dentifrice. The null hypotheses tested was that (i) the incorporation of arginine in child formula dentifrice has no effect on its remineralization potential and (ii) there is no difference in remineralization potential of Arg-NaF child formula dentifrice and 1100 ppm NaF adult formula dentifrice.

## MATERIALS AND METHOD

The study design was approved by the Institutional Review Board (IRB) (Reference number: UW 17-150). The study consisted of a series of *in vitro* experiments, which evaluated the enamel of primary teeth (incisors and molars)[24] that was subjected to artificial caries-like lesion formation and subsequent pH cycling.

### Sample size determination

*A priori* computation of sample size (n = 10/group, at $p < 0.05$, df: 3) was done using G*Power 3.1 (Franz Faul, Kiel, Germany). Based on the preliminary study, an absolute difference of 0.60 g/cm$^3$ between pre- & post-treated lesion's mineral density was arbitrated as an effect size on β/α: 4. The output parameters quantified actual power as 0.82 with critical F: 2.90, non-centrality parameter: 12.96 for the total sample size: 40 based on the input group numbers: 4.

### De-/Re-mineralizing solutions

The de-/re-mineralizing solutions were prepared using appropriate analytical-grade chemicals as per previous studies. [25,26]

*Demineralizing solution (DS):* 2.2 mM $CaCl_2$, 2.2 mM $KH_2PO4$ and 0.05 M acetic acid adjusted with 5 M KOH to pH 4.4.

*Remineralizing solution (RS):* 1.5 mM $CaCl_2$, 0.9 mM $NaH_2PO4$ and 0.15 M KCl adjusted with 5 M KOH to pH 7.0.

### Caries-like lesion formation

The extracted or exfoliated primary teeth (stored in 0.5% thymol at 4°C) were assessed using stereomicroscope (Carl Zeiss Stereo 475002, Jena, Germany) at 0.8x to rule out enamel defects or fragmentation developed (if any) before and during the preparation process. The decoronated primary teeth specimens were dual-coated with acid resistant nail varnish (Revlon®, New York, USA), only exposing a narrow window of approximately 1 × 1 mm$^2$ on sound enamel. The specimens were immersed in DS for 96 hours on an orbital shaker (Labnet, Woodbridge, USA) at 80 rpm at room temperature to create artificial caries-like lesion.

### Experimental groups

The specimens with caries-like lesions were randomly divided in the following study groups:

*Group A* (2% Arg-NaF): 2% arginine incorporated in a child formula dentifrice containing 600 ppm NaF (Colgate Kids Anticavity Toothpaste, Minions—Bubble fruit flavor, Colgate-Palmolive Company, New York, USA)

*Group B* (600-ppm NaF): A child formula dentifrice containing 600 ppm NaF (Colgate Kids Anticavity Toothpaste, Minions—Bubble fruit flavor, Colgate-Palmolive Company, New York, USA)

*Group C* (1100-ppm NaF): An adult formula dentifrice containing 1100 ppm NaF (Colgate Triple Action, Colgate-Palmolive Company, New York, USA)

*Group D* **(DIW):** Deionized water

### Dentifrice slurry preparation

Dentifrice slurry for *Group B* & *C* was prepared in 1:3 ratio of dentifrice: DIW. The 60 seconds vortexed aqueous solution was centrifuged at 4000 rpm at 25°C for 20 minutes.[26] For *Group A*, milled 2% weight/weight L-arginine monohydrochloride (Sigma-Aldrich, St. Louis, USA) was incorporated in 600-ppm NaF toothpaste. The arginine: NaF toothpaste blend was further added to DIW following the preparation protocol for *Group B* & *C*. The sediment of test groups based centrifuged slurries was discarded and the supernatant was used as a treatment solution as per previous study.[26]

### pH-cycling

A 7-day[9] pH-cycling model using White's regimen[16,27] that comprised of 1-minute treatment (5 ml) 4 times/day to simulate toothpaste exposure was used in the present study. Alongside, to simulate plaque acid challenge the specimens were immersed in DS (20 ml/specimen) 2 hours/day. The specimens were immersed in RS (10 ml/specimen) for the rest of the time. The re-/demineralizing experiments with treatments were performed on a continuously operated orbital shaker (Labnet, Woodbridge, USA) at 80-rpm at room temperature throughout the pH-cycling.

### Treatment solutions characterization

The treatment solutions were subjected to pH measurement, F estimation using ion selective electrode (Thermo Fisher Scientific, Inc., MA, USA), Na, Cl elemental analysis using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Spectro Arcos, Ametek, Kleve, Germany) to determine Na:Cl ratio (principally for *Group A* dentifrice slurry) based on calibrated standard curves.

UV-visible spectroscopy at different time points during pH-cycling were effectuated by transferring the solutions to 96-well microplate to measure the end-point absorbance in UV-visible spectra (200-900 nm at 10 nm steps) using microplate reader.[28]

## Micro-CT scan and Mineral Density (MD)

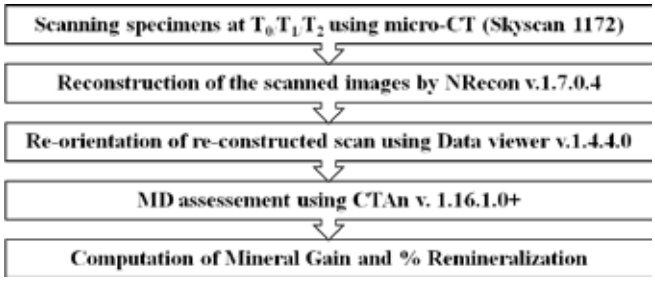
A flow of MD assessment using micro-CT is shown in Figure 1. The specimens were scanned at baseline—before artificial caries-like lesion formation ($T_0$), pre-treatment—after caries-like lesion formation ($T_1$) and post-treatment—after pH cycling ($T_2$) using micro-CT (Skyscan 1172 X-Ray, Microtomograph, Kontich, Belgium) with constant scanning parameters being exposure—4.84 seconds, resolution—7 µm, rotation–360$^0$ each 1$^0$ steps at 80kV, 100 µA with calibrated flat-field correction for isotropic resolution. The MD assessment was performed using two hydroxyapatite phantoms (of density 0.25 and 0.75 g/cm3) as a reference. Prior to the MD assessment, calibration was done with 100-stacks, each reference phantoms using respective attenuation coefficients. The scanned specimens were reconstructed using NRecon (v. 1.7.0.4 (SkyScan, Kontich, Belgium) with constant variable at 3-scanning levels being smoothing: 1, ring artifact correction: 20, beam hardening correction: 30, and dynamic range: 0–0.25. The reconstructed images at $T_0$, $T_1$ and $T_2$ were oriented to match similar planes using coronal, trans-axial and sagittal frames in DataViewer v.1.4.4.0 (SkyScan, Kontich, Belgium) for uniform orientation. Preceding to the MD assessment using CTAn v. 1.16.1.0+ (SkyScan, Kontich, Belgium), the reconstructed trans-axial 2D images at $T_0$, $T_1$ and $T_2$ were matched to identify corresponding sections for analysis. The 3D reconstructed images predicated the basis for lesion identification. The lesion identified areas (5 sections stack–7µ thickness/section at levels in duplicate) were further matched based on the pre-/post-treated 2D images with parallel $T_0$ and $T_1$/$T_2$ sections to randomly identify fields for MD assessment using uniform region of interest (ROI) approximately 50µ diameter at equidistant points. A combined mean MD per level assessment at $T_0$, $T_1$ and $T_2$ were calculated to compute mineral gain (MG) and % remineralization (% remin) from the equation:

$$MG = \Delta Z_d - \Delta Z_r$$

$$\% \text{ remin} = (\Delta Z_d - \Delta Z_r / \Delta Z_d) \times 100$$

($\Delta Z_d$—MD difference between $T_0$ and $T_1$, $\Delta Z_r$—MD difference between $T_2$ and $T_0$)

**Figure 1: Schematic flow of mineral density assessment using micro-CT**




## Lesion acid etching

The pre-/post-treated artificial caries-like lesions were acid etched using 1 N $HClO_4$ under constant agitation at 150-rpm at room temperature for 15 seconds. Then, 2 ml of TISAB II and 1 ml of 1 N NaOH was added to establish a ratio of 1:2:1. The buffered solutions were vortexed for 15 seconds prior to fluoride uptake assessment, Ca/P elemental analysis and inorganic phosphate detection.

## Enamel Fluoride Uptake (EFU)

The EFU of pre-/post-treated specimens based on a standard calibration curve was determined in triplicate as per previous studies.[29,30] For consistency, the sample solutions were stowed in 15 ml centrifuge tube (continuously stirred at 100 rpm) and the electrode was introduced in the tubes to achieve membrane-sample contact. The values obtained in mV were converted to ppm using calibration curve that were further computed to µg F.

## Ca/P element analysis

The acid-etched pre-/post-treated lesions were subjected to Ca/P element analysis using ICP-OES at 0.04 MPa and > 1000$^o$C with argon gas calibrated as per standard solutions (TraceCERT® Reference Material, Sigma-Aldrich, St. Louis, USA) for Ca ($CaCO_3$) and P ($H_3PO_4$). The aliquots were measured as parts per billion (ppb) in triplicate using standardized ICP-OES protocol.

## Inorganic phosphate detection

A high sensitivity microplate assay protocol (50 µl, sample + 30 µl, malachite green reagent A and 100 µl, distilled water + 30 µl, malachite green reagent B) was used to detect the inorganic phosphate in the treated lesion with malachite green phosphate detection kit (R & D systems, Minneapolis, USA). A 6-point standard curve subjected to 4-parameter logistic regression, was prepared using buffer medium. The optical density of the assayed samples with 96-well plate was determined at 620 nm end-point absorbance in duplicate.

## Statistical analysis

The data for the treatment solutions and primary enamel characterization were subjected to statistical analysis using SPSS v. 24 (IBM SPSS® Statistics Inc, Chicago, USA). The pH, F concentration, Na/Cl ratio, pre-/post-treatment changes in Ca & P were analyzed using Kruskal-Wallis test with Dunn's post-hoc test. The MD changes between groups at $T_0$, $T_1$ & $T_2$ were analyzed using one-way repeated measures ANOVA with Bonferroni's post-hoc test (assuming Mauchly's test of sphericity, $p = 0.075$). The mineral gain (MG), % remin, pre-/post treatment EFU and inorganic phosphate content were analyzed by one-way ANOVA with Duncan's post-hoc test. The paired sample t-tests were used to identify the mean differences in the pre- & post-treatment Ca & P content, indicating significant Ca deposition and enamel resistance to solubility.[31] The statistical significance was set at 0.05.

## RESULTS

### UV-Visible absorption spectra, pH, fluoride and Na:Cl of treatment solutions

The spectra for 2% Arg-NaF test solution was similar to that of 600-ppm NaF (**Figure 2**). There was no different characteristic peak identified in 2% Arg-NaF test solutions as compared to spectra of other treatment solutions in the study since all followed a similar course. The data obtained from pH measurement, fluoride estimation and Na:Cl of treatment solutions are presented in Table 1. The pH and fluoride concentrations of all the test solutions were significantly different from each other ($p < 0.001$). The F concentration was the highest in 1100-ppm NaF, followed by 2% Arg-NaF, 600-ppm NaF and DIW. The Na/Cl ratio of 2% Arg-NaF and 1100-ppm NaF was significantly lower than 600-ppm NaF ($p < 0.001$). However, no significant difference was observed between the two groups.

**Table 1: pH, fluoride concentrations and ICP-OES element analysis of treatment solutions**

| Groups | pH | F concentration | Na/Cl |
|---|---|---|---|
| 2% Arg-NaF | 7.48 ± 0.03 [7.48 (0.01)]$^{a}$ | 150.39 ± 6.65 [148.17 (8.88)]$^{A}$ | 1.00 ± 0.01 [1.00 (0.01)]$^{\alpha}$ |
| NaF (600 ppm) | 7.78 ± 0.01 [7.79 (0.03)]$^{b}$ | 99.89 ± 1.51 [100.13 (2.42)]$^{B}$ | 66.78 ± 10.69 [62.34 (14.73)]$^{\beta}$ |
| NaF (1100 ppm) | 7.73 ± 0.04 [7.74 (0.07)]$^{c}$ | 164.27 ± 9.66 [163.08 (16.92)]$^{C}$ | 12.63 ± 0.09 [12.63 (0.10)]$^{\alpha\Phi}$ |
| DIW | 6.25 ± 0.57 [6.22 (0.95)]$^{d}$ | 0.08 ± 0.04 [0.07 (0.05)]$^{D}$ | 15.56 ± 0.60 [15.34 (0.37)]$^{\beta\Phi}$ |
| p-value | < 0.001 | < 0.001 | < 0.001 |

Kruskal-Wallis One-way ANOVA with Dunn's post-hoc test; $p < 0.05$ is significant.

Different superscripts indicate significant differences in each column.

For, pH–lowercase letters (a, b, c, d); F concentration—uppercase letter (A, B, C, D) and Na/Cl—Greek letters (α, β, Φ) indicate column specific significant differences.

### MD assessment

The enamel MD (g/cm$^3$) assessment of the treated specimens at $T_0$, $T_1$ and $T_2$, MG and % remin are presented in Table 2. The results of one-way repeated measures ANOVA identified a significant difference in the repeated measures factor—Time ($T_1$, $T_2$ & $T_0$) ($p < 0.001$). The treatment time interaction with groups was statistically significant ($p < 0.001$).

**Table 2: Mineral Density (MD) assessment (g/cm$^3$)**

| Groups | MD (Mean ± SD) | | | | |
|---|---|---|---|---|---|
| | $T_0$ | $T_1$ | $T_2$ | Mineral Gain | % Remineralization |
| 2%Arg-NaF | 2.44 ± 0.13$^{a,\alpha}$ | 1.44 ± 0.27$^{A,\beta}$ | 1.66 ± 0.27$^{I,\lambda}$ | 0.22 ± 0.07$^{a}$ | 21.83 ± 7.12$^{a}$ |
| 600-ppm NaF | 2.44 ± 0.09$^{a,\alpha}$ | 1.40 ± 0.08$^{A,\beta}$ | 1.47 ± 0.07$^{I,\beta}$ | 0.07 ± 0.05$^{b}$ | 6.26 ± 4.97$^{b}$ |
| 1100-ppm NaF | 2.46 ± 0.13$^{a,\alpha}$ | 1.28 ± 0.22$^{A,\beta}$ | 1.62 ± 0.20$^{I,\lambda}$ | 0.34 ± 0.15$^{c}$ | 28.10 ± 11.00$^{a}$ |
| DIW | 2.44 ± 0.04$^{a,\alpha}$ | 1.17 ± 0.13$^{A,\beta}$ | 1.11 ± 0.18$^{II,\beta}$ | -0.06 ± 0.11$^{d}$ | -4.74 ± 8.82$^{c}$ |

One-way repeated measures ANOVA with Bonferonni's post-hoc test assuming Mauchly's test of sphericity ($p = 0.075$)

Lowercase letters (a)/Uppercase letters (A)/roman numerals (I, II) represent differences in each column–$T_0$, $T_1$ and $T_2$ respectively.

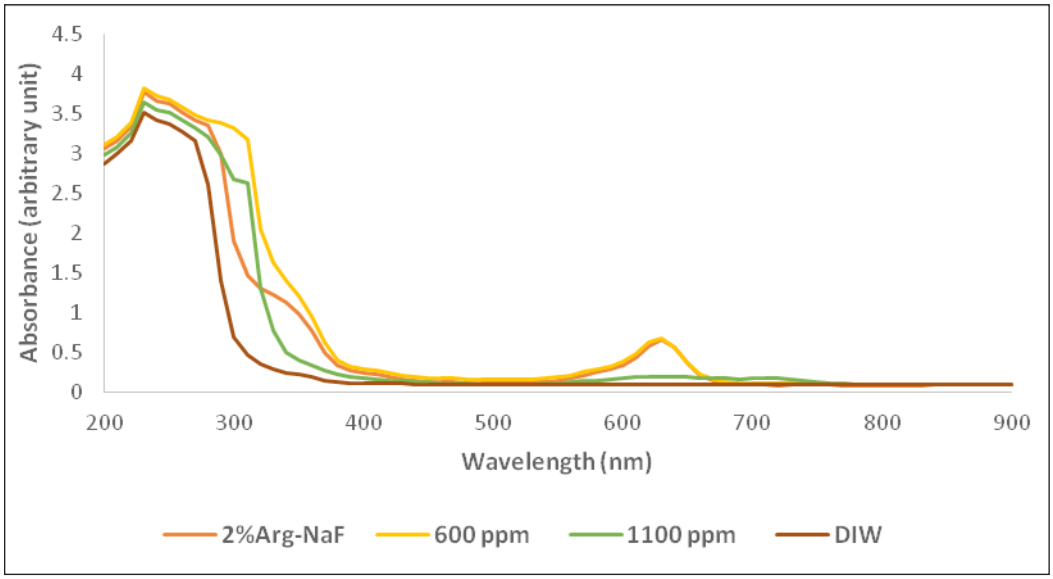
Symbols α, β and λ represent differences in each row.

Time ($p < 0.001$); Time-Group interaction ($p < 0.001$); Group ($p = 0.006$).

One-way ANOVA with Duncan's post-hoc test. ($p < 0.001$).

Different superscripts indicate significant differences in each column. The lowercase letters (a, b, c, d) indicate column specific differences without overlap being unique in description.

**Figure 2: UV-vis spectra of treatment solutions**



Comparing the different treatment groups, there was no significant difference in the mean MD among the groups at $T_0$ and $T_1$, which justifies the experimental baseline prerequisites. However, the mean MD for the 2% fArg-NaF, 600-ppm NaF and 1100-ppm NaF groups was significantly higher than the DIW groups at $T_2$ ($p<0.05$).

Comparing the different treatment time, a significant decrease in the mean MD was observed for all the groups from $T_0$ to $T_1$ ($p<0.05$). Conversely, a significant increase in the mean MD was found for the 2% Arg-NaF and 1100-ppm NaF groups from $T_1$ to $T_2$ ($p < 0.05$); while no significant change was observed for the 600-ppm NaF and DIW groups ($p > 0.05$).

The MG of 1100-ppm NaF group was the highest, followed by 2% Arg-NaF, 600-ppm NaF and DIW. The % remin for the 2% Arg-NaF and 1100-ppm NaF groups were significantly higher than the 600-ppm NaF and DIW groups ($p < 0.001$). The results of overall MD assessment suggest that the remineralization effect of 2% Arg-NaF was higher than 600-ppm NaF and similar to 1100-ppm NaF.

## EFU

Table 3. There was no significant difference in the EFU among the groups at $T_1$ ($p = 0.055$). The EFU of 2% Arg-NaF and 1100-ppm NaF groups was significantly higher than 600-ppm NaF and DIW ($p < 0.05$). However, no significant difference was found between the 2% Arg-NaF and 1100-ppm NaF groups ($p > 0.05$). Hence, the EFU results suggest that the post-treatment fluoride uptake of 600-ppm NaF was significantly increased after the incorporation of arginine, to a level similar to 1100-ppm NaF.

## Ca/P determination

The data obtained from Ca and P (in ppb) element analysis is presented in **Table 4**. No significant difference in Ca and P content was found among the groups at $T_1$ ($p > 0.05$). The Ca & P content were significantly different among the tested groups at $T_2$ ($p = 0.002$). No significant difference in the Ca and P content was found among 2% Arg-NaF, 600-ppm NaF and 1100-ppm NaF groups at $T_2$ ($p>0.05$) which could be due to the high SD of pre/post- Ca and P levels with 1100-ppm NaF treatment group. Furthermore, the paired sample t-tests revealed that 2% Arg-NaF and 1100-ppm NaF groups demonstrated significant differences in the mean pre & post-treatment Ca and P content ($p < 0.05$). Additionally, the post-treatment computed Ca/P ratio for the 2% Arg-NaF group was significantly higher than 600-ppm NaF and DIW groups ($p = 0.002$); while no significant difference was found between the 2% Arg-NaF to 1100-ppm NaF groups ($p > 0.05$). Hence, it can be advocated that arginine incorporation to 600-ppm NaF facilities calcium deposition and increases enamel resistance to solubility.

## Inorganic phosphate detection

The results for $PO4^{3-}$ (in µM) detection is presented in **Table 5**. There was no significant difference in detected $PO4^{3-}$ among the groups at $T_1$ ($p = 0.999$). The detected $PO4^{3-}$ for the 2% Arg-NaF group was significantly higher than 600-ppm NaF and DIW groups at $T_2$ ($p = 0.003$). No statistically significant difference was found between 2% Arg-NaF and 1100-ppm NaF groups ($p > 0.05$). It is noteworthy that incorporation of 2% arginine in 600-ppm NaF presented with much higher detected $PO_4^{3-}$ as compared to the other groups, indicating that arginine with fluoride might assist in phosphate deposition.

The overall results signify that arginine in 600-ppm NaF toothpaste enhances Ca, F and $PO4^{3-}$ deposition, thereby augments the remineralization potential of child formula dentifrices.

**Table 3: Enamel fluoride uptake** *(µg F)*

| Groups | 2% Arg-NaF | NaF (600 ppm) | NaF (1100 ppm) | DIW | p-value |
|---|---|---|---|---|---|
| Pre-treatment ($T_1$) | 0.06 ± 0.02 | 0.05 ± 0.02 | 0.08 ± 0.02 | 0.06 ± 0.02 | 0.055 |
| Post-treatment ($T_2$) | $0.37 \pm 0.08^{a}$ | $0.27 \pm 0.09^{b}$ | $0.38 \pm 0.17^{a}$ | $0.08 \pm 0.02^{c}$ | < 0.001 |

One-way ANOVA with Duncan's post-hoc test; $p < 0.05$ is significant.

Different superscripts (lowercase letters—a, b, and c) indicate significant differences at $T_2$. ($p < 0.001$).

**Table 4: Calcium (Ca) and phosphorous (P) element content analysis (ppb)**

| Groups | Pre-treatment ($T_1$) | | Post-treatment ($T_2$) | | |
|---|---|---|---|---|---|
| | Ca | P | Ca | P | Ca/P |
| 2% Arg-NaF | 1619.42 ± 903.79* | 1271.12 ± 822.89* | $3691.67 \pm 795.60^{a*}$ | $3063.52 \pm 649.67^{A*}$ | $1.20 \pm 0.01^{\alpha}$ |
| NaF (600 ppm) | 1498.10 ± 775.11 | 1508.94 ± 712.18 | $2555.79 \pm 519.56^{ac}$ | $2224.86 \pm 460.85^{AC}$ | $1.15 \pm 0.04^{\beta}$ |
| NaF (1100 ppm) | 1646.66 ± 1171.30* | 1315.82 ± 1011.66* | $4391.55 \pm 2915.57^{a*}$ | $3685.29 \pm 2462.44^{A*}$ | $1.19 \pm 0.02^{\alpha\beta}$ |
| DIW | 1763.84 ± 456.74 | 1474.02 ± 476.32 | $1615.67 \pm 265.71^{bc}$ | $1420.17 \pm 237.68^{BC}$ | $1.14 \pm 0.01^{\beta}$ |
| p-value | 0.947 | 0.964 | 0.002 | 0.002 | 0.002 |

Kruskal-Wallis One-way ANOVA with Dunn's post-hoc test, $p < 0.05$ is significant.

Different superscripts indicate significant difference in each column at $T_2$. ($p = 0.002$). For, Ca—lowercase letters (a, b, and c); P—uppercase letters (A, B, and C) and Ca/P—Greek alphabets (α and β) ) indicate column specific significant differences.

*–indicates significant differences between matched groups determined using paired t-test to identify increase in enamel resistance (using P content) and facilitation for calcium deposition between $T_1$ and $T_2$ for individual treatment group.

**Table 5: Inorganic phosphate detection** *(μM)*

| Groups | 2% Arg-NaF | NaF (600 ppm) | NaF (1100 ppm) | DIW | p-value |
|---|---|---|---|---|---|
| Pre-treatment ($T_1$) | 67.44 ± 36.31 | 66.18 ± 34.66 | 65.54 ± 35.83 | 66.10 ± 47.38 | 0.999 |
| Post-treatment ($T_2$) | 125.41 ± 32.19[a] | 81.50 ± 33.83[b] | 103.17 ± 31.47[ab] | 84.78 ± 21.72[b] | 0.003 |

One-way ANOVA with Duncan's post-hoc test; $p < 0.05$ is significant.

Different superscripts (lowercase letters—a, b, and c) indicate significant differences at $T_2$. ($p = 0.003$).

## DISCUSSION

The results of this *in vitro* study showed that the incorporation of 2% arginine in 600-ppm NaF child formula dentifrice significantly enhanced its remineralization potential, therefore the first null hypothesis that "the incorporation of arginine in child formula dentifrice has no effect on its remineralization potential" has to be rejected. Furthermore, no significant difference in the remineralization potential was found between 2% Arg-NaF and 1100 NaF dentifrice, therefore the second null hypothesis that "there is no difference in remineralization potential of Arg-NaF child formula dentifrice and 1100 ppm NaF adult formula dentifrice" cannot be rejected.

The comparison of the remineralization potential of 2% Arg-NaF with other treatment groups was based on the pre-/post-treatment mineral profile evaluated using micro-CT, EFU, Ca, P content and the inorganic $PO_4^{3-}$ detected. The F concentration estimated for the 2% Arg-NaF group was significantly higher as compared to other treatment groups. The rationale for such a behavior could be due to the interaction between arginine and NaF to form arginine-fluoride ionic complex, thereby making the availability of free fluoride ions as opposed to NaF that might not have been completely dissociated during the slurry preparation.

The 2% by wt. of L-arginine monohydrochloride was the concentration and arginine variant selected for the present study, since it has been demonstrated that the incorporation of 2% by wt. of L-arginine monohydrochloride into commercially available 1100-ppm NaF toothpaste significantly increased the remineralization of enamel caries-like lesion of permanent teeth.[23] It was further shown in the study that increasing the concentration of L-arginine monohydrochloride in the adult formula dentifrice had a negative effect on remineralization.

The enhanced remineralization effect could possibly be due to the interaction of NaF with L-arginine monohydrochloride, resulting in the formation of arginine-fluoride complexes in an ionic form, with subsequent formation of sodium chloride (NaCl) (Arg. HCl + NaF → Arg.F + NaCl). Results of the present study are in agreement with a previous *in vitro* study, which also showed that the combined 2.5% arginine—500-ppm NaF solution had significantly higher EFU than the 500-ppm NaF solution.[16]

Furthermore, the ionic arginine-fluoride complexes produced could also attract Ca ions to form arginine-calcium-fluoride complexes (Figure 3), facilitating the diffusion of the complexes to sub-surface enamel demineralized lesions. Interestingly, the inorganic phosphate detected for 2% Arg-NaF group were much higher than the other groups. This signifies that arginine might also form an arginine-phosphate complex (Figure 4), given the negative charge on $PO4^{3-}$ and the positive charge on guanidinium group of arginine. Therefore, the enhanced remineralization effect of 2% Arg-NaF might be due to the arginine that facilitates the deposition of calcium and phosphates as arginine-calcium-fluoride and arginine-phosphate complexes, respectively to the sub-surface enamel carious lesion. The fluorides in the complexes might further inhibit demineralization during acid attack, due to its inherent formation of alkali stable fluorapatite.

**Figure 3: Arginine-calcium-fluoride complex**

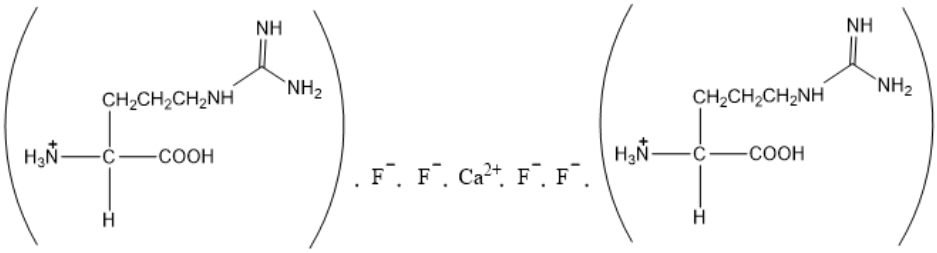
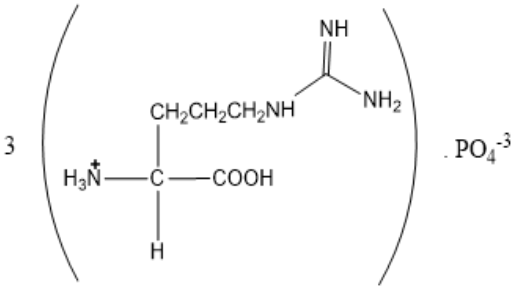



**Figure 4: Arginine-phosphate complex**



It is also quite possible that during the acid attack, the arginine complexes will release arginine that lead to its known ecological benefit. The released arginine might further enhance the arginolytic potential of the oral micro-environment, resulting in an increase in ADS activity, producing ammonia to neutralize biofilm pH.[10–13] Moreover, the release of arginine could enhance the anti-bacterial effect of NaF on oral biofilms.[32] Thus, the incorporation of 2% arginine in the child formula dentifrices reduces the risk of fluorosis associated with adult dentifrices and provides significant anti-caries benefits through its effect on biofilm and enhanced remineralization potential.

Although the study was quite comprehensive to justify its objectives, there are few inherent limitations, which can be subjugated by future investigations. Further studies should quantify the availability of arginine in treated enamel lesion by high-performance liquid chromatography, thereby confirming the presumption stated in this study. The commercially available arginine-fluoride (MFP) toothpaste in a calcium base has a very high concentration of F (1450-ppm), making it unsuitable for use in children due to the high risk of fluorosis. It has been shown in a previous study that the child formula MFP toothpaste did not significantly remineralize enamel incipient carious lesion of the primary tooth.[9] The addition of arginine in low concentrations MFP toothpaste might significantly remineralize the lesion, considering the proven *in vivo* remineralization effects of the commercially available arginine-fluoride toothpaste. Therefore, future studies should evaluate the effect of different arginine variants with low concentration NaF or MFP child formula toothpastes. The present study is *in vitro* and performed in controlled conditions whereby the complete simulation of oral conditions might not be possible. Further studies involving more robust models (microbial pH cycling) can provide better insights into the mechanistic properties of the proposed combination of arginine in fluoridated child formula dentifrices.

## CONCLUSION

Within the limitations of the present study, incorporation of 2% arginine in 600-ppm NaF child formula dentifrice enhanced the remineralization potential of artificial enamel caries, to a level comparable to 1100-ppm NaF adult formula dentifrice.

## REFERENCES


1. Petersen PE. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century—the approach of the WHO Global Oral Health Programme. Community Dent Oral Epidemiol;31:3–24. 2003.
2. Pitts NB, Zero DT, Marsh PD, et al. Dental caries. Nat Rev Dis Prim;3(May):17030. 2017,
3. Edelstein B. The Dental Caries Pandemic and Disparities Problem. BMC Oral Health;6:S2. 2006.
4. Fontana M. Enhancing Fluoride: Clinical Human Studies of Alternatives or Boosters for Caries Management. Caries Res;50(1):22–37. 2016.
5. Walsh T, Worthington H V, Glenny AM, et al. Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2010;(1):CD007868.
6. Ekambaram M, Itthagarun A, King NM. Ingestion of Fluoride from Dentifrices by Young Children and Fluorosis of the Teeth–A Literature Review. J Clin Pediatr Dent;36(2):111–22. 2011,
7. Wong M, Glenny A, Tsang B, et al. Topical fluoride as a cause of dental fluorosis in children. Cochrane Database Syst Rev;(2):CD007693. 2009.
8. Wong M, Clarkson J, Glenny A, et al. Cochrane Reviews on the Benefits / Risks of Fluoride Toothpastes. J Dent Res ;90:573–9. 2011.
9. Ekambaram M, Itthagarun A, King NM. Comparison of the remineralizing potential of child formula dentifrices. Int J Paediatr Dent;21(2):132–40. 2011.
10. Hajishengallis E, Parsaei Y, Klein MI, et al. Advances in the microbial etiology and pathogenesis of early childhood caries. Mol Oral Microbiol;32(1):1–11. 2016.
11. Burne RA, Marquis RE. Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett;193:1–6. 2000.
12. Huang X, Exterkate RA, ten Cate JM. Factors Associated with Alkali Production from Arginine in Dental Biofilms. J Dent Res;91(12):1130–4. 2012.
13. Sissons CH, Cutress TW, Pearce EI. Kinetics and product stoichiometry of ureolysis by human salivary bacteria and artificial mouth plaques. Arch Oral Biol;30(11–12):781–90. 1985.
14. González-Cabezas C, Fernández CE. Recent Advances in Remineralization Therapies for Caries Lesions. Adv Dent Res;29(1):55–9. 2018
15. Cummins D. The development and validation of a new technology, based upon 1.5% arginine, an insoluble calcium compound and fluoride, for everyday use in the prevention and treatment of dental caries. J Dent. Aug;41 Suppl 2:S1–11. 2013.
16. Cheng X, Xu P, Zhou X, et al. Arginine promotes fluoride uptake into artificial carious lesions in vitro. Aust Dent J;60:104–11. 2015.
17. Zheng X, Cheng X, Wang L, et al. Combinatorial effects of arginine and fluoride on oral bacteria. J Dent Res;94(2):344–53. 2015.
18. Yin W, Hu DY, Li X, et al. The anti-caries efficacy of a dentifrice containing 1.5% arginine and 1450ppm fluoride as sodium monofluorophosphate assessed using Quantitative Light-induced Fluorescence (QLF). J Dent;41:S22–8. 2013.
19. Srisilapanan P, Korwanich N, Yin W, et al. Comparing the efficacy of a dentifrice containing 1.5% arginine and 1450ppm fluoride to a dentifrice containing 1450ppm fluoride alone in the management of early coronal caries as assessed using Quantitaive Light-induced Fluorescence. J Dent;41 Suppl 2:S29–34. 2013.
20. Kraivaphan P, Amornchat C, Triratana T, et al. Two-year caries clinical study of the efficacy of novel dentifrices containing 1.5% arginine, an insoluble calcium compound and 1,450 ppm fluoride. Caries Res;47(6):582–90. 2013.
21. Koopman JE, Hoogenkamp MA, Buijs MJ, et al. Changes in the oral ecosystem induced by the use of 8% arginine toothpaste. Arch Oral Biol;73:79–87. 2017.
22. Wang Y, Mei L, Gong L, et al. Remineralization of early enamel caries lesions using different bioactive elements containing toothpastes: An in vitro study. Technol Heal Care;24(5):701–11. 2016.
23. Bijle MN, Ekambaram M, Lo EC, et al. The combined enamel remineralization potential of arginine and fluoride toothpaste. J Dent;76:75–82. 2018.
24. Sabel N, Dietz W, Lundgren T, et al. Elemental composition of normal primary tooth enamel analyzed with XRMA and SIMS. Swed Dent J;33:75–83. 2009.
25. Gopalakrishnan VL, Anthonappa RP, King NM, et al. Remineralizing potential of a 60-s in vitro application of Tooth Mousse Plus. Int J Paediatr Dent;27(5):356–63. 2017.
26. Kumar VL, Itthagarun A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: An in vitro study. Aust Dent J;53(1):34–40. 2008.
27. White DJ. Reactivity of fluoride dentifrices with artificial caries II. Effect on subsurface lesions. F uptake, F distribution, surface hardening and remineralisation. Caries Res;22:27–36. 1988.
28. Targino AG, Flores MA, Dos Santos VE, et al. An innovative approach to treating dental decay in children. A new anti-caries agent. J Mater Sci Mater Med;25(8):2041–7. 2014.
29. Lippert F, Hara AT, Martinez-Mier EA, et al. Laboratory Investigations Into the Potential Anticaries Efficacy of Fluoride Varnishes. Pediatr Dent;36:291–5. 2003
30. Karlinsey RL, Mackey A, Walker TJ, et al. In vitro remineralization of human and bovine white-spot enamel lesions by NaF dentifrices: A pilot study. J Dent Oral Hyg;3(2):22–9. 2011.
31. Moore J, Schneiderman E, Farmer T. Fluoride Uptake Profiles of Selected European Toothpastes into Hard Tissues and Plaque. J Clin Dent ;28(3):39–43. 2017.
32. Zhang M, He LB, Exterkate RA, et al. Biofilm layers affect the treatment outcomes of NaF and nano-hydroxyapatite. J Dent Res;94(4):602–7. 2015.