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The combined enamel remineralization potential of arginine and fluoride toothpaste

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ABSTRACT

Objective(s): This study examined the remineralization potential of arginine (Arg) in NaF toothpaste.
Methods: Fifty enamel specimens allocated to five groups (n = 10) were subjected to artificial caries formation. A 10-day pH-cycling was performed to treat specimens as per group – [1]: 2% Arg – NaF, [2]: 4% Arg – NaF, [3]: 8% Arg – NaF, [4]: NaF and [5]: deionized water. The test solutions were subjected to pH measurement, fluoride estimation, Na-Cl element analysis using ICP-EOS and FTIR analyses. Mineral density of the specimens were assessed using micro-CT; while Ca/P ratio and surface fluorine concentration were determined using energy dispersive x-ray spectroscopy (EDS) and enamel fluoride uptake (EFU) by acid-etch method.
Results: pH, fluoride concentration and Na-Cl ratio exhibited significant difference amongst groups ($p < 0.001$). FTIR analysis showed presence of free amino acids in 2% and 4% Arg-NaF group. The mean mineral gain (0.40 ± 0.07 g/cm^3) and percent remineralization ($27.91 \pm 4.66\%$) of 2% Arg-NaF group were significantly higher than the other 4 groups ($p < 0.001$). Conversely, the median Ca/P ratio for 2% Arg-NaF (1.60) was significantly higher than deionized water (1.53) ($p = 0.029$). The mean surface fluorine concentration of specimens treated with 2% Arg-NaF ($1.51 \pm 0.14\%$) was significantly higher than treatment with NaF ($1.02 \pm 0.28\%$) ($p < 0.001$). The EFU of 2% Arg-NaF group (6.84 ± 1.59 μg/cm^2) was significantly higher than NaF group (5.22 ± 1.88 μg/cm^2) ($p < 0.001$).
Conclusion: Incorporation of 2% arginine in NaF toothpaste significantly increased the remineralization of enamel caries-like lesion when compared to NaF toothpaste; while 4% and 8% arginine in NaF toothpastes were ineffective in improving enamel remineralization.
Clinical Significance: In high-risk patients, daily use of 2% arginine in NaF toothpaste might provide a synergistic anti-caries effect given the proven prebiotic benefits of arginine in caries prevention and the demonstrated remineralization effect in the present study.

1. Introduction

Dental caries is one of the most prevalent conditions with a global prevalence of 35% for all ages combined [1,2]. The pathophysiology of dental caries is due to bacterial metabolism of fermentable carbohydrates, producing acid and demineralization of dental hard tissues [3]. The caries preventive agents mainly act by inhibiting bacterial acid production or by changing the de/remineralization equilibrium [4]. Fluoride has been identified as a potent caries preventive agent with significant benefits [5–9]. Daily brushing with fluoride toothpaste is the most common topical fluoride application method. However, there are problems associated with fluoride applications, such as toxicity at high doses. The availability of fluoride over the past few decades has now led to the evolution of fluoride-resistant *S. mutans* and other oral bacterial species [10]; hence, its actions on acid-producing microbes may be diminishing. Thus, it is imperative to supplement fluoride toothpaste with potent modifiers that enhances its remineralization potential and combats acidogenic microorganisms.

Arginine is a prebiotic-based organic compound that has recently been introduced as an additive to fluoride toothpaste and other oral care products with significant anti-caries benefits [11]. Arginine is a naturally occurring amino acid in dietary proteins. The high-protein diet metabolism leads to the presence of arginine in the oral cavity. Substantial salivary arginine (available in micro-concentrations) favors

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the existence of less cariogenic biofilm by increasing oral biofilm pH owing to alkali production [12–14]. External arginine supplementation further strengthens the potential to produce oral alkali [15]. Therefore, arginine may complement the limitations of fluorides on oral biofilms.

The synergistic effects of arginine with fluorides and calcium in enamel remineralization have been reported previously [16,17]. Arginine has been added to toothpaste comprehending its potential as a biofilm modifier evident through clinical data [18]. Proteins (esp. serum albumin) enhances remineralization as a result of its affinity to adsorb fluorides [19,20]. Hence, it is quite possible that arginine (as a residual protein) might have a similar remineralization effect in incipient carious lesions. Moreover, the positively charged guanidinium group of arginine favors the attraction of highly electronegative fluorides [16].

Arginine in fluoride toothpaste is commercially available as 1.5% and 8% arginine with insoluble calcium base and 1450-ppm sodium monofluorophosphate (MFP). The 1.5% arginine-fluoride toothpaste was a priori introduced as a caries preventive agent [18]; while the 8% arginine-fluoride toothpaste with arginine bicarbonate variant, initially marketed for treatment of dentin hypersensitivity, was recently explored as a potent caries-preventive agent [21,22]. The effect of arginine with readily dissociable sodium fluoride (NaF) has been reported in several *in vitro* studies [16,23]. The arginine-NaF solution synergistically inhibits *S. mutans* and enhances *S. sanguis* in a multi-species biofilm [23]. An *in vitro* study found the remineralization effect of 2.5% arginine with 500-ppm NaF solution on artificial enamel carious lesion similar to that of control 500-ppm NaF solution; while the enamel fluoride uptake for the arginine-F solution was significantly higher [16]. Therefore, the interaction of arginine-NaF seems favorable in terms of enhancing the existing anti-caries effect of NaF.

So far, the published studies have only studied the effect of arginine in a low concentration NaF solution. No study has evaluated the interactive effect of arginine with high concentration (greater than 1000-ppm) NaF toothpaste, which is recommended for caries prevention in high caries-risk patients [6]. In addition, the optimum concentration of arginine to be incorporated in high concentration NaF toothpaste to impart effective anti-caries effect demands further evaluation. Hence, the aim of the present study was to examine the remineralization potential of arginine in NaF toothpaste. The null hypothesis tested was that the incorporation of arginine, regardless of its concentration, in NaF toothpaste has no additional remineralizing effect on artificial enamel caries-like lesions when compared to toothpaste with only NaF.

2. Materials and methods

2.1. Experimental study design

The experimental study design and ethical concerns were duly reviewed and approved by the Institutional Review Board of The University of Hong Kong – Hospital Authority Hong Kong West Cluster (Reference number: UW 17-058). A schematic representation of this study design is presented in Fig. 1. The outline represents significant steps in the experiment process. The study involved *in vitro* evaluation of enamel specimens, which underwent artificial enamel caries-like lesion formation, followed by investigations on the remineralization efficacy of three increasing arginine concentrations, supplemented to 1100-ppm F toothpaste, compared to respective positive and negative controls.

2.2. Sample power calculation

The prospective sample power calculation was done using G*Power 3.1 (Franz Faul, Germany) based on the results of a preliminary study. Considering the primary variable - mineral gain and based on the study protocol, the expected effect size was estimated to be 0.52 on standardized β/α: 4. Finally, the software computed the total sample size of 50 for 5 groups at significance of p-value < 0.05 with actual power quantified as 0.81 at critical F: 2.58, non-centrality parameter (λ): 13.52 using a priori protocol for F – tests sample power analysis.

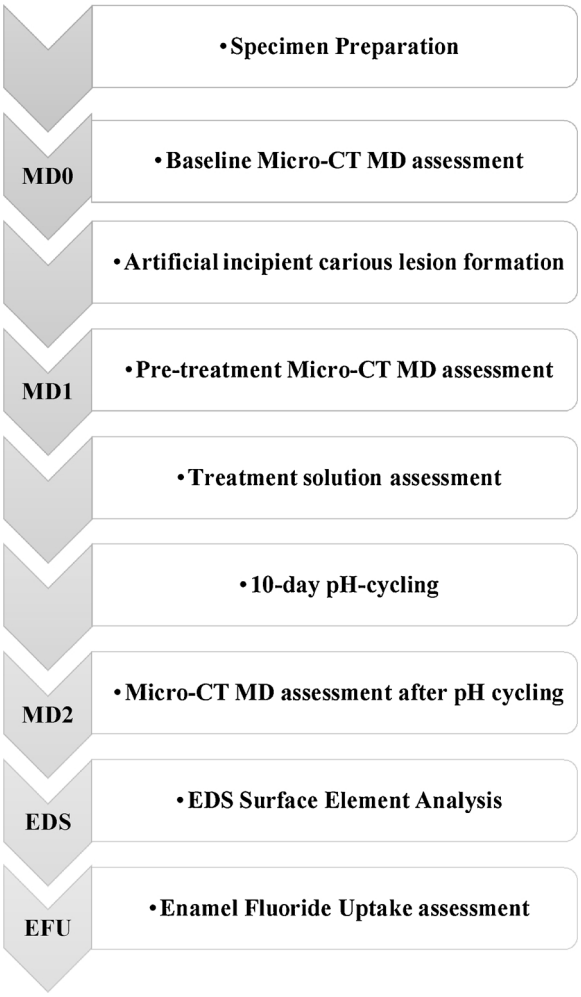


Fig. 1. Schematic representation of experimental study design.

2.3. Specimen preparation

Freshly extracted sound human third permanent molars collected after obtaining informed consent from patients were used in this study. The collected teeth were thoroughly debrided and disinfected before specimen preparation. The extracted teeth were primarily assessed using stereomicroscope (Carl Zeiss Stereo 475002, Germany) at 0.8x to rule out enamel defects like hypomineralisation, hypoplasia and fluorosis. Subsequently, the teeth were stored in 0.5% thymol solution at 4 °C. Fifty quadri-sectioned enamel specimens were prepared using 150-μm thick saw on a microtome (SYD Mikki Pulley, Japan). Initially, the selected teeth were decoronoted at the cemento-enamel junction. The obtained coronal structures were equally sectioned to acquire uniform sample outline. A minimum of 2.5 mm specimen height was maintained. A window of 3 × 3 mm^2 was covered with an equidimensional-masking strip; while other surfaces were covered with dual-applied acid-resistant nail varnish (Revlon®, New York, USA). Afterwards, the window was exposed to receive further treatment.

2.4. Demineralizing and remineralizing solutions

The demineralizing and remineralizing solutions were prepared as per the saturation concentration of hydroxyapatite minerals in saliva

similar to the previous studies [24,25]. Depending upon the solution, appropriate analytical-grade chemicals were thoroughly mixed in deionized (DI) water with the help of a magnetic bar and stirrer.

Demineralizing solution (DS): The DS contained 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4 and 0.05 M acetic acid adjusted with 5 M KOH to pH 4.4.

Remineralizing solution (RS): The RS contained 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 and 0.15 M KCl adjusted with 5 M KOH to pH 7.0.

The pH adjustment was evaluated using pH electrode (CyberScan pH 500, Eutech Instruments, Thermo Scientific, USA) calibrated to three solutions of known pH, 4.01, 7.0 and 10.01.

2.5. Artificial incipient carious lesion formation

The enamel specimens were immersed in DS (20 ml/specimen) on an orbital shaker (Labnet, Woodbridge, USA) at 80-rpm at room temperature for 96 h to subject it to demineralization. The DS was changed every 24 h. After artificial incipient caries-like lesion formation, the specimens were removed from DS and thoroughly rinsed with DI water.

2.6. Test groups

The prepared specimens for the study were randomly divided into 5 groups (10 specimens/group): Group 1: 2% arginine – NaF dentifrice slurry (2% Arg-NaF), Group 2: 4% arginine – NaF dentifrice slurry (4% Arg-NaF), Group 3: 8% arginine – NaF dentifrice slurry (8% Arg-NaF), Group 4 (positive control): NaF dentifrice slurry and Group 5 (negative control): DI water.

2.7. Dentifrice slurry formulation

The NaF toothpaste used for the study was Colgate Triple Action (Colgate-Palmolive Company, USA) with active ingredient - 0.24% NaF (0.11% fluoride) in a silica base. The arginine variant used for the study was L-arginine monohydrochloride (Sigma-Aldrich, St. Louis, USA). L-arginine monohydrochloride was available as minute crystals that were further milled to powder using ceramic mortar-pestle. Fresh dentifrice slurries were formulated using a standard arginine:NaF toothpaste:DI water ratio. Arginine:NaF toothpaste ratio differed as per the concentrations of arginine added to sodium fluoride toothpaste. The arginine incorporation was based on weight/weight determination of NaF toothpaste. Therefore, the higher the concentration of arginine, NaF toothpaste weight was relatively reduced to achieve appropriate blend. Once an absolute arginine:NaF toothpaste blend was obtained, it was integrated in DI water in the ratio - 1:3, whereby 1 part was arginine:NaF toothpaste and 3 parts were DI water. Hence, a fourth dilution of arginine:NaF toothpaste blend was achieved in the final dentifrice slurry prepared. For the positive control – NaF toothpaste slurry, a standard 1:3 ratio of NaF toothpaste to DI water ratio was followed. The test agents underwent thorough mixing and mechanical agitation for 60 s using vortex mixer (Super Mixer, Lab Line Instruments Inc., Illinois, USA) at room temperature. The thoroughly mixed solutions were then centrifuged at 4000 rpm for 20 min. (Beckman, Avanti *J*-251, California, USA) at 25 °C. The sediment was discarded and the supernatant was used during pH cycling [25].

2.8. pH-cycling

The specimens were subjected to pH cycling model for 10 days as per previous study [16]. The daily pH-cycling model involved 2 h of demineralization with four treatment phases (using test solutions) for 60 s each at room temperature. The remaining time was used to immerse the specimens in RS. The four treatment phases were two treatments before and two treatments after the 2 h demineralization phase. The first treatment was initiated 3 h before the demineralization phase, followed by the second treatment phase at 1 h interval. The third treatment phase commenced 2 h after the demineralization phase, following which the final treatment phase was effectuated at 1 h post third treatment phase. Freshly prepared DS and RS (20 ml & 10 ml/specimen respectively) were used with solutions changed every 24 h. Specimens were treated with 5 ml treatment solution per specimen at individual treatment phase. For each solution change, the specimens were thoroughly washed with DI water and soaked on dry fibreless laboratory napkins (Kimwipes™ Ex-L, Kimberly-Clark Professional, USA). The entire pH-cycling model was performed on continuously operated orbital shaker (Labnet, Woodbridge, USA) at 80-rpm at room temperature.

2.9. pH and fluoride concentration of treatment solutions

The pH of the treatment solutions was determined before each treatment phase using pH electrode (CyberScan pH 500, Eutech Instruments, Thermo Scientific, USA). The electrode was calibrated to three solutions of known pH, 4.01, 7.0 and 10.01 before every measurement. In between measurements, the electrode was thoroughly rinsed with DI water to avoid contamination. The position of the electrode immersion was standardized for pH determination. The concentrations of fluoride in the slurries were measured immediately after pH determination. 4-ml of treatment solutions were withdrawn from sterilin tubes and subjected to fluoride concentration estimation using fluoride ion selective electrode (Thermo Fisher Scientific, Inc., USA). The standard solutions of known fluoride concentrations: 1-ppm, 2-ppm, 10-ppm and 100-ppm were prepared to obtain the standard calibration curve at the pH-cycle inception. The fluoride electrode was secured in Milli-Q water in between measurements. After every measurement, the electrode was thoroughly rinsed with DI water. The fluoride electrode orientation was standardized throughout the pH-cycle. Before measuring, the treatment solutions were treated with 0.4 ml total ionic strength adjustment buffer (TISAB II) except DI water whereby DI water:TISAB II ratio was 1:1. The values were recorded as mV potential, which were converted to ppm using calibration curve ($R^2 = 0.9962$).

2.10. Element analysis of treatment solutions with Inductively Coupled Plasma – Emission Optical Spectrometry (ICP-EOS)

Six ml of treatment solutions were removed every day for sodium (Na) and chlorine (Cl) element high-resolution ICP-EOS analysis (Spectro Arcos, Ametek, Germany). The analysis was made effective at 0.04 MPa and > 1000 °C using argon gas. PlasmaCAL (Spectro Arcos, Ametek, Germany) was used to standardize the inbuilt plasma tube. Six standard solutions (0.1, 1, 10, 100, 500 and 1000 ppm) of Na and Cl elements were prepared for linear measurements using 2% HNO_3 as medium. An equivalent volume of negative control - HNO_3 was also subjected to standard measurements. The spectrum was calibrated using the standard solution measures. The treatment solutions were then drawn with in-built suction electrode to measure Na and Cl concentration in ppm using Smart Analyser Vision, 2014 v. 6.01.0943 (Spectro Analytical Instruments, GmBH, Germany), which provided spectrum for the determined Na and Cl concentrations. Three measurements were taken with every solution and an average was presented. A ratio of Na-Cl was determined to observe the relative concentration of Na to Cl.

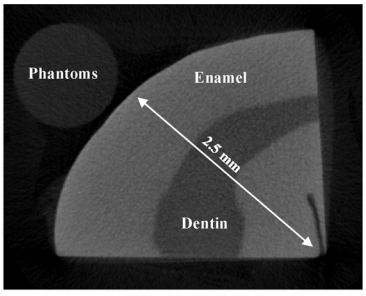
2.11. Fourier transform infrared spectroscopy (FTIR) analysis of test solutions

The qualitative FTIR analysis was performed with the test solutions to determine and verify the possible molecular organic structure in the treatment agents. The measurement mode for the analysis was percent transmittance with Happ-Genzel apodization. The resolution was set at 4 cm^{-1} with wavenumber range: 400 – 4000 cm^{-1}. Sodium chloride discs were used to hold the test solutions. A solution smear (with sterile

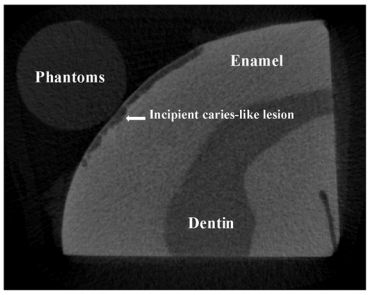
pipette) was applied between the discs that was further stabilized on a platform to insert the assembly on the recipient end of infrared spectrometer (Shimadzu IRAffinity-1S, Shimadzu Corporation, Japan). The discs were thoroughly cleaned with chloroform solution during analysis with different test agents. Randomly three samples were withdrawn from each group to undergo FTIR analysis. Prior to analysis of test solutions, a background spectrum was obtained to subtract it from the spectrum of test agents. A total of 16 scans were programmed to receive the final analysis. The spectrum obtained for all the test agents was unified on wavenumber range and intensity for comparative analysis. The relatively significant characteristic peaks were observed across the groups for further interpretation with enhanced infrared spectroscopic tools. The peaks were classified on the basis of its intensity and the respective wavenumber to observe its relevance within the spectrum.

2.12. Micro-computed tomography (Micro-CT) mineral density assessment

The mineral density (MD) of the specimens was evaluated using micro-CT scan (Skyscan 1172 X-Ray Microtomograph, Belgium) with constant scanning parameters. Flat field correction was confirmed at 9-µm before the specimens were scanned. Individual specimen was mounted on the computer-controlled turntable so that the beam of X-ray is perpendicular to the surface of interest. The scanning parameters were, voltage – 80 kV, current – 100 µA, integration time – 3.63 s, isotropic resolution – 9 µm with rotation at 360° and 1° steps. Mineral density calibration was obtained by scanning reference phantoms comprising of hydroxyapatite disks with known MD (0.25 and 0.75 g/cm^3, respectively). The three-dimensional scanned images were reconstructed using NRecon v. 1.7.0.4 (SkyScan, Belgium). The reconstruction variables were set at – smoothing: 1, ring artifact correction: 20, beam hardening correction: 30, and dyanamic range: 0 - 0.25 for all the images at any scanning level. The baseline, preoperative and postoperative reconstructed images were aligned simultaneously in DataViewer v. 1.4.4.0 (SkyScan, Belgium) to achieve uniform orientation. The processed images with trans-axial view were selected for MD assessment. The reconstructed trans-axial 2D view of baseline (Fig. 2a)

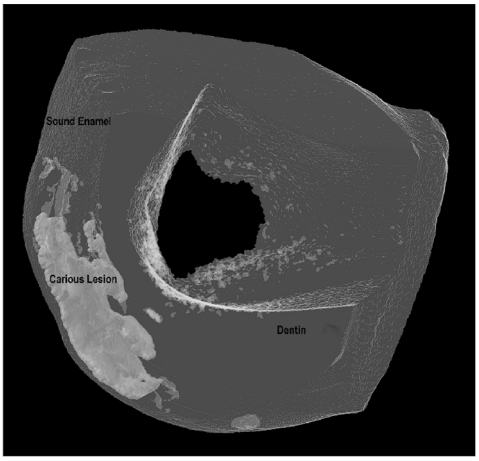


a) Baseline image of sound specimen

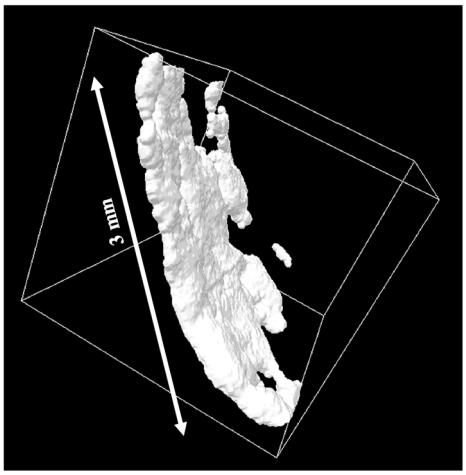


b) parallel specimen image after artificial incipient caries-like lesion formation

Fig. 2. Trans-axial view of 2D Micro-CT reconstructed images: a) baseline image of sound specimen and b) parallel specimen image after artificial incipient caries-like lesion formation.



a) Enamel specimen with artificial incipient caries-like lesion



b) Artificial incipient caries-like lesion

Fig. 3. 3D Micro-CT reconstructed images: a) enamel specimen with artificial incipient caries-like lesion and b) artificial incipient caries-like lesion.

and preoperative (Fig. 2b) parallel images were initially matched to further re-match it with parallel post-operative image. 3D reconstructed images (Fig. 3a) with artificial enamel caries-like lesions (Fig. 3b) formed the basis to identify areas for MD assessment. Mineral density was analyzed using CTAn v. 1.16.1.0+ (SkyScan, Belgium). Mineral density evaluated at three different time points (baseline, preoperative and postoperative) were labelled as T_0 – before artificial caries-like lesion formation or sound tooth structure (baseline), T_1 – after caries-like lesion formation (preoperative) and T_2 – post pH cycling (post-operative). Five sections (9-µm each) at randomly selected three levels within the window were outlined for MD evaluation. Considering the trans-axial view, a uniform region of interest with approximately 100-µm depth was used for MD assessment at three uniformly distant points. A mean MD value from each combined five sections at three random levels was calculated to obtain respective MD at T_0, T_1 and T_2. The mineral gain and percent remineralization were computed using the following equation:

$$\text{Mineral Gain} = \Delta Z_d - \Delta Z_r$$

$$\text{Percent Remineralization} = (\Delta Z_d - \Delta Z_r/\Delta Z_d) \times 100$$

(ΔZ_d – MD difference between T_0 and T_1, ΔZ_r – MD difference between T_2 and T_0)

2.13. Quantitative surface elemental analysis using Energy Dispersive X-ray Spectroscopy (EDS)

The treated specimens were further analyzed using EDS (IXRF Systems Model 550i, USA) through scanning electron microscopy (SU1510, Hitachi, Japan) at 15 kV with magnification at 250 × . The specimens were mounted on the stubs to face the specimen window perpendicular to the electron beam. A random area within the window was chosen to capture the image that was further subjected to EDS surface element analysis with Iridium Ultra 2016 v. 2.4 E software (IXRF Systems, USA). The calcium (Ca) and phosphorous (P) atomic weights were determined and their respective ratio was obtained through the software. In addition, the relative surface fluorine concentrations (wt. %) were obtained in comparison to major biological elements observed in tooth – Ca, P, carbon, nitrogen, hydrogen and oxygen. The scanning electron microscope image along with the spectrum were the recorded report for each specimen.

2.14. Enamel fluoride uptake (EFU) by acid etch method

EFU of treated specimens were determined using acid-etch method as described in previous studies [26,27]. The specimens were acid-etched with 2 ml of 1 N $HClO_4$ for 15 s under continuous agitation and stirring with magnetic bar stirrer at 150-rpm at room temperature. 1 ml of the acid-etched solution was combined with 2 ml of TISAB II and 1 ml of 1 N NaOH to establish a ratio of 1:2:1, respectively. An alternative standard calibration curve was obtained using freshly prepared standard solutions as mentioned above for fluoride concentration determination using fluoride ion selective electrode (Thermo Fisher Scientific, Inc., USA). The values obtained in ppm through calibration ($R^2 = 0.9957$) were further determined in μg/cm^2. The fluoride concentration was estimated twice and mean of the values were used for further analysis.

2.15. Statistical analysis

The data obtained were organized in MS Office Excel 2016 (Microsoft Office Professional Plus 2016, Microsoft, USA) for further statistical analysis with SPSS v. 24 (IBM SPSS® Statistics Inc, USA). Shapiro-Wilk test for normality and the Levene test for equality of variances were performed. Statistical differences among the test groups following primary assumptions (normality and equality of variances) were analyzed by one-way ANOVA with the post-hoc test, whereas for non-parametric data, the Kruskal-Wallis test followed by the pairwise comparison test was applied. Two-way ANOVA with post hoc test was used to evaluate the effect of the 2 factors, time and treatments on MD data. The statistical significance level was set at 0.05.

3. Results

3.1. Treatment solutions assessment

3.1.1. pH measurement and fluoride concentration

The pH and fluoride concentration of the treatment solutions measured during the pH-cycling is summarized in Table 1. The Kruskal-Wallis test revealed significant differences in pH and fluoride concentrations among the test groups ($p < 0.001$). Post-hoc Dunn-Bonferroni test showed that the mean pH and fluoride concentrations of 2% Arg-NaF was significantly higher than the other arginine-fluoride groups ($p < 0.05$).

3.1.2. ICP-EOS element analysis

Na and Cl concentrations (measured as ppm) of the test solutions calculated as ratio of Na to Cl concentrations is presented in Table 1. The Kruskal Wallis test demonstrated significant difference in the median Na, Cl and Na-Cl ratio among the test groups ($p < 0.001$). Post-hoc Mann-Whitney-Wilcoxon test showed that the Na:Cl ratio of 2% Arg-NaF was significantly higher than 4% Arg-NaF and 8% Arg-NaF ($p < 0.05$).

3.1.3. FTIR analysis

Fig. 4 presents the analyzed infrared spectrum of test solutions. Three characteristic peaks (2358.94, 2086.98, and 1080.14) from 2% Arg-NaF and 4% Arg-NaF were identified as significantly different from the other test solutions. Initiating from 4000 wavenumber cm^{-1} towards 400 cm^{-1}, the first distinctive peak (2358.94) falls within the range of 2280–3380 wavenumber cm^{-1} suggesting NH_3^+ stretching vibration. Similarly, the second peak (2086.98; range: 2010 – 2120 cm^{-1}) indicates presence of free amino acids. The peak at 1080.14; range: 1000 - 1110 cm^{-1} exhibits the presence of aliphatic mono-fluorinated compounds. Therefore, the interaction between L-arginine monohydrochloride and NaF seems to have made arginine available (as free amino acid) with the formation of NH_3^+ attached to common α-carbon combined to organize as aliphatic mono-fluorinated compound in 2% Arg-NaF and 4% Arg-NaF only.

3.2. Specimen analysis

3.2.1. Mineral density assessment

The enamel mineral density (MD) (mean ± SD) of the treated specimens measured in g/cm^3 before demineralization, after demineralization and following pH cycling is presented in Table 2. The computed mineral gain (MG) and percent remineralization at T_0, T_1 and T_2 are also shown in Table 2. Results of the two-way ANOVA revealed that both factors, time and treatments were statistically significant ($p < 0.001$). However, the interaction between these two factors was not significant ($p = 0.206$). No significant difference in MD was found among the groups at T_0 and T_1 ($p > 0.05$). The mean MD of the lesions treated with 2% Arg-NaF and NaF at T_2 was significantly higher than those treated with DI water ($p < 0.05$). Comparing the different time-period, a statistically significant difference in MD was observed in all

Table 1
pH, fluoride concentrations and ICP-EOS element analysis of treatment solutions.

Groups	pH	Fluoride concentrations (ppm)	Na:Cl
	Mean ± SD [Median (IQR)]		
2%Arg-NaF	7.42 ± 0.04 [7.40 (0.00)]^a	267.22 ± 1.26 [267.13 (1.77)]^A	1.89 ± 0.19 [1.77 (0.36)]$^\alpha$
4%Arg-NaF	7.15 ± 0.11 [7.15 (0.25)]^b	259.26 ± 0.07 [259.29 (0.00)]^B	1.14 ± 0.13 [1.05 (0.26)]$^\beta$
8%Arg-NaF	6.95 ± 0.05 [6.95 (0.10)]^b	213.44 ± 1.51 [213.33 (2.13)]^C	0.69 ± 0.09 [0.62 (0.16)]$^\gamma$
NaF	7.65 ± 0.11 [7.65 (0.25)]^c	206.32 ± 0.97 [206.39 (1.36)]^D	23.32 ± 3.15 [21.01 (6.07)]$^\delta$
DI Water	6.26 ± 0.25 [6.15 (0.53)]^d	0.06 ± 0.01 [0.06 (0.00)]^E	49.74 ± 17.20 [48.37 (32.07)]$^\theta$

Groups identified by different superscripts were significantly different in each column.

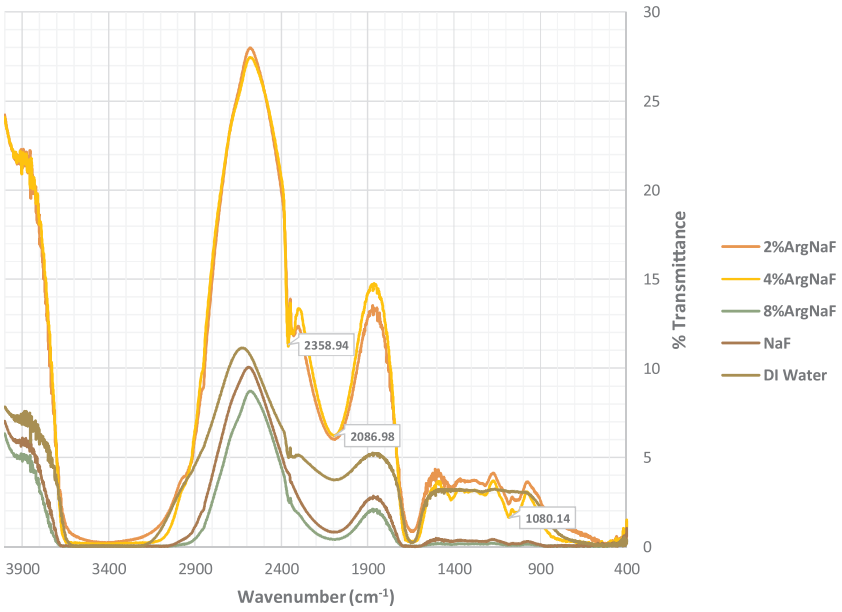


Fig. 4. Fourier Transform Infrared Spectroscopy analysis of test solutions.

the groups between T_0 and T_1 ($p < 0.05$). Apart from 8% Arg-NaF and DI water, a similar significant difference in MD was noted in all groups between T_1 and T_2 ($p < 0.05$).

One-way ANOVA identified statistically significant difference in mean MG and % remineralization among the test groups ($p < 0.001$). Scheffe's post-hoc multiple comparison analysis showed that the mean MG and % remineralization were the highest for the 2% Arg-NaF group and the lowest for the DI water group. There was no significant difference in mean MG and % remineralization among the 4% Arg-NaF, 8% Arg-NaF and NaF groups ($p > 0.05$).

3.2.2. Quantitative surface elemental analysis

The results for post-treatment quantitative surface element analysis obtained using EDS are shown in Table 3. The Kruskal-Wallis test showed a statistically significant difference in the median Ca/P ratio among the test groups ($p = 0.029$). Dunn-Bonferroni's post-hoc analysis identified significant difference between the median Ca/P ratio of specimens treated with 2% Arg-NaF and that of specimens treated with DI water ($p < 0.05$). The differences in median Ca/P ratio of specimens treated with 2% Arg-NaF, 4% Arg-NaF, 8% Arg-NaF and NaF were not statistically significant ($p > 0.05$).

One-way ANOVA displayed significant difference in the mean surface fluorine concentration amongst the treatment groups ($p < 0.001$). Tukey's HSD post-hoc test showed that the mean surface fluorine concentrations of 2% Arg-NaF and 4% Arg-NaF were significantly higher than NaF ($p < 0.05$). There was no significant difference ($p > 0.05$) in the mean surface fluorine concentration of specimens treated with 8% Arg-NaF and NaF.

3.2.3. Enamel fluoride uptake

Table 3 presents the enamel fluoride uptake (mean ± SD) measured in μg/cm^2 after 10-days pH cycling for specimens treated with different study solutions. One-way ANOVA exhibited statistically significant difference in the mean EFU of the specimens treated with the different test solutions ($p < 0.001$). Duncan's multiple range post-hoc analysis showed that the mean EFU of 2% Arg-NaF was significantly higher than NaF ($p < 0.05$). No significant difference in the mean EFU of specimens treated with 4% Arg-NaF, 8% Arg-NaF and NaF was observed.

4. Discussion

The incorporation of 2% arginine into the commercially available NaF toothpaste significantly increased its remineralization properties, as demonstrated by increased mineral gain, percent remineralization, surface Ca/P ratio with fluorine concentration, and enamel fluoride uptake. In addition, the treatment solution assessment for 2% Arg-NaF showed significantly higher fluoride concentration with neutral pH, acceptable Na-Cl ratio and availability of free amino acids. In contrast, the incorporation of 4% and 8% arginine into NaF toothpaste did not have any remineralization enhancement effect. Hence, the null hypothesis that "The incorporation of arginine in NaF toothpaste has no additional remineralization effect when compared to toothpaste with only NaF" was partially rejected.

L-arginine monohydrochloride was the arginine variant selected for the study, as the variant in combination with NaF solution has demonstrated a significant inhibition of *Streptococcus mutans* in a

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	$2.79 \pm 0.11^{a,\alpha}$	$1.36 \pm 0.18^{A,\beta}$	$1.76 \pm 0.17^{I,\chi}$	0.40 ± 0.07^{a}	27.91 ± 4.66^{A}
4%Arg-NaF	$2.81 \pm 0.10^{a,\alpha}$	$1.34 \pm 0.15^{A,\beta}$	$1.61 \pm 0.17^{I,II,\chi}$	0.26 ± 0.10^{b}	18.25 ± 7.55^{B}
8%Arg-NaF	$2.70 \pm 0.13^{a,\alpha}$	$1.37 \pm 0.18^{A,\beta}$	$1.54 \pm 0.19^{I,II,\beta}$	0.17 ± 0.05^{b}	13.51 ± 6.02^{B}
NaF	$2.89 \pm 0.06^{a,\alpha}$	$1.46 \pm 0.32^{A,\beta}$	$1.68 \pm 0.29^{I,\chi}$	0.21 ± 0.06^{b}	15.34 ± 4.07^{B}
DI Water	$2.74 \pm 0.19^{a,\alpha}$	$1.38 \pm 0.21^{A,\beta}$	$1.43 \pm 0.19^{II,\beta}$	0.05 ± 0.04^{c}	3.77 ± 2.48^{C}

Uppercase letters/lowercase letters/roman numerals represent differences in each column (T_0/ T_1/ T_2/Mineral Gain/% Remineralization). Symbols α, β and χ represent differences in each row.

Table 3
Post-treatment quantitative surface element analysis and enamel fluoride uptake.

Groups	Ca/P Ratio Mean ± SD [Median (IQR)]	Fluorine Concentration (wt. %) (Mean ± SD)	EFU (μg/cm^2) (Mean ± SD)
2%Arg-NaF	1.73 ± 0.38 [1.60 (0.12)]^{a}	1.51 ± 0.14^{A}	6.84 ± 1.59^{a}
4%Arg-NaF	1.60 ± 0.05 [1.58 (0.15)]^{a,b}	1.37 ± 0.25^{A}	6.51 ± 1.41^{a,b}
8%Arg-NaF	1.58 ± 0.11 [1.55 (0.13)]^{a,b}	1.33 ± 0.34^{A,B}	6.47 ± 2.11^{a,b}
NaF	1.60 ± 0.10 [1.59 (0.10)]^{a,b}	1.02 ± 0.28^{B,C}	5.22 ± 1.88^{b}
DI Water	1.53 ± 0.05 [1.53 (0.07)]^{b}	0.98 ± 0.26^{C}	2.30 ± 0.60^{c}

Groups identified by different superscripts were significantly different in each column.

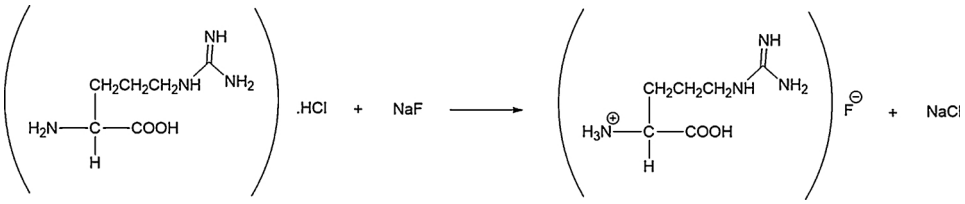


Fig. 5. Postulated interaction between L-arginine monohydrochloride and sodium chloride.

cariogenic biofilm [23]. Additionally, it is postulated that the arginine variant might interact with NaF generating sodium chloride as depicted in Fig. 5. The interaction would be possible due to the positively charged guanidinium group of arginine, which attracts the negatively charged fluoride ion (high electronegativity). Furthermore, the FTIR analysis in the present study confirms the postulated interaction by demonstrating the presence of free amino acids (per case arginine) in both 2% Arg-NaF and 4% Arg-NaF groups.

In this study, the pH of the dentifrice slurry with arginine decreased with increasing concentration of arginine. The monohydrochloride component of the arginine variant might be the reason for such a change. The 2% Arg-NaF and 4% Arg-NaF retained the pH of the treatment solutions above neutral, which might be due to the higher Na-Cl ratio as indicated by ICP-EOS element analysis. It is noted that the fluoride concentrations of all the arginine-based slurries were significantly higher than that of NaF alone. The postulated interaction between L-arginine monohydrochloride and sodium fluoride would have led to the formation of L-arginine fluoride in an ionic form, making free fluoride ion available. However, the fluoride concentration of the arginine-incorporated slurries decreased with increasing concentration of arginine, which could be attributed to the decreasing amount of NaF toothpaste (by weight) in the preparation of the respective toothpaste slurry.

In both 2% Arg-NaF and 4% Arg-NaF groups, the concentration of Na exceeds that of Cl; whereas in the 8% Arg-NaF group, the opposite is observed. The increased Cl concentration in the 8% Arg-NaF group could be attributed to arginine retention in its original form of L-arginine monohydrochloride, along with arginine-fluoride and sodium chloride formation. The overabundance of L-arginine monhydrochloride in 8% Arg-NaF might have prevented the postulated interaction between L-arginine monohydrochloride and sodium fluoride from occurring, limiting the availability of free fluoride ion when compared to 2% Arg-NaF.

In addition, the fluoride concentration estimated for the arginine-based slurries and the positive control was quite low, when compared to the labelled 1100-ppm fluoride concentration in the toothpaste used in this study. The obtained standard calibration curve and dilution factor could be the possible reasons for such findings. The standard calibration curve was obtained with a maximum of 100-ppm known fluoride concentration solution; hence, the relatively detected free fluoride ions might have been beyond its scope. The arginine:sodium fluoride toothpaste formulations were diluted in DI water to a fourth of dilution factor; therefore, the fluoride concentration values appear reasonable.

The mineral density (MD) values at T_0 (baseline) and T_1 (pre-operative) were similar across the study groups. It is noteworthy that MD values were significantly different between T_0 and T_1 in all the groups, which confirms the formation of caries-like lesions at T_1. This is in accordance with a previous study that used micro-CT for MD assessment of white spot lesions treated with various remineralizing agents [28]. Overall, the 2% Arg-NaF group presented with the highest MG and % remineralization. The high MG for 2% Arg-NaF confirms the postulated reaction between L-arginine monohydrochloride and NaF with an enhanced remineralization effect when compared to NaF.

There are several advantages in using micro-CT for MD assessment in the study. Firstly, it provides a non-invasive assessment of the specimens at different time points. Secondly, it allows a parallel assessment of the specimens, irrespective of the treatment received. Thirdly, there is no special requirement for specimen preparation, since the scanning procedure can be effectuated on intact enamel surface and finally, it provides a platform for sub-surface MD assessment, which is very critical for studying artificial incipient caries-like lesions.

Recent studies have used EDS for quantitative element surface assessment of enamel [29,30]. The EDS-based element analysis appears to be a promising non-invasive technique. In the present study, we have used Ca/P ratio and relative surface fluorine concentration as measures for surface analysis of enamel with artificial incipient caries-like lesion. The Ca/P ratio was similar among the groups treated with arginine-based slurries and NaF; while the ratio was significantly higher in 2% Arg-NaF than DI water groups.

Results of the relative surface fluorine concentration and EFU were the highest in the Arg-NaF groups, followed by NaF and the lowest in the DI water group, which imply that incorporating arginine in NaF increases fluoride uptake and surface fluorine availability, thereby enhancing enamel resistance to cariogenic challenge. The sodium chloride formed as a result of the postulated interaction between L-arginine monohydrochloride and sodium fluoride might have improved EFU as shown by previous studies [31–33]. The results for EFU in the present study are in concordance with another study, which showed that the combined 2.5% arginine – 500-ppm NaF solution had significantly higher EFU than the 500-ppm sodium fluoride solution [16].

The enhanced remineralization effect of 2% Arg-NaF when compared to NaF observed in this study is possible as arginine is a residual protein. The adsorption of albumin to fluorides attracts calcium and phosphates to form alkali stable fluorapatite [20]. The topographical polar surface area of L-arginine monohydrochloride (128 Aˆ2) is more than 10-fold smaller than albumin (1530 Aˆ2), facilitating the diffusion of arginine-fluoride for sub-surface lesion remineralization as well as formation of a reservoir of arginine-fluoride for release during acid attack [16].

The study has limited itself to the use of micro-CT, EFU and EDS for

surface characterization of enamel specimens. Non-destructive characterization techniques, like high kinetic energy - HIKE X-ray photoelectron spectroscopy (XPS), provide better sub-surface elemental analysis. This study has only evaluated the combined remineralization effect of one arginine variant *i.e.* L – arginine monohydrochloride with NaF toothpaste. The effect of other arginine variants with NaF or MFP toothpaste should be further investigated. Projecting the availability of arginine-fluoride combination in a commercial dentifrice, increasing the arginine concentrations in the test formulations has reduced the NaF mass by weight, accordingly. Future studies evaluating the synergistic remineralizing effect of arginine with other topical fluoride formulations should aim to match the fluoride concentration in the test groups to reduce the number of variables. As the next step of investigation, it is also recommended to evaluate the antimicrobial potential of the tested concentrations of arginine in this study on multi-species biofilm.

5. Conclusion

Within the limitations and based on the findings of the present *in vitro* study, the following conclusions are drawn:

1 The incorporation of 2% arginine in NaF toothpaste significantly increased the remineralization of incipient enamel caries-like lesion when compared to NaF toothpaste.
2 Increasing the concentration of L – arginine monohydrochloride in sodium fluoride toothpaste reduced the remineralization effect as compared to that of 2% arginine – NaF.

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