Synergistic Effect of Arginine on Remineralization Potential of Fluoride Varnish and Nanohydroxyapatite on Artificial Caries Lesions: An In Vitro Study

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ABSTRACT

Aim and objective: To evaluate the synergistic effect of arginine on the remineralizing potential of fluoride varnish and nanohydroxyapatite.

Materials and methods: A total of 100 teeth were taken; of them, 50 teeth were allotted for microhardness test and 50 teeth for scanning-electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDX) analysis. Fifty teeth used for hardness testing were sectioned to obtain 100 samples, and the baseline hardness values were measured. Samples were allotted into five groups (n = 20): group I: arginine; group II: fluoride varnish; group III: nanohydroxyapatite; group IV: arginine + fluoride varnish; group V: arginine + nanohydroxyapatite. Microhardness values were measured after 96 hours of demineralization and then again after application of remineralizing agents (pH cycling) for 10 days to check for gain in microhardness. The other 50 samples were subjected to SEM-EDX analysis for evaluating gain in the mineral content after demineralization and after application of the remineralizing agents. The collected data were subjected to statistical analysis using SPSS software version 22.0.

Results: The maximum mean microhardness values were observed in group IV and group V. There was no statistical significance between them. Similarly, maximum mineral gain was seen in groups IV and V. A significant increase in fluoride gain was seen in group IV.

Conclusion: Arginine has a synergistic effect on remineralization potential of fluoride varnish and nanohydroxyapatite.

Clinical significance: The incorporation of arginine into fluoride varnishes and nanohydroxyapatite significantly increased their remineralization potential.

Keywords: Arginine, Energy-dispersive X-ray analysis, Fluoride varnish, Microhardness, Nanohydroxyapatite, Remineralization.

The Journal of Contemporary Dental Practice (2020): 10.5005/jp-journals-10024-2915

INTRODUCTION

Dental caries is a chronic, multifactorial disease characterized by demineralization of the calcified tissues of the tooth. Every tooth in the oral cavity is continuously put under the dynamic process of demineralization and remineralization. The saliva supersaturated with calcium and phosphorous plays an important role in the natural remineralization process of the teeth. When there is a shift in equilibrium toward net demineralization due to acids produced by plaque bacteria, it leads to caries.¹,² The worldwide estimation of people affected by oral diseases is 3.58 billion, of which permanent tooth caries is the most prevalent. The estimated number of people who suffer from permanent teeth caries is 2.4 billion people; and primary teeth caries is 486 million.³

It is more acceptable to treat dental caries at an early stage with noninvasive remineralizing agents than invasive cavity preparation and restoration. Remineralizing agents prevent further demineralization and promote remineralization of the existing incipient lesions.⁴ Till date, it is well established that fluoride-containing agents are the best option for the remineralization of incipient caries lesions.⁵–⁷ Fluoride used in dentifrice, professional topical fluoride application, and community water fluoridation are considered as major routes of administration that result in remineralization.⁸ Anticariogenic activity of fluorides is due to the inhibition of bacterial enzyme responsible for acid production and by the formation of acid-resistant fluorapatite crystals.⁹ Synthetic nanohydroxyapatite is a recent biocompatible and bioactive material that has a crystalline structure similar to enamel crystals.¹⁰

Recent studies showed a greater potential for remineralization of caries with the use of nanohydroxyapatite particles.¹¹,¹² The efficacy of fluoride alone as a remineralizing agent is limited. Thus, newer strategies such as addition of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) to fluoride were developed to improve its remineralization potential.

Both negatively charged amino acids (AAs) and positively and negatively charged AAs play a crucial role in hydroxyapatite (HA) mineralization in both the bone and the teeth. These AAs mediate the interaction of calcium and phosphorous ions necessary for HA precipitation.¹³,¹⁴ Previous studies reported that the addition of arginine to dentifrices resulted in improved remineralization compared to nonarginine toothpastes.¹⁵,¹⁶ Professional applications...
of remineralizing agents were found to be more effective than self-applications in the form of toothpaste.13

Arginine is an essential AA present in saliva at the concentration of 50 μM. Oral microbial flora metabolizes arginine into ornithine, ammonia, and carbon dioxide. Ammonia raises the pH of saliva, thereby inhibiting demineralization of teeth. The alkaline nature of arginine interferes with the formation of cariogenic biofilm.13 Arginine contains both negatively charged COO⁻ and positively charged NH₄⁺ that play significant role in the mineralization of HA in the bone and in the teeth. Negatively charged COO⁻ attracts calcium ions and positively charged NH₄⁺ attract phosphate ion into collagen matrix and promote crystallization and growth of HA crystals.13,14

Previously published literature has evaluated the effect of arginine in fluoride and nonfluoride-based dentifrices. However, till date, no study has assessed the combination of arginine with fluoride varnish and HA for professional application. This study was conducted to evaluate the synergistic effect of arginine on the remineralizing potential of fluoride varnish and nanohydroxyapatite.

In the present study, the null hypothesis tested is, no synergistic effect is seen with the addition of arginine to fluoride varnish and nanohydroxyapatite for remineralization of artificial carious lesion. The crowns of fifty teeth were cut longitudinally in the mesiodistal direction into two halves with a diamond saw (Glassland, Hebei, China: Lot no. 596445) under constant water cooling to obtain 100 sections for microhardness testing (Flowchart 1). Samples were divided randomly into five groups based on remineralization treatment received: group I: arginine; group II: fluoride varnish; group III: nanohydroxyapatite; group IV: arginine + fluoride varnish; group V: arginine + nanohydroxyapatite (Table 1).

Sample Collection

One hundred and five maxillary anterior teeth extracted due to periodontal pathologies were collected. Debris was cleaned from the surfaces of all teeth and the samples stored at room temperature in 0.9% saline. Five teeth were excluded from the study due to caries, restorations, cracks, hypoplastic lesions, developmental anomalies, and discoloration due to pulpal necrosis. Teeth were sectioned 2 mm below the cementoenamel junction separating the roots. Of 100 samples, 50 teeth were used for hardness analysis and remaining 50 teeth for EDX analysis.

Vickers Hardness Testing

The crowns of fifty teeth were cut longitudinally in the mesiodistal direction into two halves with a diamond saw (Glass land, Hebei, China: Lot no. 596445) under constant water cooling to obtain 100 sections for microhardness testing (Flowchart 1). Samples were mounted in acrylic resin molds of 1-inch diameter and 1.5 cm height, with the labial surface of crowns exposed and parallel to the floor. Samples were polished progressively with silicon carbide disc (800, 1,000, 2,400 grit), until a flat surface was obtained for accurate measurement of hardness. On the bottom of acrylic mounting, samples were numbered zero to hundred to compare hardness scores of each sample at baseline, after demineralization and remineralization.

Baseline Microhardness

One hundred crown samples were used for hardness evaluation, and the baseline microhardness values were measured at four points on the polished surface of each tooth using a Vickers Hardness Testing Machine (400 Series, Wilson Wolpert, Germany; Model no. 0214788) with a force of 200 g applied for 15 seconds, and the average was taken for each sample (Flowchart 1). Following the measurement of baseline hardness values, 100 samples were divided randomly into five groups based on remineralization treatment received: group I: arginine; group II: fluoride varnish; group III: nanohydroxyapatite; group IV: arginine + fluoride varnish; group V: arginine + nanohydroxyapatite (Table 1).

Preparation of Demineralization and Remineralization Solutions

The demineralization and remineralization solutions were prepared by following the protocols described by Ten Cate and Duijsters.18 The demineralization solution was composed of 50 mM acetic acid, 2.2 mM Ca(NO₃)₂, 2.2 mM KH₂PO₄ and 0.1 ppm NaF (pH = 4.5). The remineralizing solution was composed of 1.5 mM CaCl₂, 0.9 mM NaH₂PO₄, 0.15 M KCl with a pH of 7.0.

Artificial Caries Formation

The samples from each group were submerged in the demineralizing solution for 96 hours, and this solution was replaced every 24 hours. After 96 hours of demineralization, samples were rinsed with saline, and the microhardness values were recorded.

pH Cycling

All groups were subjected to pH cycling for 10 days. Every cycle involves 3 hours of demineralization two times a day, followed by 2 hours of remineralization. The remineralizing agents were applied on the demineralized areas and left to dry for 15 minutes before first demineralization cycle in all groups. In the second demineralization cycle, remineralizing agent application is done both before and after demineralization in all groups, following which all samples are left overnight in the remineralizing solution. Demineralization and remineralization solutions were replenished every day. After 10 days of pH cycling, microhardness values were measured.

Energy-dispersive X-ray Spectroscopy Analysis

Fifty samples were used for scanning-electron microscopy (SEM) with EDX analysis, and all of them were allotted to five experimental groups (n = 10) based on the remineralizing agent applied (Table 1). All groups were subjected to demineralization for 96 hours, and SEM-EDX (Shimadzu EDX 7000, Japan) analysis was done for the evaluation of calcium, phosphorous, and fluoride ions (Flowchart 2). Then, pH cycling was done with the application of the respective remineralizing agent to teeth in all groups for 10 days. After completion of pH cycling, SEM-EDX analysis was done to check for gain in mineral and fluoride content. The gain in the calcium, phosphorus, and fluoride elements was analyzed by peaks in weight% and volume% in EDX analysis.

Statistical Analysis

All the data in the microhardness test and SEM-EDX test were tabulated and transferred to SPSS software version 22.0 (IBM, New York, USA). The statistical analysis was done by keeping the power of study at 85%, alpha error (%) = 5, and effect size at 1.2.
Synergistic Effect of Arginine on Remineralization

The Journal of Contemporary Dental Practice, Volume 21 Issue 9 (September 2020)

Table 1: Groups and materials used in the study

| Groups          | Remineralizing agents                          | Manufacturers                                      |
|-----------------|-------------------------------------------------|----------------------------------------------------|
| Group I         | 10% Arginine: 1 g of arginine mixed with 10 mL distilled water | Sigma Aldrich, USA, 11009-25G-F, Lot # BCBX4892   |
| Group II        | Mi Varnish: 5% NaF, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) | GC Corporation, Itabashi-Ku, Tokyo, Japan          |
| Group III       | 10% Nanohydroxyapatite: 1 g of nanohydroxyapatite mixed with 10 mL distilled water | Sigma Aldrich, USA, 900194-25G, Lot # MKCG2129    |
| Group IV        | 10% Arginine added to Mi Varnish combination    | Experimental combination                           |
| Group V         | 10% Arginine added to 10% Nanohydroxyapatite   | Experimental combination                           |

Table 2 shows mean microhardness values at baseline, after demineralization and remineralization. One-way ANOVA test shows that statistically there was no significant difference in the mean baseline microhardness values among all groups (p = 0.7989). After demineralization, mean hardness values were similar in all groups, and no significant difference was noted (p = 0.9875). After remineralization, group V (10% arginine-containing Mi Varnish) showed the highest hardness value, followed by group IV (10% arginine-containing HA). Pairwise comparison showed that there was statistically no significant difference between the mean remineralization hardness values of groups I, II, and III (Table 3). A significant increase in the remineralization hardness values was seen in groups IV and V compared to groups II and III, respectively (Table 3). However, there was statistically no significant difference between group IV and group V.

Table 4 shows a total mineral gain and fluoride gain after remineralization. The highest mineral gain was seen in group IV, followed by group V (Fig. 1). Pairwise comparison showed that there was a significant rise in mineral gain values in groups IV (41.13%) and V (36.73%) compared to groups II (28.38%) and III (23.55%), respectively (Tables 4 and 5). The percentage of

Results

York, USA) for statistical analysis. The intergroup comparisons were made using one-way ANOVA which was followed by Tukey’s post hoc test for pairwise comparisons.
Synergistic Effect of Arginine on Remineralization

**Discussion**

Saliva supersaturated with calcium and phosphorous ions is a natural source of remineralization, where the bioavailable forms...
Synergistic Effect of Arginine on Remineralization

The Journal of Contemporary Dental Practice, Volume 21 Issue 9 (September 2020)

of Ca$^{2+}$ and PO$_4^{3-}$ that diffuse into decalcified crystal voids and remineralize incipient caries lesions. However, saliva alone cannot remineralize incipient caries lesions in persons with increased caries activity due to the low calcium and phosphorus ion concentration gradient between saliva and decalcified areas. This leads to the development of various remineralizing agents, of which fluorides are most widely used. Previous literature showed that dentifrices and varnish-containing fluoride in the concentration of 1,000–1,500 ppm were found to be effective in remineralizing incipient caries. Fluorides at higher concentrations (5,000 ppm) should be applied to remineralize teeth with high caries activity and root caries. Recent research has shown fluorides as a neurotoxic chemical at higher concentration. This led to the development of newer strategies to potentiate the efficacy of fluoride by keeping its concentration low. Thus, this study evaluated the effect of addition of arginine to fluoride varnish and nanohydroxyapatite on their remineralization potential.

In the current study, arginine, when applied alone as a remineralizing agent, showed the least improvement in hardness values and mineral gain values compared to all other groups. Significant increase in hardness and mineral gain values was noted with arginine-fluoride varnish (group IV) and arginine-HA (group V), when compared to fluoride varnish (group II) and HA (group III), respectively. This shows that there was a synergistic effect of arginine when added to fluoride varnish and HA, thereby potentiating their capacity. Thus, the null hypothesis was rejected.

Biomineralization of bone and teeth is initiated by phosphorylated noncollagen proteins present in the extracellular matrix. Electrically charged AA domains of these proteins attract Ca$^{2+}$ and PO$_4^{3-}$ and promote precipitation of HA. Amino acids with the negatively charged COO$^-$ group have greater potency in

**Table 5: Pairwise comparisons of mineral gain (Ca + P) and F gain**

| Groups             | Mineral gain (Ca + P) | F gain |
|--------------------|-----------------------|--------|
| Group I vs group II| $p = 0.1200$          | $p = 0.8300$ |
| Group I vs group III| $p = 0.9400$         | $p = 1.0000$ |
| Group I vs group IV | $p = 0.0001^*$       | $p = 0.0001^*$ |
| Group I vs group V  | $p = 0.0001^*$       | $p = 1.0000$ |
| Group II vs group III | $p = 0.4400$       | $p = 0.8100$ |
| Group II vs group IV | $p = 0.0001^*$       | $p = 0.0001^*$ |
| Group II vs group V  | $p = 0.0400^*$       | $p = 0.7600$ |
| Group III vs group IV | $p = 0.0001^*$      | $p = 0.0001^*$ |
| Group III vs group V  | $p = 0.0001^*$       | $p = 1.0000$ |
| Group IV vs group V   | $p = 0.5300$         | $p = 0.0001^*$ |

*p ≤ 0.05 is statistically significant

Fig. 1: Comparison of five groups with mean mineral gain in EDX scores of Ca + P

Fig. 2: Comparison of five groups with mean gain in EDX scores of fluoride
promoting HA precipitation compared to positively charged AA domains. However, arginine contains both negatively charged COO− and positively charged NH4+ domains, thus facilitating the attraction of both Ca2+ and PO43− ions which results in HA precipitation. This explains the synergistic effect of 10% arginine in groups IV and V. Cheng et al. also reported that fluoride dentifrices containing 2.5% arginine showed greater remineralization potency compared to only fluoride dentifrices.

Interaction between positively charged guaninium group of L-arginine and sodium fluoride in group IV results in the formation of L-arginine fluoride in ionic form that is deposited in the crystal voids of decalcified areas. Simultaneously negatively charged COO− group of L-arginine fluoride attracts calcium ions into crystal voids. This explains the high calcium and fluoride gain in group IV when compared to all the other groups.

Wang et al. reported that NaF remineralizing agent showed high hardness values after 12 days of pH cycling followed by 15% and 10% nanohydroxyapatite application.24 In the current study, fluoride agents showed better remineralizing potential than nanohydroxyapatite. Lower hardness and mineral gain values in group V compared to group IV could be attributed to the formation of disoriented HA crystals. Wang et al. reported that the presence of glycine promoted the formation of well-oriented rod-like HA crystals that resulted in improved nanohardness and elastic modulus values as compared to that HA remineralization in the absence of glycine. In the current study, nanohydroxyapatite was mixed with distilled water. This could be the reason for the lower values of microhardness and mineral gain in group V compared to group IV.

In this study, the synergistic effect of arginine on the remineralization potential of fluoride varnish and HA was evaluated using indirect methods (SEM-EDX and Vickers hardness test). Further studies with more direct methods such as polarized light microscopy, electron probe analysis, and micro-CT scanning are warranted for more accurate analysis.

**Conclusion**

Within the limitations of the study, the addition of arginine to fluoride varnish and nanohydroxyapatite significantly increased their remineralization potential. Further studies have to be conducted to evaluate HA crystal growth and crystal morphology during the process of tooth remineralization.

**Clinical Significance**

The incorporation of arginine into fluoride varnishes and nanohydroxyapatite significantly increased their remineralization potential. As fluorides are considered harmful at high doses, this combination utilizing arginine potentially achieves adequate remineralization at lower level of fluorides.

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