https://doi.org/10.33472/AFJBS.6.11.2024.1578-1586

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

EVALUATION OF THE REMINERALIZING ABILITY OF AMINO ACIDS USING SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS

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Article Info

Volume 6, Issue 11, July 2024

Received: 21 May 2024

Accepted: 27 June 2024

Published: 12 July 2024

doi: 10.33472/AFJBS.6.11.2024.1578-1586

ABSTRACT:

Background: Multiple peptides have been suggested for use in enamel remineralisation. Despite showing very good results in vitro, they have not been able to translate well into clinical practice due to their high cost, the risk of disintegration of the peptide by hydrolysis in the oral environment, and their unavailability as a commercial product. The aim of this study was to assess the remineralisation potential of the individual amino acids on demineralised enamel.

Materials and Methods: 15 maxillary incisors were sectioned to produce 15 enamel slices of 5 mm x 5 mm each. They were then immersed in 0.1M acetic acid at pH 4 for five days in order to mimic demineralised carious lesions. Five demineralised specimens each of enamel respectively were then chosen as baseline samples for Scanning Electron Microscopy(SEM) and Energy dispersive X ray analysis (EDX). The remaining 10 samples each of enamel were again randomly divided into 2 groups - Group 1 - Glutamic acid + remineralizing solution and Group 2 l-aspartic acid and l-serine + remineralizing solution. The samples were immersed for 48 hours. The samples were then retrieved and subjected for SEM and EDX analysis post treatment.

Results: In the baseline SEM images clearly showed demineralised enamel with loss in the prismatic structure of enamel. In the SEM images taken after immersion in glutamic acid, granular deposition of the calcium phosphate in each of the enamel prisms. In the SEM images of the aspartic acid-serine group, deposition of calcium and phosphate was observed but less than that of the glutamic acid group. The EDX results showed a 4% increase in calcium content from baseline in the glutamic acid group. The Aspartic acid-serine group had a 3% increase in calcium levels.

Conclusion: From the results of this study, glutamic acid showed a better remineralisation potential in comparison to aspartic acid and serine after subjection to demineralisation.

Keywords: Aspartic acid, Demineralization, Remineralization, Glutamic acid, Serine

1. INTRODUCTION

The pathophysiology of dental caries is not just a gradual and continuous depletion of the mineral content of the tooth, but a dynamic process marked by cycles of demineralization and remineralization. The advancement or regression of carious lesions depends on the interplay between factors that promote demineralization (such as cariogenic bacteria, fermentable carbohydrates, and impaired salivary function) and protective elements (like antibacterial agents, adequate saliva production, and remineralizing ions), which collectively determine the shift towards either demineralization or remineralization.[1] Remineralization, which results in a net increase in mineral content, is a reparative procedure in which calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions from saliva and plaque are deposited into the inside demineralized tooth structures. Fluorapatite, which has significantly increased resistance to subsequent acid attacks, is formed when free fluoride (F^-) ions are introduced into the oral cavity. This process helps in the integration Ca^{2+} and PO_4^{3-} ions into the crystal structure.[2] Traditional fluoride-based remineralization replaces the hydroxyl ions of hydroxyapatite with fluorapatite, making them more resistant to acid assault. [3] With numerous systematic reviews attesting to the effectiveness of fluoride products in preventing dental caries, fluoride still remains the gold standard in caries prevention. [3][4][5][6] However, emerging epidemiological data indicate a concerning trend, with caries experience plateauing or even increasing in some population groups with regular fluoride use. [7][8][9] The increasing fluoride exposure from multiple sources increases the risk of fluorosis in children, which results in mottling or the halo effect, causing an unesthetic appearance. [10] High-concentration topical fluoride use also increases the risk of occult caries due to surface-only remineralization. [11] This brings about a need for new-age remineralizing systems, which attempt to shift from reparative to regenerative therapy. [12]Multiple peptides, like P11-4 and 8DSS,bioactive glass, CPP-ACP etc., have been attempted as scaffolding materials for enamel regeneration.[13][14] Despite showing very good results in vitro, they have not been able to translate well into clinical practice due to their high cost, the risk of disintegration of the peptide by hydrolysis in the oral environment, and their unavailability as a commercial product.

The role of amino acids in remineralization is a developing area of research with implications for various fields, including dentistry, nutrition, and biomedical engineering. [15] Amino acids are the building blocks of proteins and play a crucial role in the formation and maintenance of mineralized tissues such as bones and teeth.[16] They have been shown to enhance the uptake of calcium and phosphate ions into the tooth structure, promoting the repair of demineralized areas.[17] In addition, specific amino acids such as arginine have been found to have a direct impact on the regulation of oral pH, creating an environment that is more conducive to remineralization and less favorable for the growth of cariogenic bacteria.[18]

Furthermore, amino acids can also influence the composition and structure of the tooth's surface, leading to improved resistance against acidic challenges and promoting the formation of a protective layer that hinders further demineralization.[19] Understanding the specific mechanisms by which amino acids contribute to the remineralization process is essential for the development of targeted strategies for caries prevention and treatment. The initiation of hydroxyapatite (HA) nucleation primarily stems from a group of phosphorylated negatively charged non-collagenous proteins (NCPs) linked with the extracellular matrix (ECM). These proteins possess charged amino acid (AA) domains that attract calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions, thereby elevating the local level of supersaturation to a point where nuclei of critical size can form, subsequently facilitating the growth of HA crystals. Within the acidic domain of NCP, there is a notable prevalence of negatively charged AAs, such as aspartic acid

(Asp), glutamic acid (Glu), and phosphoserine (PSer) and they have been linked to HA mineralisation in bone and dentin. The presence of these AAs seems to be critical for interaction with both Ca^{2+} and PO_4^{3-} ions ultimately leading to HA precipitation. [20]
The present study aims to evaluate the effect of the l-glutamic acid, l-serine and l-aspartic acid in a remineralization solution on enamel specimens. The null hypothesis states that there is no significant increase in the calcium and phosphate content in amino acid treated specimens in comparison to the demineralised enamel.

2. MATERIALS AND METHODS

Sterilised Thomas PP narrow mouth bottles (Thomas Scientific, Malaysia) were used to prepare stock solutions of 0.1 M acetic acid, NaF, and l-glutamic acid, l-aspartic acid and l-serine.To prevent microbial growth, the bottles were sterilized using the standard autoclaving method at 121℃ and a pressure of 15 psi for 15 minutes.The demineralizing solution utilized was 0.1 M acetic acid adjusted to pH 4.0 by the addition of drops of 1 M NaOH, with pH calibration conducted using a pH meter. The phosphate buffer saline solution employed in the study was prepared according to the manufacturer's instructions (Oxoid, U.K), wherein one tablet of phosphate buffer saline was dissolved in 100 ml of deionized water and autoclaved at 121℃ and 15 psi pressure for about 10 minutes. This solution was stored at room temperature until needed, with its pH adjusted to 7.0, and served as the negative control. l-glutamic acid, l-aspartic acid, and l-serine were obtained from Sigma Aldrich, India. The base of the remineralizing solution used was 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl, and 1.0 mM NaN_3 in the prepared phosphate-buffered saline solution. The prepared amino acids were then added to the prepared remineralizing solution to obtain the final remineralization solution for each group.

Group 1: 100 µg/mL glutamic acid in 2 mL of the prepared base solution
Group 2: 100 µg/mL l-aspartic acid and 200 µg/mL l-serine in 2 mL of the prepared base solution.
Preparation of extracted teeth: After the ethical committee's approval, 15 extracted sound maxillary incisors were obtained after obtaining written consent from the patients. The teeth were stored in a phosphate-buffered saline solution after washing and the removal of periodontal tissues by scaling. The teeth were used without any prior treatment (demineralization or acid-etching). The teeth were then sectioned into standardized thin enamel slices of dimensions 5mm x 5 mm, and these slices were then used for the demineralization-remineralization study. All the 15 specimens each of enamel were immersed in a buffered demineralization solution (2.2 mN $CaCl_2.2H_2O$, 2.2 mM KH_2PO_4, 0.05 M acetic acid; pH adjusted to 4.5 with 10 M KOH)[21] for five days resulting in artificial caries-like lesions. Five demineralised specimens each of enamel respectively were then chosen as baseline samples for Scanning Electron Microscopy(SEM) and Energy dispersive X ray analysis (EDX). The remaining 10 samples each of enamel were again randomly divided into 2 groups and immersed in the respective remineralising solutions for 48 hours. The samples were then retrieved and subjected for SEM and EDX analysis post treatment. (Figure 1)

3. RESULTS

SEM Analysis

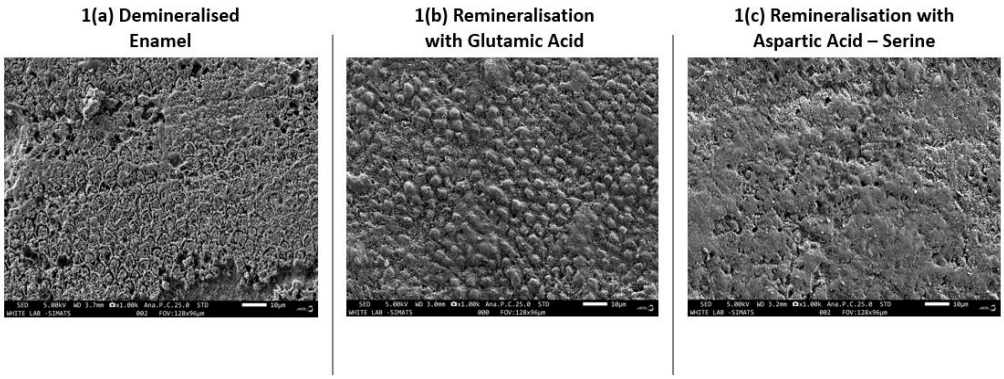
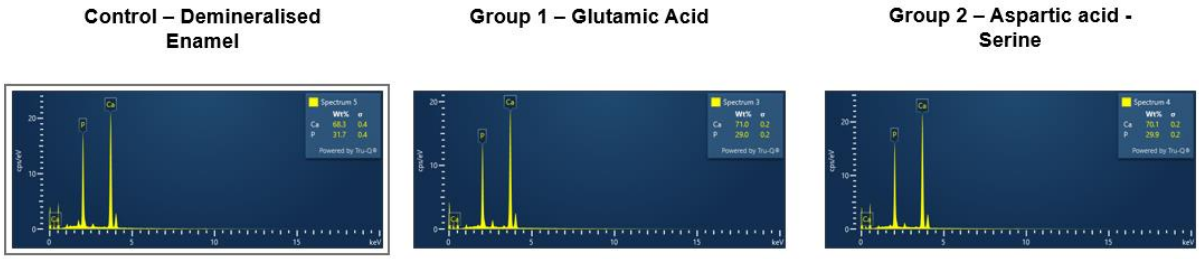


Figure 1 - Fig.1(a) represents the SEM image of enamel after demineralisation. Fig.1(b) represents the SEM image of remineralised enamel when subjected to Glutamic Acid. Fig.1(c) represents the SEM image of remineralised enamel when subjected to remineralisation with aspartic acid-serine combination.

The demineralized enamel showed prism structures with many larger pores, resembling fish scales. The glutamic acid group showed dense deposition of crystals in the demineralised enamel prism areas while the serine group showed an irregular occlusion of calcium and phosphate as supported by the EDX analysis. (Figure 2)

Figure 2: EDX Analysis



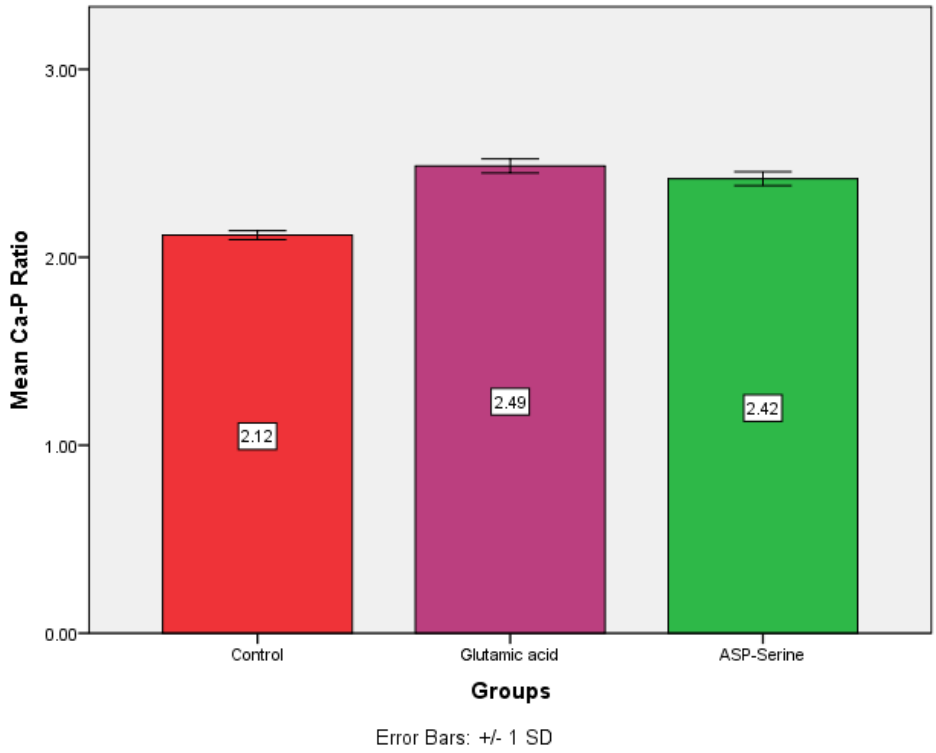
Statistical Analysis

SPSS software (IBM Corp, SPSS Inc, Chicago, IL, USA) version 23 was used for statistical data analysis. Mean Ca-P ratio in all groups was assessed. Normality was assessed using the Shapiro-Wilk test, and as the results were found to be parametric, the means were compared using one way ANOVA was applied at a 95% confidence interval. Post-hoc analysis was done using Tukey's test. (Table 1)

Groups	Mean Ca-P ratio	P value
Control	2.12 ± 0.581	Glutamic acid - p=0.000*
		Aspartic acid - Serine - p=0.000*
Glutamic acid	2.48 ± 0.037	Control - p=0.0008*
		Aspartic acid - Serine - p=0.019*

Aspartic acid - Serine	2.42 ± 0.037	Control - p=0.000*
		Glutamic acid - p=0.019*

Table 1 - Represents the mean Ca-P ratio in the enamel samples in all three groups.



Graph 1 - Represents the mean Ca-P Ratio in the enamel specimens after demineralisation, remineralisation with glutamic acid and remineralisation with Aspartic acid-Serine.
The results show significant difference in the Ca-P ratio in all three groups(p value < 0.05), with glutamic acid showing the highest Ca-P ratio, followed by Aspartic acid-Serine group.
Graph 1 Represents the mean Ca-P Ratio in the enamel specimens after demineralisation,

4. DISCUSSION

Researchers have delved into the impact of smaller biological molecules like amino acids and peptides (short amino acid chains) on the crystallization of hydroxyapatite, important for bone and dental health. Amino acids are fundamental components of proteins. Negatively charged ones such as aspartic acid, glutamic acid, and phosphoserine are notably abundant within acidic domains of non-collagenous proteins (like osteopontin, bone sialoprotein, dentin matrix protein 1, and dentin phosphophoryn) and play a part in HA formation in bones and teeth. Moreover, contemporary studies indicate that collagen's hole zones contain both positively and negatively charged amino acids, which facilitate HA formation within the fibrils by interacting with calcium and phosphate ions necessary for HA to precipitate. [20][22] Amino acids have been able to generate a crystal structure by altering the spatial structure, increasing the spatial effect, and decreasing the nucleation activation energy, thereby resulting in faster uptake of calcium and phosphate ions from the saliva. The salivary homeostatic mechanism, which involves statherin and proline-rich protein in normal calcium phosphate metabolism, preserves the integrity of HAP crystals, the most stable form of enamel under physiological conditions.[23]

Enamel contains trace amounts of organic macromolecules, which are rich in functional groups like -NH3+ cations and -COO− anions that can interact with inorganic ions, thus inducing the crystalline process.[24]

The difficulty in enamel remineralization stems from the fact that ameloblasts undergo apoptotic degeneration after deposition of the enamel matrix onto the predentin in the advanced bell stage of tooth formation. This acellularity makes it difficult for true enamel regeneration despite the availability of multiple remineralization agents like fluorides, CPP-ACP, etc.[25] The unique arrangement of crystals, like elongation in the c-axial direction, which are bundled together to form enamel prisms in high aspect ratio, gives enamel its superior strength, toughness and optical properties. [26] The enamel prisms are also geometrically aligned in an S-course from the dentin to the enamel surface. [27] Thus, there is a difficulty in synthesizing a material that can replicate such a well-aligned microstructure on a large scale. The crystal organization and control of mineralisation are done by enamel matrix proteins such as amelogenin, enamelin, etc. However, these proteins are difficult to extract and purify and also carry the risk of contamination and denaturation. Amelogenin is shown to constitute between 15-20% glutamic acid, and this brought about the idea of using amino acids and peptides in enamel remineralization. Previous literature has shown that the presence of l-glutamic acid in statherin plays a key role in attachment of statherin to enamel, thereby giving its protective effect.[28] Poly-gamma glutamic acid has been shown to inhibit the dissolution of hydroxyapatite crystals even more than salivary statherin.[29] The objective of our study was to see if demineralized enamel when coated directly in l-glutamic acid showed better uptake of salivary calcium and phosphate. 8DSS peptide, which contains a repeated sequence of aspartate and serine, has been shown to initiate and modulate the deposition of hydroxyapatite due to its high affinity to calcium as it has highly negatively charged ends. [30] It is said that the anionic ends of serine and aspartic acid mediate faster deposition of amorphous calcium phosphate from saliva.

Remineralization may not occur, or it may be limited, in the absence or scarcity of residual crystals. The mineral composition of the carious lesion's surface layer plays a significant role in determining the quality of the remineralization process, influencing factors such as its location within the lesion and the density of mineral deposition. The calcium phosphate ratio seen in sound enamel is 2.1 The present study was done with the objective of finding out if the amino acids by themselves can enhance remineralization. The results of the present study has shown an increase in the calcium content in both demineralised enamel in glutamic acid and in aspartic acid-serine groups. This rejects the null hypothesis and therefore supports that the presence of amino acids helps in faster adsorption and deposition of calcium ions onto demineralized enamel surfaces. The SEM results also clearly show amorphous deposition onto the enamel surface in both the groups, thus validating the increase in calcium content seen in EDX analysis. The effect of pH on the binding affinity of calcium to enamel surfaces treated with the amino acids, whether or not there is a synergistic effect of these amino acids with fluorides, and if the mineralization is comparable to that achieved by peptide are to be assessed further which can be considered as the limitation of the present study.

5. CONCLUSION

The presence of glutamic acids, aspartic acid-serine combinations help in better uptake of calcium ions in demineralised enamel. This brings great potential inorder to develop biomaterials for remineralization. Further the addition of these to conventional toothpastes could further enhance the remineralization potential.

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