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AGAROSE HYDROXYAPATITE COMPOSITE HYDROGEL FOR ENAMEL SURFACE BIOMIMETIC REMINERALIZATION (AN IN-VITRO STUDY)

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ABSTRACT

This study focused on developing a biomimetic agarose-hydroxyapatite (AG/HA) composite hydrogel and its evaluation for enamel repair efficacy. Sixty-four human molar slices were allocated into four groups: Control, Etched, AG hydrogel-treated, and AG/HA composite hydrogel-treated (n=8), with assessments at different intervals (4, 7, and 21 days). Enamel demineralization was induced using 37% phosphoric acid, followed by application of AG and AG/HA hydrogels. The remineralization outcomes were evaluated using energy dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), Vickers microhardness testing, and scanning electron microscopy (SEM). Statistical analysis was performed by one-way ANOVA and Tukey post hoc test. The findings revealed that the AG/HA significantly enhanced the calcium-to-phosphate (Ca/P) ratio and restored enamel hardness and crystal microstructure to levels comparable to control group, unlike the AG, which exhibited limited reparative effects. As a conclusion, AG/HA biomimetic hydrogels are effective in achieving rapid and sustained remineralization of enamel, closely replicating natural enamel structure.

KEYWORDS: Agarose hydrogels, Biomimetic enamel remineralization, Enamel demineralization, Hydroxyapatite.

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INTRODUCTION

Dental enamel is the highly mineralized outermost layer of the tooth, providing structural reinforcement against masticatory forces and serving as a critical barrier against biochemical and microbial challenges within the oral cavity. This mature enamel is a sophisticated composition primarily composed of inorganic substances (about 96% by weight), along with a minor portion of organic matter and water (about 4% by weight), devoid of any cells or collagen [1]. The principal mineral in enamel is hydroxyapatite (HA) with its chemical formula Ca10(PO4)6(OH)2 has garnered significant interest due to its close similarity in both chemical composition and crystal structure to the inorganic components found in natural bone and other hard tissues present in mammals [2].

Enamel remineralization process aims to replenish the lost ionic component of the enamel through various strategies depending on early detection. During a remineralization approach, inorganic calcium and phosphate are added from the external environment onto the demineralized enamel [3]. Throughout this dynamic process, if demineralization outweighs remineralization, it can lead to the formation of cavities and caries lesions. Before cavities form, white spot lesions emerge as the initial clinical indication of tooth decay, which can potentially be halted or reversed through remineralization. Thus, diagnosing these lesions is an attempt to remineralize it whenever possible rather than drilling a cavity in a carious tooth and restoring with artificial materials would be beneficial [4].

In recent years, caries research has increasingly emphasized the development of non-invasive approaches to manage early caries lesions by promoting remineralization to conserve tooth structure. Remineralization can naturally take place through saliva's buffering properties or be induced biologically using remineralizing agents [5].

Various types and concentrations of remineralizing agents containing fluoride, calcium, and phosphate ions are commercially accessible. These agents release active ions that form stable bonds with the crystalline enamel structures, leading to the creation of new crystals and the reconstruction of damaged ones. Fluoride ions are essential in inhibiting enamel demineralization, primarily by the formation of fluorapatite in enamel. This process occurs in the presence of calcium and phosphate ions, which are liberated during the demineralization of enamel by organic acids produced by cariogenic bacteria in dental plaque [6].

Enamel reconstruction by the biomimetic remineralization scheme imitates natural mineralization processes is a smart conservative approach for treatment of the early stage eroded enamel, white spots, and/or non-cavitated lesion as well as for the prevention of cavity progression. The biomimetic tooth repair methods were initially influenced by the successful creation of hydroxyapatite through biomineralization [7]. This process depends on an organic self-assembling matrix that directs enamel remineralization by forming scaffolds where HA crystals can nucleate and grow within a remineralizing environment, eventually creating structures similar to natural enamel. Various remineralizing agents, such as artificial and natural saliva, simulated body fluids, bioglass slurries, and hydroxyapatite, are commonly employed to restore the functionality of dental enamel [8].

It was reported that agarose can create an effective biomimetic microenvironment for human enamel and dentin remineralization, particularly in repairing non-carious enamel loss when applied as a covering with ionic and non-ionic agarose hydrogel. Agarose, a natural polysaccharide derived from red algae, is effective in forming thermo-reversible hydrogels that simulate organic gel matrices, facilitating the growth of prism-shaped HA crystals. Its numerous anionic hydroxyl (-OH) groups

enable agarose to act as an excellent scaffold in tissue engineering, promoting the self-assembly of HA nanocrystals into nanorod structures. Due to its superior biocompatibility, cost-effectiveness, and adaptable properties, agarose is well-suited for various biomedical applications. Although preliminary research shows promising potential for agarose hydrogels in human enamel remineralization, more comprehensive studies are necessitated to elucidate its biomimetic properties and clinical stability [9].

Building on previous findings, this study aimed to explore the potential of integrating agarose with hydroxyapatite to enhance the remineralization of human enamel. The research focused on developing a biomimetic agarose-hydroxyapatite (AG/HA) composite hydrogel and assessing its effectiveness in promoting enamel repair. The null hypothesis states that incorporating agarose with hydroxyapatite in the composite hydrogel has no significant effect on enamel remineralization or repair compared to the control.

MATERIALS AND METHODS

Teeth Slices Preparation

Sixteen extracted human permanent third molars were collected and visually inspected. Teeth with any visible caries, restorations, cracks, stains, or white spot lesions were excluded. (Molars extracted following the standard procedures for extractions at Faculty of Dentistry, Alexandria University). The teeth were thoroughly cleaned to remove debris, calculus, and soft tissues, then stored in 10% formalin until further use. The collected molars were disinfected by treating them with 3 wt% sodium chloride (NaClO) and phosphate-buffered saline (PBS) [10]. Prior to cutting, the teeth were embedded in methyl methacrylate resin blocks and mounted on a microtome equipped with a water-cooled diamond saw to be sectioned longitudinally. Four slices (1-1.5 mm thick) with an outer face of the tooth crown made of enamel were selected from each tooth. Two slices were taken buccolingually and the other two mesiodistally [9], resulting in a total of 64 slices. After cutting, the tooth slices were ultrasonically cleaned for 2 minutes, rinsed, and stored in deionized water.

Sample Size and Grouping

Sample size was calculated based on using Med Calc statistical software. The minimum sample size required was 64 teeth slices divided into 8 slices in each group as follows: Control group (n=8), Etched group (n=8), AG hydrogel treated enamel group (n=24), and AG/HA composite hydrogel treated enamel group (n=24). For both treated enamel groups (AG and AG/HA groups), teeth slices were randomly divided into (3) subgroups regarding follow up periods including 4, 7 and 21 days, (n=8 each).

Enamel Demineralization

Fifty-six of the prepared slices were designated to simulate carious lesions. These slices were acid-etched with 37% phosphoric acid for 60 seconds, then rinsed three times, each for 2 minutes, with a generous amount of deionized water in an ultrasound bath. At the end of the procedure, a white chalky lesion developed on the surface of the specimens, indicating early enamel caries. The teeth slices were then dried and stored at 4°C prior to remineralization[9].

Preparation and Characterization of Enamel Remineralization Regimens

Preparation of Agarose Hydrogel

A buffer solution was prepared, comprising 0.04 mol dm^-3 Tris (hydroxy methyl amino methane) (Oxford Co, Maharashtra**,** India) and 0.02 mol dm^-3 acetic acid, adjusted to pH 8 at 25°C. Subsequently, a 1% agarose (CD Bioparticles, NY, USA) was prepared using the buffer solution (w/v%) and moulded using a gel tray [11].

Preparation of Agarose\Hydroxyapatite (AG/HA) Composite Hydrogel

Agarose hydrogel was prepared as mentioned previously. Next, twelve wells were cast on both the positive and negative sides of the 1% agarose gel. On the positive side, 20μl of CaCl2 (Alfa Aeser, Massachusetts, USA) solution (0.04 cm^3 of 0.2 mol dm^-3) were loaded into each well. Conversely, on the negative side, 20μl of Na2HPO4 (Alfa Aeser, Massachusetts, USA) solution (0.04 cm^3 of 0.12 mol dm^-3) were loaded into each well of the gel. The gel was submerged in the buffer solution, and a potential of 100 V was applied at room temperature in the electrophoresis apparatus for 45 minutes [11]. Following electrophoresis, the AG/HA composite hydrogel was immersed in a disodium hydrogen phosphate aqueous solution to neutralize the inner pH (8), and then in physiological saline to remove any Ca and P ions in solution. The resulting AG and AG/HA composite hydrogels were filled into syringes, ready for injection [12].

The produced AG and AG/HA composite hydrogels were laboratory characterized for phase identification with X-ray Diffraction (XRD) (Bruker D2 PHASER diffractometer, Cambridge, UK) used at 30 KV and 10mA, using a Cu tube (λ = 1.54 Å) within a 2 Theta (2θ) range of 5 to 80°. The hydrogel was poured into a well-plate, frozen at -80°C for 2 hours. Following freezing, the hydrogel was subjected to lyophilization using a LY-10N freeze dryer (Taisite) at a consistent temperature of -80°C and a vacuum pressure of 0.3 Pa for 24 hours.

Biomimetic Enamel Remineralization Protocol

The treated enamel groups, AG and AG/HA composite hydrogels underwent biomimetic remineralization using hydrogel-based double-diffusion systems (DDS) and artificial saliva (AS). Each demineralized enamel slice was first coated with a 1M CaCl2 solution for 15 minutes, then immediately covered with a second layer of either AG or AG/HA composite hydrogel and left to react for 2 hours in a dryer at 37°C [9].

The dried tooth slices were placed in a vial containing 30 ml of artificial saliva (AS) to promote biomimetic growth of enamel-like structures at 37°C. They were then incubated for follow-up periods of 4, 7, and 21 days. After each immersion period, the tooth slices were removed, lightly rinsed with deionized water, and air-dried to prepare them for laboratory characterization.

In Vitro Assessment

In vitro evaluation was performed for control, etched and biomimetic treated enamel groups (AG and AG/HA). The chemical composition of HA and the Ca/P ratio of the samples were analysed using Energy Dispersive X-ray Spectroscopy (EDX). (JEOL JSM-IT200 instrument Tokyo, Japan). Crystal phase microstructure was conducted by XRD, operating at 30 KV and 10mA, equipped with a Cu tube (λ = 1.54 Å). The analysis covered a 2 Theta (2θ) range from 5 to 800°C. Microhardness evaluation was conducted using a Vickers microhardness tester (HMV-2000, Shimadzu, Japan) equipped with a diamond indenter shaped as a right pyramid with a square base and an angle of 136 degrees between opposite faces. The device was calibrated to apply a load of 1 kg to each specimen. Specimens were examined for surface morphology using Scanning Electron Microscopy (SEM) (JEOL JSM-IT200, Tokyo, Japan) operating at 20 KV. The enamel slices were first ultrasonically cleaned for 30 seconds and then sputter-coated with gold using ion sputtering device. For both treated enamel groups (AG and AG/HA), the in vitro evaluation was conducted over three follow-up periods at 4, 7, and 21 days.

Ethical Approval

The ethics committee of the Faculty of Dentistry at Alexandria University, Egypt, reviewed and approved the ethical clearance application for this research in accordance with the national regulations and ethics code (IORG0008839 - IRB 00010556). The ethical approval for this study is identified by the code: 0976-0912024.

Statistical Analysis

The data underwent analysis using SPSS software. For quantitative data, descriptive statistics such as range (maximum and minimum), mean, and standard deviation were calculated. T-tests were used for intergroup comparisons, with significance set at the 5% level. Additionally, one-way ANOVA was performed to compare multiple groups.

RESULTS

The XRD pattern of the prepared AG/HA composite hydrogel is shown in figure (1). The XRD spectrum of the AG/HA composite hydrogel, following freeze-drying, revealed the organized alignment of crystalline domains. Prominent diffraction peaks were observed corresponding to the (002), (211), (112), (300), and (202) crystallographic planes at 2θ values of 25.7°, 31.8°, 32.2°, 32.9°, and 34.7°, respectively, indicating the characteristic structural features of hydroxyapatite.

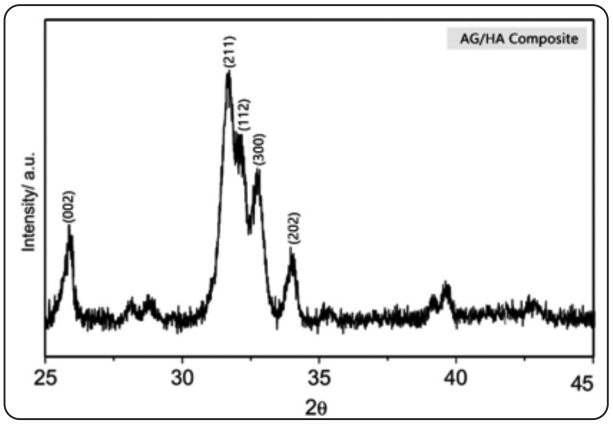

Fig. (1) XRD pattern of the prepared AG/ HA composite hydrogel

The EDX analysis, including the Ca/P ratios for the control, etched, and biomimetic enamel-treated groups (AG and AG/HA), is presented in Tables 1, 2, and 3. The control group exhibited a mean Ca/P ratio of 1.67, while the etched group showed a significantly lower mean Ca/P ratio of 1.31 (P = 0.0012). As detailed in Table 1, the AG-treated group demonstrated mean Ca/P ratios of 1.34, 1.36, and 1.38 at 4, 7, and 21 days of follow-up, respectively. Compared to the control group, the AG-treated group showed a statistically significant decrease in the mean Ca/P ratio at all three follow-up periods: 4 days (P =0.001*), 7 days (P =0.0023*), and 21 days (P =0.0017*). However, no statistically significant differences were observed between the AG-treated and etched groups at the 4-day (P =0.082) and 7-day (P =0.061) follow-up points. In contrast, a significant difference emerged between the AG-treated and etched groups after 21 days (P =0.031).

TABLE (1) Comparison of Ca/P ratios measured by EDX analysis for the AG group relative to the control and etched groups at 4, 7, and 21 days.

	Agarose Group N=24			**Etched Group (n=8)**	**Control Group (n=8)**
	AG-4 Day (n=8)	**AG-7 Day (n=8)**	**AG-21 Day (n=8)**		
Range	1.3-1.37	1.31-1.38	1.36-1.39	1.3-1.34	1.63-1.67
Mean	1.34	1.36	1.38	1.31	1.67
SD	0.02	0.03	0.01	0.02	0.02
ANOVA			18.98		
P value			0.001*		
P (Control # other groups)	0.001*	0.0023*	0.0017*	0.0012*	
P (Etched # other groups)	0.082 N.S.	0.061 N.S.	0.031*		

P was significant if ≤ 0.05 ***N.S. = Not significant*** **** Significant difference***

A comparison of the mean Ca/P ratio for the AG/HA-treated group with the control and etched groups is shown in Table 2. The AG/HA-treated group displayed mean Ca/P ratios of 1.67, 1.66, and 1.68 at 4, 7, and 21 days of follow-up, respectively. No statistically significant differences in the Ca/P ratio were found when compared to the control group at any follow-up period, including 4 days (P =0.685), 7 days (P =0.744), and 21 days (P =0.705). Conversely, a comparison with the etched group revealed a statistically significant increase in the mean Ca/P ratio for the AG/HA-treated group at all three follow-up periods: 4 days (P =0.001), 7 days (P =0.0016), and 21 days (P =0.0013).

In comparing the mean Ca/P ratios between the two biomimetic enamel-treated groups (AG and AG/HA) over the 4, 7, and 21-day follow-up periods, the results consistently showed a statistically significant increase in the mean Ca/P ratio for the AG/HA-treated group compared to the AG-treated group at all time points: 4 days (P =0.0021), 7 days (P =0.0016), and 21 days (P =0.0042), as presented in Table 3.

TABLE (2) Comparison of Ca/P ratios measured by EDX analysis for the AG/HA group relative to the control and etched groups at 4, 7, and 21 days.

	Agarose/HA Composite Group (N=24)			**Etched Group (n=8)**	**Control Group (n=8)**
	AG/HA-4 Day (n=8)	**AG/HA-7 Day (n=8)**	**AG/HA-21 Day (n=8)**		
Range	1.62-1.68	1.64-1.69	1.65-1.69	1.3-1.34	1.63-1.67
Mean	1.67	1.66	1.68	1.31	1.67
SD	0.02	0.02	0.02	0.02	0.02
ANOVA			17.58		
P value			0.001*		
P (Control # other groups)	0.685 N.S.	0.744 N.S.	0.705 N.S.	0.001*	
P (Etched # other groups)	0.001*	0.0016*	0.0013*		

P was significant if ≤ 0.05 ***N.S. = Not significant*** **** Significant difference***

Table (3): Comparison of EDX-measured Ca/P ratios between the AG and AG/HA biomimetic enamel-treated groups at 4, 7, and 21 days.

	Agarose Group	**Agarose/HA Composite Group**	**t-test - *P* value**
4-Days			
Range	1.3-1.37	1.62-1.68	
Mean	1.34	1.67	4.21
SD	0.02	0.02	0.0021*
7-Days			
Range	1.31-1.38	1.64-1.69	
Mean	1.36	1.66	3.96
SD	0.03	0.02	0.0016*
21-Days			
Range	1.36-1.39	1.65-1.69	
Mean	1.38	1.68	4.06
SD	0.01	0.02	0.0042*

The crystal phase microstructure assessment of control, etched, and biomimetic treated enamel surfaces with AG and AG/HA hydrogels was detected by XRD patterns as shown in figure (2: A-D). The control enamel surfaces distinctly exhibit the characteristic peaks of natural enamel HA minerals at 2θ values of 25.7°, 29.3°, 31.8°, 32.19°, 33.31°, 34.7°, 49.5°, and 52.82°, corresponding to the (002), (210), (211), (112), (300), (202), (213), and (004) crystal planes, respectively, as detailed in JCPDS no. 09-0432, (Figure 2-A). In contrast, etched enamel surfaces lack the characteristic HA diffraction patterns, indicating significant mineral loss and disruption of the crystalline structure in the artificially demineralized enamel, (Figure 2-B). The XRD patterns of AG-treated enamel surfaces at 4, 7, and 21 days display a consistent absence of the characteristic (002) peak and show reduced sharpness and intensity in the other peaks. The diffraction lines of the newly formed layer are significantly broader compared to normal enamel, and closely resemble those of the etched surface, (Figure 2-C). Contraries, the XRD pattern of enamel surfaces treated with AG/HA composite hydrogel at 4 days follow up period shows significantly sharper and more intense characteristic peaks, particularly the (002) peak. This indicates that the HA crystals are well-formed and properly aligned along their C-axis. The peaks at 2θ = 32.19, 33.31, and 34.7 are notably sharper and more defined, reflecting improved crystallinity following biomimetic remineralization. Furthermore, the same XRD patterns were observed after the 7- and 21-day follow-up treatment periods, (Figure 2-D).

The Vickers microhardness mean values are detailed in Tables 4-6. The control group exhibited a microhardness of 351.3, while the etched group showed a significant decrease to 261.0 (P<0.0001). For enamel surfaces treated with AG hydrogel, as outlined in Table 4, the mean microhardness values were 262.2 at 4 days, 265.3 at 7 days, and 284.4 at 21 days. Compared to the etched group, no significant difference was observed at 4 days (P=0.99) or 7 days (P=0.96), whereas the increase at 21 days was statistically significant (P=0.007). All time points in the AG group displayed a significant difference compared to the control group (P<0.0001). For surfaces treated with AG/HA, as shown in Table 5, the mean microhardness values were 347.7, 348.8, and 350.1 at the 4-, 7-, and 21-day marks, respectively. These values were not significantly different from the control at 4 days (P=0.94), 7 days (P=0.98), and 21 days (P=0.99), but there was a significant difference compared to the etched group at all time points (P<0.0001). Table 6 shows that the mean microhardness values revealed a significant difference between the AG/HA group and the AG group at all time points (P<0.0001).

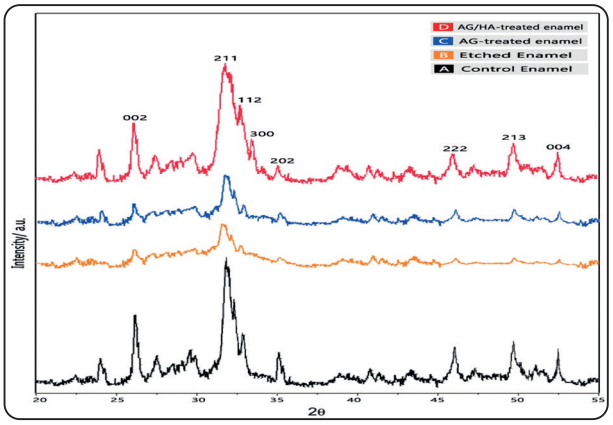

Fig. (2): (A-D) XRD patterns of control enamel surfaces (A), etched enamel surfaces (B), AG treated enamel surfaces (C), and AG/HA-treated enamel surfaces (D).

Scanning Electron Microscopy (SEM) analysis enables the evaluation of the morphology of both control and demineralized enamel surfaces, as shown in Figures 3 and 4, respectively. Furthermore, the characteristics observed after biomimetic remineralization treatment achieved by applying biomimetic remineralizing hydrogels (AG and AG/HA) over periods of 4, 7, and 21 days are illustrated in Figures (5: A-C and 6: A-C). SEM images at 1000x magnification were obtained for control, etched, and both biomimetically treated enamel surfaces.

TABLE (4) Comparison of Vickers microhardness values for the AG group against the control and etched groups at 4, 7, and 21 days.

	Agarose Group (N=24)			**Etched Group (n=8)**	**Control Group (n=8)**
	AG-4 Day (n=8)	**AG-7 Day (n=8)**	**AG-21 Day (n=8)**		
Range	239.0-279.2	241.1-282.2	270.7-300.1	235.0-281.3	343.0-357.4
Mean	262.2	265.3	284.4	261.0	351.3
SD	14.4	13.3	9.8	17.6	6.4
ANOVA			12.9		
P value			<0.0001*		
P (Control # other groups)	<0.0001*	<0.0001*	<0.0001*	<0.0001*	
P (Etched # other groups)	0.99 N.S.	0.96 N.S.	0.007*		

P was significant if ≤ 0.05 *N.S. = Not significant* ** Significant difference*

TABLE (5) Comparison of Vickers microhardness values for the AG/HA group relative to the control and etched groups at 4, 7, and 21 days.

	Agarose/HA Composite Group (N=24)			**Etched Group (n=8)**	**Control Group (n=8)**
	AG/HA-4 Day (n=8)	**AG/HA-7 Day (n=8)**	**AG/HA-21 Days (n=8)**		
Range	334.5-354.4	337.2-356.4	340.0-357.3	235.0-281.3	343.0-357.4
Mean	347.7	348.8	350.1	261.0	351.3
SD	7.0	6.9	6.1	17.6	6.4
ANOVA			9.8		
P value			<0.0001*		
P (Control # other groups)	0.94 N.S.	0.98 N.S.	0.99 N.S.	<0.0001*	
P (Etched # other groups)	<0.0001*	<0.0001*	<0.0001*		

P was significant if ≤ 0.05 *N.S. = Not significant* ** Significant difference*

TABLE (6) Comparison of Vickers microhardness values between the AG and AG/HA biomimetic enamel-treated groups at 4, 7, and 21 days.

	Agarose Group	**Agarose/HA Composite Group**	**t-test *P* value**
4-Days			
Range	239.0-279.2	334.5-354.4	5.65
Mean	262.2	347.7	<0.0001*
SD	14.4	7.0	
7-Days			
Range	241.1-282.2	337.2-356.4	5.32
Mean	265.3	348.8	<0.0001*
SD	13.3	6.9	
21-Days			
Range	270.7-300.1	340.0-357.3	4.08
Mean	284.4	350.1	<0.0001*
SD	9.8	6.1	

The SEM images of control enamel revealed normal featured represented by a uniform, smooth surface with little to no pits or scratches, as demonstrated in Figure 3. In contrast, the demineralization process using 37% phosphoric acid resulted in a loss of surface integrity, characterized by an irregular rod-end structure and increased porosity. Additionally, fragmented and discontinuous enamel crystals were observed, as shown in Figure 4.

For enamel surfaces treated with AG hydrogel, the SEM images continued to display micropores, cavities, and a honeycomb-like structure. The surfaces showed substantial roughness with numerous interspaces and frequent porosities. The SEM images of the AG group indicated damage to the enamel crystalline structure that the gel did not repair following 4- and 7-days. However, it was noteworthy that some of the pores and cavities became occluded after a 21-day follow-up, as shown in Figure (5: A-C).

Regarding the AG/HA biomimetic-treated enamel surfaces, the SEM images revealed significant and rapid morphological changes within the first 4 days, which continued over the 7- and 21-day follow-up periods. These alterations reflect effective repair of cavities and defects, leading to smoother and more uniform enamel surfaces. The previously porous areas were fully concealed, and the crystals appeared to cluster together to form a continuous apatite layer on the demineralized surface. This provides clear evidence of mineral deposition attached to and integrated within the lesion, as shown in Figure (6: A-C).

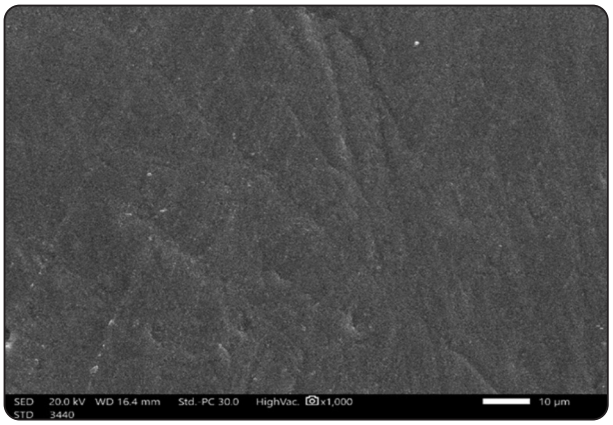

Fig. (3) SEM image (1000x) of the control enamel surface showing its typical characteristics, including a uniform and smooth surface with minimal imperfections.

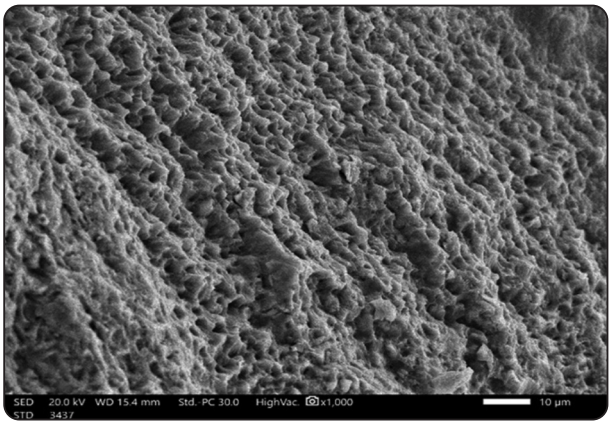

Fig. (4) SEM image (1000x) of the etched enamel surface, demonstrating a loss of surface integrity with an irregular rod-end structure and increased porosity.

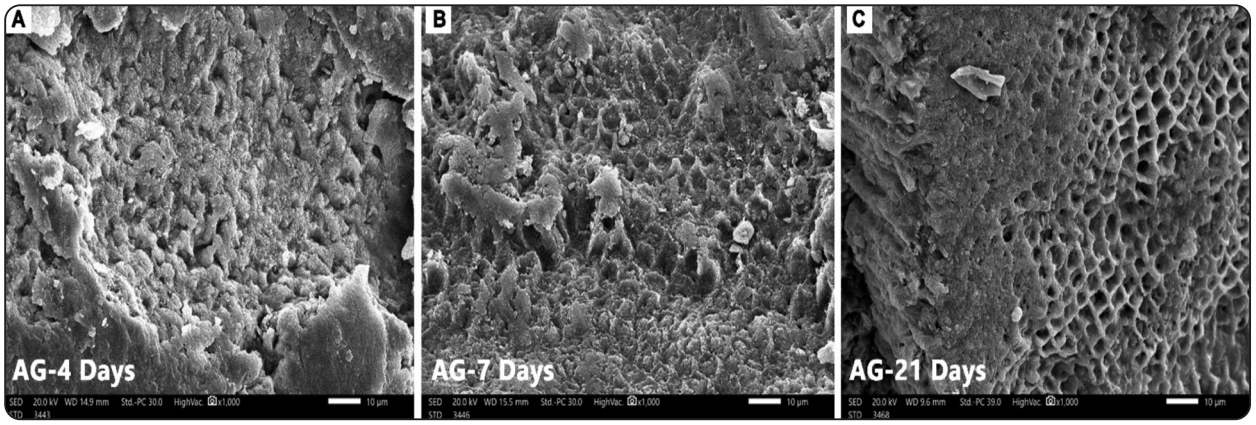

Fig. (5) (A-C) SEM images (1000x) of AG-treated enamel surfaces at 4, 7, and 21 days. The images display persistent micropores, cavities, and a honeycomb-like structure. Damage to the enamel crystalline structure was evident at 4 and 7 days, with partial occlusion of pores and cavities by 21 days.

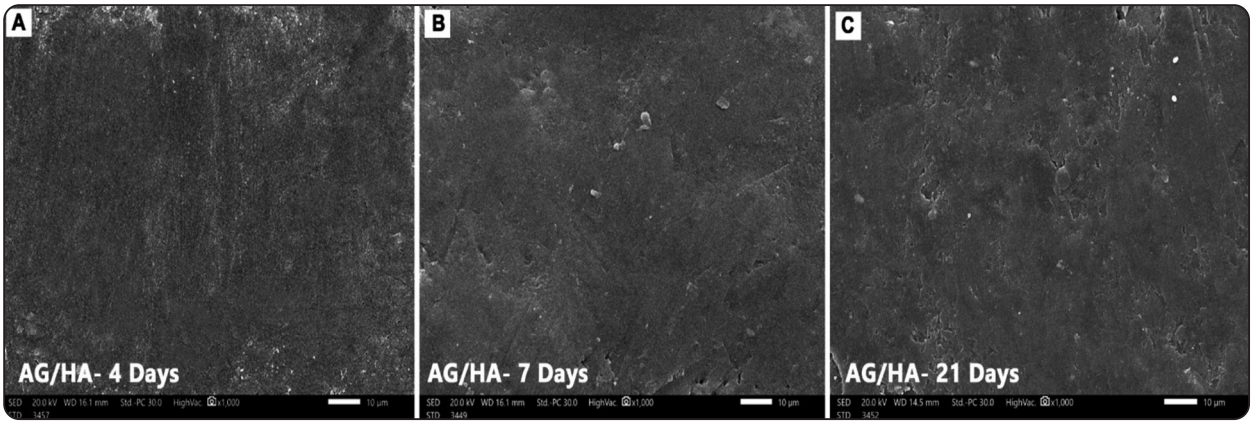

Fig. (6) (A-C) SEM images (1000x) of enamel surfaces treated with AG/HA biomimetic hydrogel at 4, 7, and 21 days. The images show significant and speedy morphological differences after 4 days of treatment, including effective repair of cavities and defects. These improved features persist through the 7- and 21-day intervals, leading to surfaces that are progressively more homogeneous and smoother. The porous regions are effectively occluded, with a continuous apatite layer forming across the entire treated enamel surface.

DISCUSSION

Enamel demineralization, a common dental issue impacting both the health and appearance of patients, is a concern for both dental professionals and individuals. The primary chemical component of enamel, hydroxyapatite, is susceptible to dissolution when exposed to acid, leading to enamel demineralization [13]. The pathogenetic mechanism of dental enamel demineralization undoubtedly requires the presence of a structurally organized dental biofilm, which converts food glucose into acidic products [14].

Dental caries is conventionally managed using restorative materials like resin-based composites. However, these restorative approaches are regarded as invasive techniques that can compromise the integrity of the natural tooth structure. Consequently, non-invasive treatment modalities are being explored as preferable alternatives to traditional restorative methods [15].

Biomimicry is an interdisciplinary field that emulates nature's optimal biological methods and strategies using principles from chemistry, physics, mathematics, and engineering to create innovative synthetic materials and organs. Human enamel and dentin are formed through a process regulated by an organic matrix. Inspired by this natural mechanism, a biomimetic, cell-free strategy for dental tissue regeneration has been developed. This approach uses the gel-like organic matrix as a template to control mineral crystallization via molecular interactions between polymers and minerals. In this system, ions or clusters bind to the organic surface, forming amorphous particles that subsequently organize and promote crystallization [16].

Agarose, a natural polysaccharide composed of repeating d-galactose and 3,6-anhydro l-galactose units, functions as an organic matrix template for biomimetic mineralization. It forms agarose fibre-nanoscale-amorphous calcium phosphate complexes that act as precursors for mineralization. The agarose hydrogel serves as a reservoir for these mineral precursors, with its confined network ensuring uniform and controlled complex sizes. This leads to the aggregation of amorphous particles, resulting in the calcification of demineralized collagen fibrils and the occlusion of dentinal tubules [9].

Hydroxyapatite (HA) is the most stable calcium phosphate at physiological pH and is widely used for its ability to promote cell adhesion and proliferation, but it is brittle and has low porosity. Mineralizing agarose hydrogels with HA improves

its mechanical properties and wettability. Various in situ mineralization methods are used to create more homogeneous HA-containing hydrogels. One common method is the wet alternate soaking process, which involves repeated immersion of thin hydrogels in calcium and phosphate solutions; however, this method is time-consuming and limited to thin gels. Another method precipitates HA by adding calcium solution dropwise or adjusting the pH within an agarose solution containing phosphate. This approach is faster but can be more complex and may lack control over the calcium phosphate phase or complicate the incorporation of active ingredients. On the contrary, electrophoresis is used to enhance ion transport through the gel, speeding up HA formation and allowing directed ion migration [17,18].

Building on prior findings, this study sought to develop an agarose-hydroxyapatite (AG/HA) composite hydrogel using electrophoresis and to assess its biomimetic remineralization potential in comparison to agarose hydrogel.

The XRD pattern of the AG/HA composite hydrogel indicated a high presence of HA (100%). This result is consistent with earlier studies demonstrating successful AG/HA composite formation using electrophoresis[11). The in-vitro analysis conducted in this study demonstrated significant variations among the subgroups treated with remineralizing agents, leading to the rejection of the null hypothesis. The Ca/P ratios of AG group were like etched values, indicating minimal or no remineralization. Significant differences between etched and AG groups were observed at 21 days, supporting previous findings that AG hydrogel remineralizing effect improves over time [11]. While the Ca/P ratios of the AG/HA remineralized surfaces were comparable to those of natural enamel surfaces at 4-,7- ad 21 days follow up. These results indicate that HA was successfully formed on the treated enamel surfaces, demonstrating the effectiveness of the biomimetic remineralization achieved with the AG/HA composite hydrogel regimen.

Similarly, in this study, XRD was utilized to examine normal enamel, etched enamel, and both remineralized groups over three follow-up periods. Sharp, prominent HA peaks were observed in the AG/HA group, closely resembling natural enamel, which indicates successful remineralization. Additionally, the (002) peak was intensified, demonstrating the 'preferred orientation' of crystals, which is a characteristic crystallographic alignment within normal enamel where crystals align vertically along the c-axis. These findings are supported by the Vickers microhardness analysis, which demonstrated that the AG/HA group achieved a full reversal of demineralization, restoring the microhardness of natural enamel as early as 4 days and maintaining it throughout the follow-up period up to 21 days [19].

All previous laboratory results regarding elemental composition, crystal microstructure, and microhardness were corroborated by SEM analysis, which revealed the morphology of normal and demineralized enamel, as well as the changes observed after treatment with AG and AG/HA composites hydrogels. AG treatment resulted in numerous porosities and damage to the enamel crystalline structure that was not fully repaired. By 21 days, some pores and cavities were occluded. Conversely, AG/HA treatment achieved uniform coverage of cavities and defects, smoothing the surface and filling gaps between enamel HA crystals. The AG/HA composite hydrogel effectively covered and integrated mineral aggregates into the lesions, resulting in a homogenous surface with minimal porosity.

Subsequently, the in vitro assessment results of the current study disclosed that the AG/HA composite hydrogel demonstrated a rapid and pronounced biomimetic remineralization capability within 4 days post-treatment. The newly formed mineralized layer exhibited substantial stability, persisting even after exposure to rinsing, ultrasonic agitation for 7 days, and throughout the 21-day observation period. These results align with Chris Ying CAO and Quaun-Li's research which showed dentin remineralization in an AG hydrogel microenvironment using a

two-layer hydrogel approach for creating enamel prism-like tissue. XRD analyses confirmed that the AG/HA composite's remineralization closely resembled normal enamel, with sharp peaks indicative of successful remineralization [20]. A similar conclusion was reached in the study by El-Bedewy et al., which evaluated the regenerative efficiency of different agarose-based hydrogels. The addition of EMD to the agarose hydrogel led to the formation of regenerated crystals and the complete masking of dental erosion [21].

CONCLUSION

Within the limitation of this study, it could be concluded that AG/HA biomimetic hydrogels are effective in remineralizing demineralized enamel and mimicking natural enamel structures. Subsequently, The AG/HA composite hydrogel emerges as a powerful tool for non-invasive enamel repair and caries prevention.

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AUTHOR CONTRIBUTIONS

Abeer El Manhaly contributed to the conceptualization, methodology, investigation, and resource acquisition. Abdel Ghaffar Magraby was responsible for methodology and validation. Wagih Abdel Alim handled validation and formal analysis. Sherif Kandil provided conceptualization and supervision. Seham Hanafy contributed to conceptualization and validation. Dawlat Mostafa was involved in conceptualization, methodology, investigation, data curation, and visualization. Abeer El Manhaly and Dawlat Mostafa prepared the original draft, while all authors participated in reviewing and editing the manuscript.

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

REFERENCES

1. Mansoor A, Moeen F, Hussain A, Din SU, Khan MT, Said F. Age related changes in physiology of normal human tooth enamel: A review. Pak J Physiol. 2020;16:35-40.
2. Maqbool M. Substituted hydroxyapatites for antibacterial applications (Doctoral dissertation, Friedrich-Alexander-Universitaet Erlangen-Nuernberg (Germany)). 2021.
3. Ramadoss R, Padmanaban R, Subramanian B. Role of bio-glass in enamel remineralization: Existing strategies and future prospects-A narrative review. J Biomed Mater Res B Appl Biomater. 2022;110:45-66. doi: 10.1002/jbm.b.34904.
4. Mohabatpour F, Chen X, Papagerakis S, Papagerakis P. Novel trends, challenges and new perspectives for enamel repair and regeneration to treat dental defects. Biomater Sci. 2022 14;10:3062-3087. doi: 10.1039/d2bm00072e.
5. Orsini G, Tosco V, Monterubbianesi R, Orilisi G, Putignano A. A new era in restorative dentistry. In: Longhi S, Monteriù A, Freddi A, Aquilanti L, Ceravolo MG, Carnevali O, et al (eds). The First Outstanding 50 Years of "Università Politecnica delle Marche" Research Achievements in Life Sciences.1st ed. Springer. 2020:319-34.
6. Vitiello F, Tosco V, Monterubbianesi R, Orilisi G, Gatto ML, Sparabombe S, Memé L, Mengucci P, Putignano A, Orsini G. Remineralization efficacy of four remineralizing agents on artificial enamel lesions: SEM-EDS investigation. Materials (Basel). 2022;15:4398. doi: 10.3390/ma15134398.
7. Baioumy YM. Deciphering pH Effects on the Peptide-guided Nucleation and Growth of Hydroxyapatite and Peptide-Ion Interactions for Dentin Hypersensitivity Treatment. Thesis (Master's). University of Washington; 2020.
8. Arifa MK, Ephraim R, Rajamani T. Recent advances in dental hard tissue remineralization: a review of literature. Int J Clin Pediatr Dent. 2019;12:139-44. doi: 10.5005/jp-journals-10005-1603.
9. Muşat V, Anghel EM, Zaharia A, Atkinson I, Mocioiu OC, Buşilă M, Alexandru P. A chitosan–agarose polysaccharide-based hydrogel for biomimetic remineralization of dental enamel. Biomolecules. 202;11(8):1137. doi: 10.3390/biom11081137.
10. Coelho A, Amaro I, Apolónio A, Paula A, Saraiva J, Ferreira MM, Marto CM, Carrilho E. Effect of cavity disinfectants on adhesion to primary teeth—a systematic review. Int J Mol Sci. 2021;22(9):4398. doi: 10.3390/ijms22094398.

11. Kimura K, Kamitakahara M, Yokoi T, Ioku K. Formation process of hydroxyapatite granules in agarose hydrogel by electrophoresis. Cryst Growth Des. 2018;18:1961-6. doi: 10.1021/acs.cgd.7b01154.

12. Watanabe J, Kashii M, Hirao M, Oka K, Sugamoto K, Yoshikawa H, Akashi M. Quick-forming hydroxyapatite/agarose gel composites induce bone regeneration. J Biomed Mater Res A. 2007;83:845-52. doi: 10.1002/jbm.a.31435.

13. Yan J, Yang H, Luo T, Hua F, He H. Application of amorphous calcium phosphate agents in the prevention and treatment of enamel demineralization. Front Bioeng Biotechnol. 2022;10:853436. doi: 10.3389/fbioe.2022.853436.

14. Tasios T, Papageorgiou SN, Papadopoulos MA, Tsapas A, Haidich AB. Prevention of orthodontic enamel demineralization: a systematic review with meta-analyses. Orthod Craniofac Res. 2019;22:225-235. doi: 10.1111/ocr.12322.

15. Malcangi G, Patano A, Morolla R, De Santis M, Piras F, Settanni V, Mancini A, Di Venere D, Inchingolo F, Inchingolo AD, Dipalma G. Analysis of dental enamel remineralization: a systematic review of technique comparisons. Bioengineering (Basel). 2023;10:472. doi: 10.3390/bioengineering10040472.

16. Dawasaz AA, Togoo RA, Mahmood Z, Ahmad A, Thirumulu Ponnuraj K. Remineralization of dentinal lesions using biomimetic agents: a systematic review and meta-analysis. Biomimetics (Basel). 2023;8:159. doi: 10.3390/biomimetics8020159.

17. Witzler M, Ottensmeyer PF, Gericke M, Heinze T, Tobiasch E, Schulze M. Non-cytotoxic agarose/hydroxyapatite composite scaffolds for drug release. Int J Mol Sci. 2019;20:3565. doi: 10.3390/ijms20143565.

18. Cao Y, Mei ML, Li QL, Lo EC, Chu CH. Agarose hydrogel biomimetic mineralization model for the regeneration of enamel prismlike tissue. ACS Appl Mater Interfaces. 2014;6:410-20. doi: 10.1021/am4044823.

19. Wu X, Xu Q, Li Q, Cao Y, Chu CH. Remineralization of Dentin in hydrogel microenvirment aided by Electrophoresis. Journal of Dental Research. 2013.

20. Cao CY, Li QL. Scanning electron microscopic analysis of using agarose hydrogel microenvironment to create enamel prism-like tissue on dentine surface. J Dent. 2016;55:54-60. doi: 10.1016/j.jdent.2016.09.015.

21. El-Bedewy DA, Hussein AM, Rashed AA, Zayed MM. Enamel Prism-Like Tissue Regeneration Using Different Agarose-Based Hydrogels as Preventive Treatment Approaches for Dentin Erosion. Egypt Dent J. 2024 Apr 1;70:2047-64. doi: 10.21608/EDJ.2024.265135.29