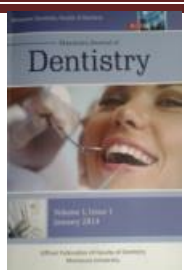

Chemical Stability of the Biomineralized Initial Enamel Caries

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Abstract:

Key words: Initial enamel caries like lesions, Biomimetic mineralization, agarose hydrogel mineralization system, Scanning Electron Microscopy [SEM]-Energy Dispersive X-ray [EDAX], surface microhardness.

Purpose: To evaluate mineral content, microhardness, and microstructure of the enamel like structure using agarose hydrogel system and its stability under further acid attack.

Materials & Methods: Twenty-four human 1st premolars were collected. 3 × 3 mm enamel windows were created in the middle third of the buccal surfaces of all teeth on which initial enamel caries like lesions were created . These were treated with agarose hydrogel system. Twelve specimens were analyzed with EDAX and enamel microhardness was measured throughout the study steps. The enamel microstructure was examined under SEM for the remaining specimens.

Results: ANOVA test revealed a highly statistically significant difference (p=0.000) among the mean wt. % values of (Ca), (P) content, (Ca/P) ratio and VHN values recorded in all the study steps.

The statistical correlation between the wt. % of Ca & micro hardness values revealed a significant positive relation in all study steps ($P \leq 0.05$) except after further acid attack, where no significant relation was found ($p = 0.07$). While the correlation between the (P) wt. % & micro hardness revealed a significant positive relation in sound and untreated enamel ($p \leq 0.05$), and no significant relation was found in the treated enamel and that subjected to further acid attack ($p \geq 0.05$). The statistical correlation between the Ca/P ratio & micro hardness was a significant positive relation ($p \leq 0.05$) in all study steps. SEM findings supported the previous results.

Conclusions: The tested agarose hydrogel mineralization system promoted an in vitro biomimetic mineralization and enamel prism like tissue formation on the induced enamel caries like lesions However, it was unstable under further acid attack.

Introduction

Dental enamel is a highly mineralized tissue made up of approximately 95% substituted hydroxyapatite, 4% water, and 1% organic macromolecules.[1] It consists of nanorod-like hydroxyapatite (HA) crystals arranged into well-organized microarchitectural units called enamel prisms,[2] which protects teeth from fractures and acid attack.[3] Oral cavity has a dynamic environment; if conditions are favorable both remineralization and demineralization can occur simultaneously.[4]

The enamel demineralization process begins when these acids lower the pH of to less than 5.5 (critical pH). A white spot lesion is the initial detectable evidence of enamel demineralization in the subsurface region of the tooth and is characterized by low calcium and phosphate content. This will progress into frank cavitation if the bacterial colony is not timely removed from the tooth surface.[5]

The non-invasive management of incipient lesion could be performed by remineralization. Calcium and phosphate in the saliva and plaque permit the recovery of some lost enamel mineral content. Remineralization can thus negate the need for invasive dental treatment modalities. Several remineralizing agents can diffuse or deliver calcium and phosphate ions into the subsurface.[6] Oral healthcare products containing fluoride or casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) were reported as effective in remineralizing enamel but none of these commercially available products have the potential to promote the formation of organized apatite crystals. [7]

In clinical dentistry, there is a challenge to design and fabricate biomaterials that can mimic the tooth both in form and function.[8] Biomimetics is the study of formation, structure or function of biologically produced substances and materials (such as silk or conch shells) and biological mechanisms and processes (such as protein synthesis or mineralization) for the purpose of synthesizing similar products by artificial mechanisms that mimic natural structures.[9]

Agarose hydrogel biomimetic mineralization system was developed by loading agarose (natural polysaccharide) with calcium and phosphate ions to act as an organic matrix template for biomimetic mineralization[10] and was reported to successfully induce enamel-like tissue without using enamel protein on the surface of the enamel and dentin under laboratory condition. It was reported that the modulus of elasticity and the nano hardness of the enamel prism like tissue were similar to those of natural enamel[11], however its stability to further acidic challenge was not tested. Additionally, the composition of dental enamel, in particular its mineral content, is an important factor to withstand such attacks.[12]

Therefore, the microstructure and mineral content of biomineralized lesions after an acid impact is worth to study.

Materials and Methods:

Twenty-four sound, freshly extracted human 1st premolars were collected, cleaned from debris, and soft tissue, disinfected with 3% sodium hypochlorite, rinsed with phosphate-buffered saline and stored in saline in an incubator at 37°C till the time of the experiment for a maximum of one month. Modeling wax pieces measured 3 × 3 mm were placed in the middle third of the buccal surfaces of all teeth. All around the crown surfaces of the teeth were coated with two layers of a transparent acid resistant nail varnish; the wax was then removed exposing 3 × 3 mm enamel windows. To create initial enamel caries like lesions, specimens were immersed separately in test tubes containing 10 mL of demineralizing solution (2.2mMol calcium chloride, 2.2mMol sodium phosphate, 0.05M acetic acid ,1M potassium hydroxide) (pH 4.2) at 37 °C for 96 hours.[13] These were treated with agarose hydrogel mineralization system. The teeth were placed in blocks of red compound, fresh mix of Ca Cl_2 hydrogel was first applied to the prepared windows on the enamel surfaces using plastic syringe and kept for approximately 4 hours till gelation using celluloid crowns covering the buccal and occlusal surfaces. A layer of Na_2HPO_4 hydrogel was then added carefully. The combined hydrogels were kept on the enamel surfaces for 4 hours and kept for the rest of day in artificial saliva. This procedure was repeated three times weekly (every other day) for 4 weeks (representing 48 hours of material application).

Twelve specimens were analyzed with EDAX for their calcium (Ca) & phosphorus (P) content and microhardness of the enamel surface was measured with Vickers hardness tester using vertical load of 300g for 10 seconds through the sequential steps of the study. The (Ca) & (P) wt. % values as well as the Ca/P ratio were collected, tabulated, and statistically analyzed using (SPSS version 20). While the enamel surface topography of the remaining twelve specimens was evaluated under SEM throughout the different testing steps (n=3/each step) (Table2).

Results:

High (Ca) wt. % value (61.95) was recorded in sound enamel, this was reduced to (56.03) in the untreated enamel lesions, when treated with agarose hydrogel it was increased to (59.57), while after further acid attack it was reduced to (54.62). Similarly, high (P) (wt.%) value (32.40) was recorded in sound enamel, reduced to (30.18) in the untreated enamel, increased to (30.53) after treatment, while after further acid attack it was reduced to (28.78). Considering the (Ca/P) ratio, it was (1.91) in sound enamel, reduced to (1.85) in the untreated enamel, increased to (1.94) after treatment while it was reduced to (1.89) after acid attack .Similar findings were found regarding the microhardness, high value (394.58) was found in sound enamel , reduced to (272.67) in the untreated enamel, while after treatment it was increased to (320) while it was reduced to (236) after further acid attack .

ANOVA test revealed a highly statistically significant difference (p=0.000) among the mean wt. % values of (Ca), (P) content &Ca/P ratio recorded in the different steps of the study. Also a highly statistically significant difference (p=0.000) among the mean VHN value was recorded. The statistical correlation between the% of Ca & micro hardness of enamel using Pearson correlation test revealed a highly significant positive relation ($p \leq 0.000$) in all study steps except in the specimens subjected to demineralization after treatment where no significant relation was found ($p = 0.070$). While the statistical correlation between the(P) % & micro hardness revealed a highly significant positive relation ($p \leq 0.000$) in sound and demineralized enamel with no significant relation between them in the agarose treated enamel and that subjected to further demineralization ($p \geq 0.05$). The statistical correlation between the Ca/P ratio & micro hardness revealed a significant positive relation between them in all study steps ($p \leq 0.000$). SEM findings supported the previous results revealing the clear formed hexagonal rod like parallel apatite crystals that was unstable after further demineralization.

Discussion:

One alternative strategy to overcome the challenges of conventional treatments of initial enamel lesions is to regrow an enamel-like layer directly onto the original enamel surface. This provides a tight chemical contact to the natural substrate utilizing the on-trend tissue engineering concept to regenerate rather than repair.[14]

Numerous attempts had been made to prepare enamel-like materials using biomimetic systems that contain nano-apatite or different organic materials used as analogues to organic matrix mainly in the form of slurries, solutions, or pastes.[15]

Agarose hydrogel was used to mimic the gel-like organic matrix environment to induce enamel-like tissue. Several studies concluded that agarose hydrogel biomimetic material acted as enamel organic matrix to control the size and form of the formed hydroxyapatite crystals through the interaction between hydroxyl group of agarose and calcium. In addition, it acts as a mineral reservoir for continuing remineralization.[11]

The strength and consistency of the hydrogel can be adjusted by varying the concentration and molecular weight of the agarose making it easy to transfer to clinical use and this was done in the current study. The transportation of ions through the organic matrix and the interactions between the ions and the organic matrix are crucial in the regulation of the enamel-mineralization process.[16]Fluoride was also reported to have an effect on crystal growth and was found to be enhance remineralization.-3[17] So, about 500 ppm fluoride was incorporated to Na_2HPO_4 agarose hydrogel.[11]

The topography of the enamel surface was examined using SEM, while the EDAX analysis was carried out to provide accurate quantitative analysis for Ca and P content of the investigated enamel. In addition, enamel microhardness was measured using Vickers hardness tester with a vertical load of 300g for 10 seconds.[18]

In general, the results of this study came in agreement with others.[10] SEM images of sound enamel revealed a smooth aprismatic layer, this finding was supported by the EDAX analysis and microhardness test showing high Ca, P, Ca/P

ratio and microhardness values. This could be explained by the unique physico-chemical properties of sound enamel due to its high content in hydroxyapatite, the parallel arrangement of individual elongated apatite crystals into enamel prisms, and the interwoven alignment of perpendicular prisms in a picket-fence resembling three-dimensional order. Together, these characteristics result in a biomaterial of great hardness and physical resilience.[19-23]

Regarding the untreated enamel surfaces, SEM images revealed rough porous enamel surface (Figure.V-6A, B) that could be explained by superficial dissolving of the surface enamel (the glassy outer shell) and the removal of the inorganic components from the enamel. This was supported by Kamath et al[24] reporting that the demineralization of enamel leads to dissolution of HA and diffusion of Ca/P ions toward the enamel surface creating a subsurface demineralization of approximately 150 µ width with an intact surface simulating an early enamel lesion. The concentration of both Ca and P in the demineralization solution was at 50% of saturation level, causing dissolution of only enamel subsurface This came in agreement with those reported in previous studies[10,11,15,25]and was supported by the current EDAX analysis recording a significant decrease in Ca and P content after demineralization. In addition, the present decreased enamel microhardness came in agreement with the study conducted by Lata et al [26]reporting that the surface microhardness values of the enamel specimens were decreased after demineralization.

Treatment of the induced initial carious enamel surfaces with agarose hydrogel for 96 hours presented enamel prism-like tissue with hexagonal rod like parallel apatite crystals. This came in agreement with others [11,27]reporting that agarose hydrogel acted as enamel organic matrix to control the size and form of the formed hydroxyapatite crystals through the interaction between hydroxyl group of agarose and calcium. In addition, it acts as a mineral reservoir for continuing remineralization.[11]While the present EDAX analysis revealed a significant increase in Ca value denoting precipitation of minerals on the enamel surface. Also microhardness of enamel was increased after treatment of the induced initial enamel caries like lesions but was still lower than that of sound enamel. These finding came in agreement with other study reporting that the lower microhardness than sound enamel could be attributed to incomplete compaction of the formed crystals on enamel surface.[28,29]

During treatment with agarose hydrogel system the mineralizing precursor of agarose fiber−mineral complex was formed. The organic matrix can prevent premature crystal−crystal fusion, control the subsequent phase transformations, and control the nucleation and growth of the crystals. The mineral precursors in the hydrogel were very small and less readily crystallized.

This may have contributed to the strong attractive interaction between the agarose polymer and the inorganic surface, which can in turn arrest nucleation and change the shape and size of the primary clusters.[11] Furthermore biomineralization process is an organic matrix particle-mediated nonclassical crystallization pathway.[10]The organic matrix controls the mineral crystallites through the

molecular interaction between the polymer and minerals with a sequestering mechanism.[30]The amorphous primary particles that are formed by ion or cluster binding at the organic surface can undergo coupled matrix-mediated mesophase transformations, resulting in oriented crystallization with iso-oriented mosaic textures.[31] When the currently treated enamel surface was subjected to further acid attack, SEM images revealed further loss of enamel prisms forming porous enamel structure while under high magnifications some specimens revealed partial removal of the performed enamel structure leaving a non-uniform enamel surface **,** and was supported by the EDAX results recording a reduction in the Ca & P content as well as reduced microhardness values (Tables V.1,3,5).This may be due to further dissolving of the newly formed enamel like prisms and the removal of the inorganic components , denoting that the formed enamel like structure is unstable,

On the other hand Zhou et al [32] disagreed with the current results reporting a high chemical stability of the regenerated enamel apatite structure .This may be due to their utilized different techniques where they proposed a novel way to chemically synthesize the enamel structure by mimicking the interaction of (ACP) and acidic proteins during the formation of natural enamel and infiltrated the newly formed enamel apatite structure with dental resin instead of protein found in normal enamel in order to enhance its chemical stability which resulted in a regenerated artificial composite enamel with higher stability against acid erosion.

Also Wang et al .[33] disagreed with the current findings where they reported a facile way to prepare cross-arranged enamel-like apatite (HA and fluorine-substituted HA (FHA) with both prismatic and interprismatic structure by using natural enamel as a template to serve as both mineral deposition substrate and template-directing reagent and found that the newly grown enamel-like layer (FHA) was more resistant to acid etching.

This confliction states that the acid-resistance of the newly formed enamel like structure using the tested agarose hydrogel needs further studies under different application times or further additions.

Conclusions:

Under the limitations of this in-vitro study, the following could be concluded:

- Agarose hydrogel mineralization system have a remineralizing potential to treat initial enamel caries like lesions.
- The formed enamel like structure is not stable under further acid attack.

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Table1: The experimental design of the study:

Tests / steps	Microstructure SEM	Microhardness &Mineral content
Sound enamel	(n=3)	(n=12)
Untreated enamel lesion	(n=3)	
Treated enamel lesion	(n=3)	
acid attack after treatment	(n=3)	
Total number	24	

Table 2: Statistical analysis of the mean (Ca) values (wt.%) throughout the steps of the study.

Mineral content / Steps	(Ca) value (wt.%)			
	Mean± S.D	Min – Max	ANOVA test	
			F	P-value
Sound enamel	61.95±1.56	59.43—64.20	296.573	0.000**
Untreated enamel lesion	56.03±1.58	53.80—58.30		
Treated enamel	59.57±1.45	57.60—61.70		
Acid attack after treatment	54.62±1.35	52.60—57.20		

Table 3 : Statistical analysis of the mean (wt.%) values of (P) content throughout the steps of the study.

Mineral content / Steps	(P) values (wt.%)			
	Mean±S.D	Min – Max	ANOVA test	
			F	P-value
Sound enamel	32.40±1.09	30.63—34.38	62.842	0.000**
Untreated enamel lesion	30.18±1.13	28.50—31.95		
Treated enamel	30.53±1.01	29.20—32		
Acid attack after treatment	28.78±1.23	27—30.70		

Table 4: Statistical analysis of the mean (wt.%) values of (Ca/P) content throughout the steps of the study.

Mineral content / Steps	(Ca/P) ratio values			
	Mean±S.D	Min – Max	ANOVA test	
			F	P-value
Sound enamel	1.91±0.04	1.85—1.97	9.210	0.001*
Untreated enamel lesion	1.85±0.03	1.80—1.90		
Treated enamel	1.94±0.05	1.83—2.02		
Acid attack after treatment	1.89±0.06	1.79—1.97		

Table 5 : Statistical analysis of (VHN) throughout the steps of the study.

Mineral content / Steps	(VHN) value			
	Mean±S.D	Min – Max	ANOVA test	
			F	P-value
Sound enamel	394.58±11.96	382—421	254.283	0.000**
Untreated enamel lesion	272.67±9.77	255—290		
Treated enamel	320.50±11.47	303—347		
Acid attack after treatment	236±13.54	218--268		

Table 6: Statistical correlation between Ca% & enamel micro hardness

Steps	R	p-value
Sound enamel	0.909	0.000**
Untreated enamel lesion	0.819	0.001*
Treated enamel	0.669	0.012*
Acid attack after treatment	0.530	0.070

Table 7 : Statistical correlation between (P) wt.% & enamel micro hardness

Steps	r	p-value
Sound enamel	0.848	0.000**
Untreated enamel lesion	0.742	0.006*
Treated enamel	0.517	0.085
Acid attack after treatment	0.549	0.065

Table 8 : Statistical correlation between Ca/P ratio & enamel micro hardness

Steps	r	p-value
Sound enamel	0.993	0.000**
Untreated enamel lesion	0.860	0.000**
Treated enamel	0.811	0.001*
Acid attack after treatment	0.734	0.007*

Figure 1: Bar chart showing the mean (Ca) values (wt.%) throughout the steps of the study.

Figure 2 : Bar chart showing the mean) P(values (wt.%) throughout the steps of the study.

Figure 3 : Bar chart showing the mean (wt.%) values of (Ca/P) content throughout the steps of the study.

Figure 4 : Bar chart showing the mean (VHN) throughout the steps of the study.

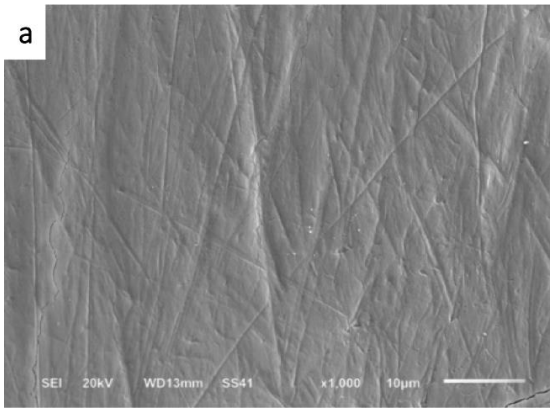

Figure 5: SEM micrograph showing mature sound enamel surface with a smooth aprismatic layer at (X1000).

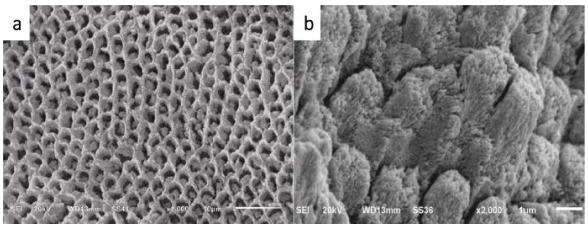

Figure 6 A: SEM micrograph of untreated enamel surface (X1000) showing obvious dissolution of elements of enamel crystals.

Figure 6 B: SEM micrograph of untreated enamel surface (X2000) showing dissolution of the interprismatic substance.

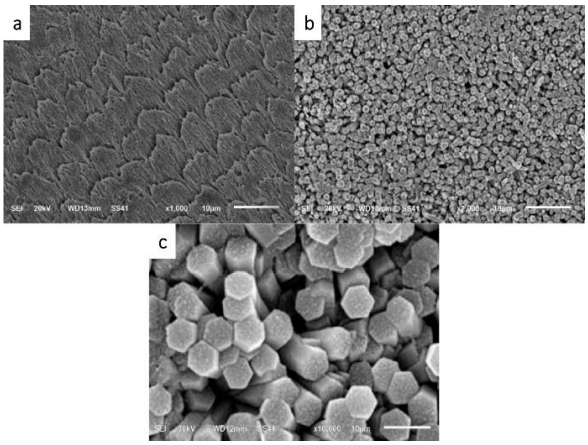

Figure 7 A: SEM micrograph of treated enamel surface (X1000) showing enamel prism-like tissue.

Figure 7 B: SEM micrograph of treated enamel surface (X2000) showing enamel prism-like tissue.

Figure 7 C: SEM micrograph of treated enamel surface (X10000) showing hexagonal rod like parallel apatite crystals .

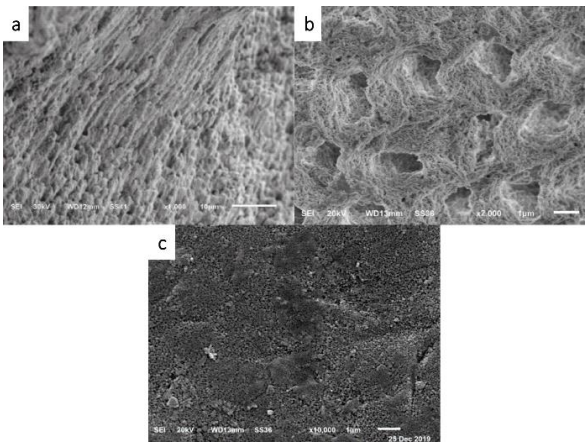

Figure 8 A: Scanning electron micrograph of enamel specimen exposed to further acid attack (X1000) showing porous enamel structure.

Figure 8 B: Scanning electron micrograph of enamel specimen exposed to further acid attack(X2000) showing further loss of enamel prisms forming porous enamel structure.

Figure 8 C: Scanning electron micrograph of enamel specimen exposed to further acid attack (X10,000) showing partial removal of performed enamel structure leaving a non-uniform surface of enamel (arrows showing remaining performed enamel like structure).

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