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Effect of Sodium Hypochlorite (NaOCl) for Pretreatment of Early Demineralized Enamel Lesions in Enhancing the Remineralization Capacity of Self-assembling Peptide (In-vitro Study)

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ABSTRACT

Background: Deproteinizing enamel with sodium hypochlorite proved its positive effect when applied before one of the recent remineralizing agents (self-assembling peptide) which is considered an ideal enamel biomimetic approach. ***Objectives:*** To investigate the effect of sodium hypochlorite on the remineralizing capacity of the self-assembling peptide when applied before versus after acid etching of enamel; and the ability of the self-assembling peptide to sustain the remineralizing effect. ***Materials and Methods:*** Artificial enamel lesions were created on the proximal surfaces of 64 sample. Specimens were randomly put into two groups (N=32) according to NaOCl sequence of application (before/after acid etching). Group S1 (NaOCl applied before acid etching as recommended by the manufacturer); Group S2 (NaOCl applied after acid etching). After that, enamel samples were subjected to a pH-cycling for 1 month, followed by 3 months. Surface microhardness (SMH) was assessed at baseline, after demineralization, after remineralization, after pH-cycling for 1 month, and 3 months. SMH values were analyzed using ANOVA, the percentage of change of SMH was calculated based on the baseline SMH; the lower the value of the percentage of change the better the remineralizing capacity. ***Results:*** Upon comparing the values of percentage of change of microhardness at different timings we found that the lowest statistically significant percentage of change belonged to group S2. ***Conclusion:*** The sequence of NaOCl application had a statistically significant effect when applied after acid etching on the remineralization capacity of self-assembling peptide during different times of measuring leading to better re-hardening action and sustainable remineralizing action.

Keywords: Remineralization, self-assembling, peptide, deproteinization, microhardness.

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INTRODUCTION

Caries is reckoned as a continuous dynamic process.[1] Enamel caries always starts through a process of subsurface demineralization leaving a microporous surface of lost minerals in-between the hydroxyapatite crystallites.[2,3] Hence, emerged the concept of minimally invasive dentistry as a very conservative way of intervention against enamel demineralization and any further breakdown where we can treat affected enamel lesions as early as possible.[2,4] Remineralization process takes place when the supersaturated saliva redeposits the lost minerals back to fill the micropores, or through different external approaches, techniques, and agents for remineralization.[4,5] As time passes by, our knowledge regarding the different ways of remineralization have improved; leading us to a better understanding of the concepts of biomimetic regeneration and the more recent technologies that goes on the other side from fluoride-mediated remineralization.[4] The most recent remineralizing technology is the self-assembling peptide P11-4 which is considered as an ideal enamel regenerative approach. Its composition has a great effect on calcium ions affinity, grabbing these ions and depositing them on a de novo needle-shaped hydroxyapatite mesh work leading to better in-depth penetration remineralization of demineralized lesions. This new approach presents the natural regeneration process of the lost enamel tissue (biomimetic remineralization).[6-8]

Analysis of many in-vitro studies and data regarding the action of self-assembling peptide showed that the presence of P11-4 fibers in the depth of the lesions led to faster hydroxyapatite deposition and formation, with great increase in microhardness of the remineralized subsurface lesions.[9-11] P11-4 has shown very optimistic results as a biomimetic mineralizing agent in in-vivo studies and clinical trials. This includes the ability to reverse early occlusal and proximal lesions that are more resistant to fluoride remineralization.[12-16] Its mode of application as stated by the manufacturer is to first ensure a clean enamel surface, then start the surface pretreatment by applying sodium hypochlorite (3%) followed by acid etching with phosphoric acid gel (35-37%). Pretreating enamel surface with sodium hypochlorite prior to acid etching acts as a deproteinizing agent removing the organic elements and the acquired salivary pellicle from the surface; this significantly increases enamel's surface retention up to 94.47% and enhances penetrative depths, also improves

the quality of type I and II etched enamel patterns.[17,18]

The idea of deproteinizing enamel with 5.25% NaOCl before acid etching with phosphoric acid (H_3PO_4) and its influence on the patterns of etching and bonding of the adhesives to tooth structure has been investigated.[17,18] On the other side, the application of sodium hypochlorite after acid etching during the process of surface pretreatment has been discussed in literature proving its effect in enhancing the shear bond strength values, increasing the penetration and retention of adhesive resins by 20.1% compared to the conventional way of only acid etch the enamel surface, and affects the surface roughness of enamel surface with the minimum percentage of surface loss.[19-22]

Upon all these findings regarding using sodium hypochlorite as an enamel surface pretreatment and self-assembling peptide remineralization capabilities, it was found thought-provoking to study the effect of applying sodium hypochlorite as a deproteinizing agent after acid etching of demineralized sound enamel structure with the aim of minimizing loss of surface enamel, exposure of more reactive enamel, and creation of porosities that may allow for more rapid uptake of remineralizing solution as assessed by surface microhardness (SMH) testing.

Literature regarding the use of sodium hypochlorite after acid etching in enamel remineralization as a surface pretreatment for increased penetration were found to be deficient, and further studies are found to be in need.[19-22]

METHODOLOGY

A total of 32 sound extracted maxillary and mandibular human molars were collected from "The National Institute of Diabetes and Endocrinology" in Cairo, Egypt; they were extracted mainly from diabetic patients for clinical indications, and patients were informed that their teeth will be used in research purposes; approval of the Research Ethics Committee was granted, with ethical issue approval number of (MIU-IRB-1718-053). The selected teeth were washed under running water and cleaned from any debris and attached soft tissue, then autoclaved at Misr International University's tooth bank at 121°C under 15 psi pressure for 15 minutes, and immersed in distilled water which was daily renewed to prevent their dehydration until being tested.[23]

The collected molars were decoronated to facilitate the work on their crowns; decoronation was done under the cementoenamel junction by approximately 2

mm and perpendicular to the long axis using a water-cooled Sectioning IsoCut Wafering Blade mounted on IsoMet 4000 linear sawing (Buehler, Illinois, USA). After that, crowns were divided longitudinally in a buccolingual direction into two halves (mesial half and distal half) using a Sectioning IsoCut Wafering Blade mounted on IsoMet 4000 linear sawing (Buehler, Illinois, USA) to get a total of 64 enamel samples. Each tooth has been considered a control for itself; and a window preparation was done on each sample to localize the demineralized working test area.

Each mesial and distal half of a tooth was embedded in pink self-cured acrylic resin cylinders with their surfaces facing upward, exposed and parallel to the horizontal plane slightly above the acrylic resin surface by 2 mm, Figure (1).

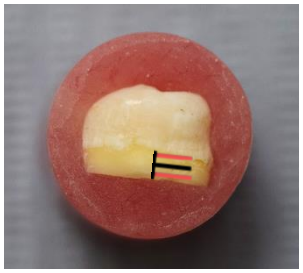

Figure (1): Tooth Decoronated under Cementoenamel Junction by 2 mm, and Embedded in Pink Self-cured Acrylic Resin Cylinder

Teeth surfaces were slightly flattened and polished to create flat parallel enamel surfaces, using OptiDisc Finishing and Polishing System (Kerr Corporation, California, USA) on low speed, the discs were sequentially used, to aid in the window preparation procedures and to facilitate for SMH measurements.[24]

After samples were prepared, a 4 × 5 mm window of exposed enamel was created in the middle of the sample surface by using adhesive paper, and the sample was rendered resistant to acid attack by applying a double uniform coats of nail varnish,[24] Figure (2).

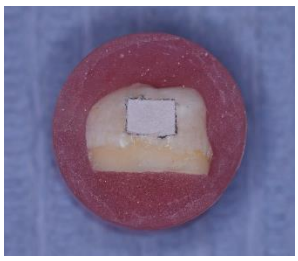

Figure (2): A 4 × 5 mm Window using Adhesive Paper

Baseline surface microhardness (B-SMH) was measured with Vickers Wilson Tukon 1102 Hardness Testing Machine (Buehler, Illinois, USA), with a Vickers diamond indenter, Figure (3).

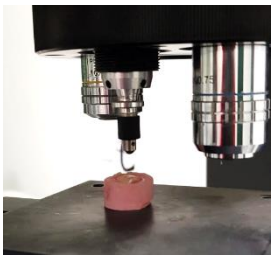

Figure (3): Vickers Diamond Indenter in-action

The (100g) load was applied smoothly without impact, forcing the indenter into the

working window test area of the specimen oriented perpendicularly to the enamel surface. The indenter is held in place for (10 seconds). The Vickers hardness (HV) is calculated using this equation:[25] **HV = 1854.4L/d^2.**

All readings were performed by the same examiner using the same calibrated machine. In each reading, three indentations were made and their average was taken to represent each specimen's hardness value.[24,25]

In order to produce visualized uniform artificial white spot lesions, all enamel samples were individually immersed in the demineralizing solution in room temperature for 96 consecutive hours (4 days), where the lesions were daily checked and the solutions were daily renewed. After lesion formation, all enamel samples were carefully rinsed under running water; and surface microhardness values (After D) were checked as done for B-SMH.[26]

All of the 64 enamel samples were randomly divided into two main groups of 32 specimens each, based on the sequence of NaOCl application as a deproteinizing agent whether before or after acid etching during the process of Regenamel Curodont Repair^{TM} (self-assembling peptide) remineralizing agent application:

1) **Group (S1):** NaOCl is applied before acid etching as recommended by the manufacturer (Control Group).
2) **Group (S2):** NaOCl is applied after acid etching application and surface rinsing and drying (Test Group).

The process of materials application with the different sequences of NaOCl application were as follows:

- Drying the working window area with air from the air/water syringe.

For the first group (S1) where NaOCl is applied before acid etching (according to manufacturer's instructions):

- Application of 3% sodium hypochlorite with a cotton bud, keeping the surface wet, Figure (4).

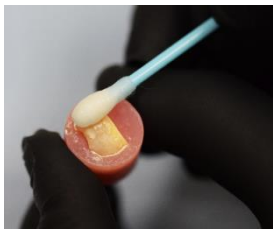

Figure (4): Application of 3% Sodium Hypochlorite with a Cotton Bud

- Water rinse and air dry the window area.
- Application of 37% phosphoric acid etching with a syringe directly on the window area and keep it work for 20 seconds, Figure (5).
- Water rinse the etching gel for 20 seconds to ensure its complete removal and then air dry the window area.

For the second group (S2) where NaOCl is applied after acid etching:

- Application of 37% phosphoric acid etching with a syringe directly on the window area and keep it work for 20 seconds, Figure (5).

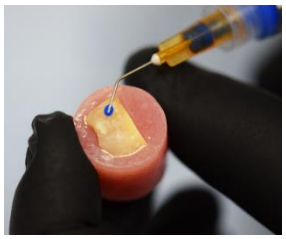

Figure (5): Application of 37% Phosphoric Acid Etch with a Syringe

- Water rinse the etching gel for 20 seconds to ensure its complete removal and then air dry the window area.
- Application of 3% sodium hypochlorite with a cotton bud, keeping the surface wet, Figure (4).
- Water rinse and air dry the window area.
- Application of Regenamel Curodont Repair™ solution through pushing the two parts of its plastic applicator for the applicator attached sponge to be soaked in the solution, Figure (6), and then rubbing it against the working window area for a minute, Figure (7), keeping it wet and let it diffuse deep into the lesion for five minutes according to manufacturer's instructions followed by an after remineralization SMH test.

Each enamel sample was pH-cycled separately in a separate container and passed through the alternating phases of demineralization and remineralization; after each phase enamel samples were rinsed thoroughly under running deionized water and were put in the next phase.

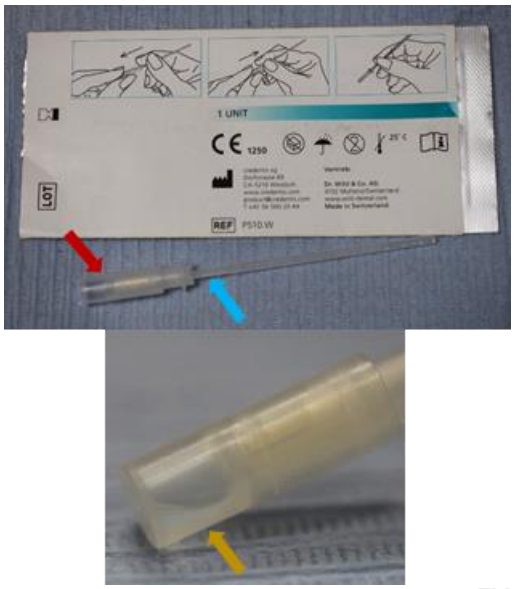

Figure (6): Regenamel Curodont Repair™ Applicator (Red Arrow: The Part that Contains the Solution. Blue Arrow: The Part being pushed where the Sponge is attached. Golden Arrow: Self-assembling Peptide Solution)

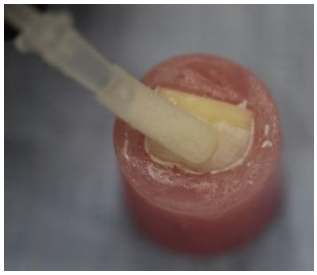

Figure (7): Regenamel Curodont Repair™ Applicator Sponge Application

The pH-cycling step was conducted at room temperature and samples were

occasionally gently rotated to allow an artificial pellicle-like layer to form. The treatment schedule was followed daily for one month for the first SMH measurement, followed by another pH-cycling sequence for three consecutive months and a final SMH measurements were taken, Table (1).

Each enamel specimen was assessed three times post treatment application in the following times:

A) After remineralization. (After R)

B) After pH-cycling for 1 month/ 30 days. (After pH/1)

C) After pH-cycling for 3 months/ 90 days. (After pH/3)

(The total SMH testing times for each enamel sample were five times)

Table (1): pH-cycling Daily Treatment Sequence of the Study

Time	***pH-cycling Phases***
4 Hours (10 a.m. – 2 p.m.)	Demineralization in Demineralizing Solution (Acid Challenge)
6 Hours (2 p.m. – 8 p.m.)	Remineralization in Artificial Saliva
4 Hours (8 p.m. – 12 a.m.)	Demineralization in Demineralizing Solution (Acid Challenge)
10 Hours (12 a.m. – 10 a.m.)	Remineralization in Artificial Saliva

RESULTS

Statistical Analysis:

The percentage of change of SMH was calculated according to the equation below based on the baseline SMH as the positive control to represent the remineralizing capacity of the tested material in the different test periods. The lower the value of the percentage of change the better the remineralizing capacity.

$$\frac{\text{Baseline SMH} - \text{After demineralization}}{\text{After remineralization} - \text{After demineralization}} \times 100 = \text{Percentage of SMH changes}$$

The mean and standard deviation values were calculated for each group in each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests, data showed parametric (normal) distribution.

Repeated measure Analysis of Variance (ANOVA) was used. Paired sample t-test was used to compare between two groups in related samples. Independent sample t-test was used to compare between two groups in non-related samples.

Three-way ANOVA tests were used to test the interactions between different variables. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® (IBM® Corporation, NY, USA)

SPSS® (SPSS® Inc., an IBM Company) Statistics Version 20 for Windows.

1) Effect of NaOCl Sequence of Application:

Data in Table (2), and Figure (8) shows that when comparing the two groups S1 and S2 we find that regarding the after remineralization values there is a significant difference between the two groups where the P-value is =0.011, with the lowest percentage of change values belong to S2 group, and there is a significant difference between the two groups in the after pH-cycling for one month values where the P-value is <0.001, and a significant difference was also found between the values after pH-cycling for three months where the P-value is <0.001, with a lower percentage of change found in S2 group; indicating that S2 group showed improved surface microhardness changes, thus, better remineralizing action when compared to S1 group.

2) Effect of Aging on the Remineralization Capacity:

Data in Table (3), and Figure (8) shows the values of percentage of change of microhardness for both groups at different timings, we find that through the period of the study the lowest percentage of change belonged to group S2 where sodium hypochlorite is applied after acid etching. Regarding the effect of pH-cycling on the remineralization capacity values decrease with time, this indicates active remineralizing action and sustained self-assembling peptide activity that improves by time.

Table (2): The Mean and Standard Deviation (SD) Values of Percentage of Change of Microhardness of Different Sequence of Application

Variables	After R		After pH/1		After pH/3	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
NaOCl - Acid Etching	22.07	3.12	17.19	3.52	10.36	4.64
Acid Etching – NaOCl	18.42	4.43	11.76	2.33	3.26	0.95
P-value	**0.011***		**<0.001***		**<0.001***	

***;** significant (P<0.05) **ns;** non-significant (P>0.05)

Table (3): The Mean and Standard Deviation (SD) Values of Percentage of Change of Microhardness at Different Timings

Variables	NaOCl - Acid Etching		Acid Etching - NaOCl		P-value
	Mean	SD	Mean	SD	
After R	22.07 ^{aA}	3.12	18.42 ^{aB}	4.43	**0.033***
After pH/1	17.19 ^{bA}	3.52	11.76 ^{bB}	2.33	**<0.001***
After pH/3	10.36 ^{cB}	4.64	3.26 ^{cC}	0.95	**<0.001***
P-value	**<0.001***		**<0.001***		

***;** significant (P<0.05) **ns;** non-significant (P>0.05)
Means with the same small letters in the same column indicates significant difference, means with the same capital letters in the same row indicates significant difference.

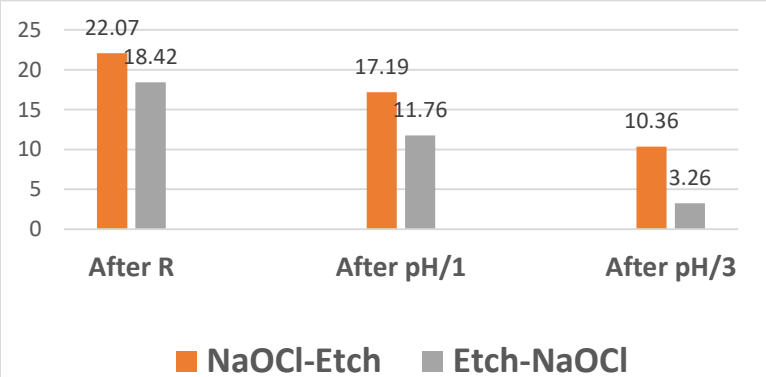

Figure (8): Bar Chart Representing the Effect of Different Sequence of Application and the Aging Effect on Percentage of Surface Microhardness Changes

DISCUSSION

The ultimate goal in modern dental practicing is to conserve the dental tissues throughout any treatment phase.[4,27-29] One of the dental conservative approaches is minimally invasive remineralization measures. Enamel remineralization has greatly progressed recently; and, the continuous progressive work of research regarding tissue engineering and material science in enamel remineralization resulted in the introduction of a successful ideal guided enamel biomimetic regenerative approach called "self-assembling peptide (P11-4)". It mainly acts by substituting the dissolved enamel matrix with new biomimetic matrix that favors in-depth natural remineralization of demineralized

enamel lesions.[4,29-31] Self-assembling peptide consists of 11 amino acids that assemble in a 3-dimensional predetermined fibrillar scaffolds where there is high acidic pH. It diffuses into the lesion body of the initial carious lesion due to its low viscosity, to form an elastomeric viscous nematic gel.[32,33]

Self-assembling peptide application process as stated by the manufacturer consists of, first, deproteinizing the enamel surface with 3% NaOCl for 20 seconds, followed by acid etching. Applying NaOCl aids in removing the organic elements and the acquired enamel pellicle from the surface, and increase the penetrative depths of any applied solution.

Enamel etching transforms the smooth enamel surface into an irregular surface with a high surface energy (72 dynes/cm) more than twice that of unetched enamel. Low viscosity self-assembling peptide wets the high energy surface and is drawn into the micro porosities by capillary action.[34] The action of phosphoric acid on the enamel surface occurs mostly on its mineralized part, i.e., its inorganic matter. Unfortunately, phosphoric acid does not eliminate the organic matter on the enamel surface, which comprises of less than 1% but can be effective in enhancing the etching pattern.[35]

The presence of the acquired enamel pellicle, comprised with organic elements lead to a poorly defined acid etching, results into decreased material infiltration. To achieve good penetration, proper enamel conditioning is a must. Justus et al. (2010)[36] suggested the use of 5.25% NaOCl as a non-invasive method to eliminate the organic pellicle. It has been seen that NaOCl exhibits a dynamic balance that when it comes in contact with organic material, several chemical reactions take place, i.e., fatty acids react with sodium hydroxide creating soap and glycerol, amino acids react with sodium hydroxide creating salt and water, and also reacts with hypochlorous acid creating chloramines and water. These reactions occur simultaneously and synergistically leading to liquefaction of organic tissues.[35]

The aim of this in-vitro study was to assess the remineralizing capacity of self-assembling peptide through measuring the final surface microhardness when NaOCl surface pretreatment is applied before versus after acid etching of enamel with artificial white spot lesion, along a period of one month followed by three consecutive months of pH-cycling to further assess the aging factor and the ability to sustain a remineralizing action.

Surface microhardness indentation provides a relatively simple, non-destructive and rapid method of assessment in demineralization and remineralization studies.[26] In the current study the remineralizing capacity of self-assembling peptide when applied to artificially demineralized enamel lesion was measured through the percentage of change of surface microhardness analysis when compared to baseline readings, where the group that shows less percentage of change indicates better enhancement in surface microhardness, meaning better remineralization action. Therefore, the microhardness values for each specimen were measured five times.

The final results of the present study revealed that both treatment applications significantly promoted the remineralizing capacity of self-assembling peptide when applied to artificially demineralized enamel lesions, and as a result increased enamel surface microhardness at the different measuring times (after remineralization, after pH-cycling for one month, and after pH-cycling for three months) proving sustainability and increased action of self-assembling peptide on the long run; the results of the current study were in agreement with Kamal et al. (2018),[26] Stoleriu et al. (2019),[27] Üstün and Aktören (2019),[37] who found that self-assembling peptide improved the remineralization of white spot lesions due to minerals deposition over self-assembling peptide scaffolds acting as a nucleator for new hydroxyapatite formation; on the other hand the results contradicts those of Golland et al. (2017)[38] who found that the application of self-assembling peptide didn't significantly increase the remineralization capacity of demineralized lesions due to the formation of irregular apatite crystals that didn't promote remineralization.

Regarding the effect of the aging procedures of pH-cycling on the sustainability of the remineralizing action of self-assembling peptide results showed that both groups showed improvement, while the group with the most enhanced remineralizing capacities with notable significant differences was group S2 (where sodium hypochlorite is applied after acid etching) after three months of pH-cycling. These results were in agreement with Welk et al. (2020),[39] Jablonski-Momeni and Heinzel-Gutenbrunner (2014).[40]

The significant enhanced remineralizing capacity (lower percentage of change) of group S2 can be attributed to the moderate effect of sodium hypochlorite on enamel surface as a deproteinizer with its low

concentration (3%) and the short application period (20 seconds) minimizing the loss of surface enamel when applied after acid etching, and exposing more reactive enamel with high surface energy;[22] and right after the duration of NaOCl application (20 seconds) when the pH of the solution starts to decrease below 8.0, P11-4 spontaneously self-assembles to produce 3D nematic gel to start the remineralization action where it increases in neutral pH.[41] This leads to a higher net gain in mineral density content, i.e., remineralization action and less amount of lost minerals with time with the single application of Regenamel Curodont Repair™. So, the more time passes by, the more remineralization action takes place over the self-assembling peptide network formed, giving it the ability to diffuse into the subsurface lesion and aggregate through the whole volume of the lesion up to the surface.[34,42]

With the nature of this study and the data collected, the null hypothesis in which no difference between applying NaOCl before or after acid etching as a surface pre-treatment on the final remineralizing capacity of self-assembling peptide and the surface microhardness was rejected. And, the null hypothesis in which time does not have an effect on the remineralization capacity of self-assembling peptide was rejected.

CONCLUSION

Under the conditions of this study it was concluded that both treatment applications significantly improved the remineralizing capacity of white spot lesions; the sequence of NaOCl application when applied after acid etching had a significant effect on the remineralization capacity of self-assembling peptide promoting better re-hardening action; and, time lapse had a positive effect proving sustainability on the long run for both groups.

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