SURFACE MICROHARDNESS OF BLEACHED TEETH ENAMEL FOLLOWING DIFFERENT REMINERALIZING APPROACHES (IN-VITRO STUDY)

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ABSTRACT

INTRODUCTION: In-office bleaching is a conservative and effective esthetic dental procedure, but it has a serious effect on the enamel matrix structure's integrity, altering its surface microhardness (SMH). Therefore, the use of different remineralizing approaches (reparative or regenerative) after vital bleaching procedures is recommended to improve the SMH of the bleached enamel.

AIM OF THE STUDY: The purpose of this study was to see how different remineralization approaches influenced the SMH of the bleached enamel.

MATERIALS AND METHODS: Crowns of 57 extracted human maxillary central incisors were bleached with a chemically activated bleaching gel containing 40% hydrogen peroxide. Then, based on the remineralizing agent that would be used after bleaching, they were randomly categorized into 3 groups (n=19). Group, I was self-assembling peptide (CURODONT™ REPAIR, Credentis AG, Switzerland), Group II was casein phosphopeptide stabilized amorphous calcium phosphate 'CAP-ACP' ACP' (GC Tooth Mousse, GC Corporation, Tokyo, Japan) and Group III was sodium fluoride (Whitesmile mousse, Whitesmile GmbH; Germany). The specimen's SMH was measured before bleaching, after bleaching, 24 hours, and 1 week after remineralization using a Vickers microhardness tester.

RESULTS: There was a statistically significant increase in Vickers Hardness Number (VHN) across the three study groups only 24 hours following remineralization. The highest VHN was recorded for Gp I (317.30), followed by Gp II (304.63), followed by Gp III (284.02), with the statistical significance only between group I and group III (0.005*). Group III presented the highest VHN 1week after remineralization (312.33) in relation to baseline VHN (287.06).

CONCLUSIONS: The best results were obtained with the self-assembling peptide over both CCP-ACP and sodium fluoride 24 hrs. from its application in SMH recovery.

KEYWORDS: Bleaching, Self- assembling peptides, CPP-ACP, Fluoride, Surface Microhardness.

RUNNING TITLE: Different remineralizing approaches and microhardness of bleached enamel.

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INTRODUCTION

Teeth bleaching is a convenient, simple, and minimally invasive esthetic procedure to enhance the appearance of discolored teeth (both vital and non-vital). The majority of dental bleaching products contain hydrogen peroxide (HP) or carbamide peroxide (CP) as their principal ingredient. They both work by oxidizing the bulky chromophore molecules that produce tooth discoloration. They turn them into colourless shorter-chain molecules by decomposing into oxygen and free radicals, transforming chromophores into carbon dioxide and water (1).

Despite the great esthetic benefit of dental bleaching procedures, their resultant oxidation-reduction processes might harm the integrity of

both organic and inorganic components of dental enamel (2). Morphological changes with erosion and craters, increased surface porosity within and between prisms, prisms exposure, reduction in the aprismatic layer, surface microhardness (SMH) changes, and changes in calcium/phosphate ratio (CaP) are the most significant alterations in enamel following bleaching (1). Da Costa Soares et al (3), reported that these changes are mild to moderate in nature, and can be reversed by post-bleaching remineralization (RM) regimens.

Although saliva has a protective remineralizing effect upon bleached enamel, the net salivary remineralization is slow and it only affects the outer 30 µm of the tooth enamel, providing surface-only remineralization (4). Therefore, several methods have been proposed to remineralize, generate, or regenerate the demineralized dental enamel in combination with the physiological salivary ions. They all aim to restore the lost mineral content by suppling calcium $(Ca)^{2+}$ and phosphate $(PO_4)^{3-}$ ions to improve the bleached enamel integrity (5).

Philip (4) categorized remineralization technologies into; reparative approaches and regenerative biomineralization approaches. Reparative approaches are effective only in remineralizing enamel without promoting the formation of organized apatite crystals. They include topical fluorides and fluoride boosters. While regenerative approaches are effective in replacing the diseased dental enamel with biologically hierarchical enamel microstructure. Achieving this replacement was very challenging because mature enamel is acellular and incapable of self-resorbing or remodelling.

Topical fluorides have always been considered the ″gold standard″ of reparative remineralizing materials, this is due to their ability to generate the more acid-resistant fluoroapatite crystals (FAP) (6). Kemaloğlu et al. (7) reported that ″mineral loss in enamel bleached with 38% HP is dramatically reduced when 2.1 % sodium fluoride was used″. The main limitation in fluoride-based agents is that they produce only superficial remineralization (8).

Casein Phosphopeptide-amorphous Calcium Phosphate (CPP-ACP), is a bioactive fluoride booster reparative remineralization agent that can synergize fluoride efficacy (4). CPP-ACP nano-complexes disseminate into the lesion's body and release high levels of the weakly bound $(Ca)^{2+}$ and $(PO_4)^{3-}$ ions, which would then deposit into the crystal voids, resulting in lower demineralization and improved remineralization of the demineralized enamel (9). Borges et al (10), found that the SMH value of groups bleached with peroxides incorporated into the CPP-ACP paste was statistically higher than the baseline value.

Self-assembling peptide (SAP- P_{11-4}) is a smart nano-bio material, that was designed to induce biomimetic enamel regeneration in cases of initial carious lesions and subsurface enamel demineralization. In response to low pH (below 7.4), the low viscosity isotropic liquid of (SAP- P_{11-4}) rapidly converts into an elastomeric nematic gel, then self-assembles into an elastomeric 3D fibrillar scaffolds, that have a high affinity to tooth minerals, serving as an ideal template for HAP nucleation, and promoting in-depth remineralization (11).

The formed scaffolds chemically bond to the tooth surface when $(Ca2+)$ is attracted to the anionic groups in the P_{11-4} side chains. This attraction activates new HAP precipitation promoting in situ mineral deposition. They also simulate the effect of proteins from the extracellular matrix in the biological mineralization phase of odontogenesis, as they can control HAP crystals' deposition and growth (12). Thus, promoting enamel regeneration via $(Ca)^{2+}$ attraction and nucleation inducing de novo HAP precipitation (13).

Farhana and Shetty (14) also studied the effect of (SAP- P_{11-4}) on enamel SMH after bleaching in vitro. They concluded that bleaching lowered enamel's SMH substantially, and (SAP-P_{11-4}) had a significant difference in remineralizing bleached enamel surface.

There are several techniques used to measure the amount of demineralization or remineralization affecting bleached dental enamel ; such as surface microhardness (SMH), scanning electron microscopy (SEM) with energy dispersive x-ray (EDX), microradiography, polarized light microscopy, plasma mass spectroscopy, confocal laser microscopy, and others (15). In this study SMH was used.

In light of the above information, it is of interest to evaluate the impact of various remineralizing approaches (reparative & regenerative) on the SMH of the bleached tooth enamel. The null hypothesis is that no difference exists between variable remineralizing approaches on the SMH of bleached tooth enamel.

MATERIALS AND METHODS

1. Sample size estimation and inclusion criteria

The sample size was estimated based on the following assumptions: confidence level= 95% and study power= 80%. The mean surface microhardness for enamel treated with self-assembling peptide P11-4 (Curodont Repair) was 283.67 ± 18.41 (16). The mean surface microhardness, when treated with casein phosphopeptide amorphous calcium phosphate (CPP-ACP), was reported to be 429.97 ± 41.4 (17). When fluoride gel was used, a remineralizing agent, the surface microhardness of enamel was reported to be 340 ± 76.6 (18).

To ensure adequate power across all comparisons, the sample size was based on the differences between self-assembling peptide P_{11-4} and fluoride gel mean surface microhardness because it produced the greatest sample size across the pairs of groups. The sample size was calculated to be 17 specimens per group. This will be increased to 19 to make up for laboratory processing errors. The total sample size= number of groups × number per group=3× 19= 57.

Software: Sample size was based on Rosner's method (19) calculated by Gpower 3.1.

The ethical committee at the Faculty of Dentistry, Alexandria University accepted this in vitro investigation. A total of 57 sound maxillary central incisors extracted for periodontal reasons were used in this study. The included teeth were extracted

from patients ranging in age from 12 to 30 years (20), free of any visible cracks, hypoplastic defects, fluorosis, or caries (16). Materials in table 1 were used in this study **(Table 1).**

Table (1): Materials composition and manufacturer.

Material	Composition	Manufacturer
In-office chemically activated bleaching gel (Whitesmile whitening 40% YF (32% mixed).	Aqua Hydrogen peroxide (40%) Silica Glycerol Organic amines Polyglycols Chlorophyll	Whitesmile GmbH; Germany
Fluoride Mousse (Whitesmile mousse)	4.2% potassium nitrate 1450 ppm sodium fluoride 30% Xylitol	
Curodont Repair	Self-assembling peptide (P$_{11-4}$ oligopeptide solution)	Credentis AG, Switzerland
GC Tooth Mousse	Casein Phosphopeptide – Amorphous Calcium Phosphate (CPP-ACP)	GC Corporation, Tokyo, Japan
Artificial saliva solution (20)	Na$_3$PO$_4$ (3.90 mM) NaCl (4.29 mM) KCl (17.98 mM) CaCl$_2$ (1.10 mM) MgCl$_2$ (0.08 mM) H$_2$SO$_4$ (0.50 mM) NaHCO$_3$ (3.27 mM) Distilled water (pH 7.2)	Department of Chemistry, Faculty of Pharmacy, Alexandria University , Egypt

2. Specimens' preparation and storage

The included teeth were scraped with a hand scaler and thoroughly cleansed to ensure that any tissue remains were removed. Their roots were cut 2mm below the cemento-enamel junction using a double side-cutting course diamond disc (SS White, New Jersey, USA) (21). All specimens were then autoclaved at 121°C/20 psi for 40 minutes in autoclave bags as recommended by Centres for Disease Control and Prevention (CDC) to ensure their complete sterilization (22).

The crown specimens were embedded in self-curing acrylic resin (Acrostone, cold cure, Egypt) with their labial surface facing upward using a custom-made copper mold (10). After resin setting the acrylic blocks were finished and polished using different girts of silicone carbide burs (FORDOM®, Bethel, Connecticut, USA) on a low-speed handpiece to obtain a smooth surface for more accurate measurements.

The specimens were stored in laboratory prepared artificial saliva solution at 37°C during the entire experiment. The solution was renewed daily for better simulation of the oral cavity conditions (23).

3. Specimens' grouping

The specimens were divided randomly using the research randomizer website (https://www.randomizer.org/), into 3 main groups

(n=19) based on the remineralizing agent that will be applied after the bleaching procedure. Group I was SAP- P$_{11-4}$ (**CURODONT REPAIR**), Group II was CPP-ACP (**GC Tooth Mousse**), and Group III was sodium fluoride (**Whitesmile mousse**). Then they were color-coded using nail polish and labeled on their bottoms according to the different study groups.

4. Surface microhardness measurement

Surface microhardness testing was done using Vickers micro-hardness Tester (**Wilson® Hardness, TUKONTM1102, Buehler Company, Germany, 2015**) under 200gm load, and 15 seconds dwell time. The measurements were conducted at baseline, and repeated immediately after bleaching, 24 hours, and 1 week after remineralization (21). On each specimen, three indentations were made (21) (**Figure 1b).** The length of the indentations at each depth was recorded for each specimen to obtain its Vickers Hardness Number (VHN). Then, their average of the VHNs was calculated.

5. Bleaching agent application

A chemically activated bleaching gel was applied on the labial surfaces of the specimens by dispensing the material using its dual-barrel syringe after being mixed by the attached mixing tip. It was left for 15 minutes then removed using a high suction tip according to the manufacturer instructions. This procedure was repeated three times yielding a total of 45 minutes of bleaching. Bleached specimens were rinsed with deionized water for 30 seconds (24), then stored in artificial saliva solution at 37°C until being retested (23).

6. Application of remineralizing agents

The three tested remineralizing agents (**CURODONT REPAIR**), (**GC Tooth Mousse**), and (**Whitesmile Mousse)** were applied over the labial surfaces of the specimens after their bleaching (21).

For **Group I,** one drop of **CURODONT REPAIR** solution was applied onto the bleached enamel surface using CURODONT™ REPAIR applicator and allowed to stand for 5 minutes for solution diffusion according to manufacturer's instructions (25).

For **Group II**, the sample surface was dried, and a sufficient amount of **GC Tooth Mousse** was applied on the tooth surface with a gloved finger and left undisturbed for 4 minutes according to the manufacturer's instructions (26)**.**

For **Group III, Whitesmile mousse** was applied on the labial surfaces of the bleached specimens using its syringe tip and left for 10 minutes according to the manufacturer's instructions, then the specimens were rinsed (21) with deionized water for 30 seconds (24).

All specimens were stored in artificial saliva at 37°C (23) for 24 hours before being retested (3).

7. Statistical analysis of data

The data were fed into a computer and evaluated with the IBM SPSS software for MacBook (Version 28.0) (Armonk, NY: IBM Corp). The normality of the distribution of data was verified using the Kolmogorov-Smirnov test. The mean, standard deviation, and plots (histogram and boxplots) were used to describe quantitative data. Significance was inferred at **p-value <0.05**.

Means and standard deviation (SD) was calculated for surface microhardness *between the three study groups*, and the comparison was done using **one-way ANOVA** for normally distributed variables (VHN) followed by **Tukey's Post hoc** if the results were statistically significant. Comparisons *within each group* were done using **repeated measures ANOVA**; then numerous pairwise comparisons with Bonferroni adjusted significance levels were performed.

RESULTS

Among the three study groups, one way ANOVA test revealed a statistically significant increase in VHN only 24 hours after remineralization (p-value 0.006*), while VNH at baseline, after bleaching, and 1 week after remineralization didn't show a statistically significant difference (p-values, 0.32, 0.10, 0.09 in order) **(Table 2)**. The highest VHN was recorded for Gp I (317.30), followed by Gp II (304.63), followed by Gp III (284.02) 24h after remineralization **(Figure 2)**, with the statistical significance only between group I and group III (p-value 0.005*) as revealed by Tukey's Post hoc test**.**

Table (2): Comparison of the mean VHN values at the different study times among the study groups.

	Group I Curodont Repair	Group II GC Tooth Mousse	Group III Whitesmile Mousse	F	p
	Mean (SD)				
Baseline	304 (37.04)	291.29 (38.93)	287.06 (26.12)	0.32	1.162
After Bleaching	280.17 (35.56)	264.82 (39.38)	256.07 (28.50)	0.10	2.339
24 hrs. after (RM)	317.30 (24.87)	304.63(36.52)	284.02 (30.15)	0.006*	5.622*
1 week after RM	290.96 (37.34)	284.71(42.99)	312.33 (37.67)	0.09	2.565
F₀	9.321*	18.017*	42.884*		
p₀	0.002*	<0.001*	<0.001*		

SD: Standard deviation
F: **F for One way ANOVA test**
p: p value for comparing between **the three studied groups**
F₀: F test (ANOVA) with repeated measures
p₀: p value for comparing between **the three studied periods in each group**
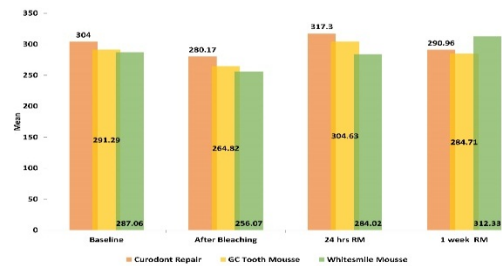*: Statistically significant at $p \leq 0.05$

Figure (2): Mean VHN among study groups.

Within each group, repeated measures ANOVA test **(Table 2)** revealed a statistically significant difference in VHN at the four different time points of the study (baseline, after bleaching, 24 hours after, and 1 week after RM) in all groups (p= 0.002*, <0.001*, &<0.001*) for groups I, II & III respectively. Post hoc- pairwise comparisons of VHN at the different time points **(Table 3)** revealed that the decrease in VHN after the bleaching was statistically significant versus the baseline values in all groups (p= < 0.001*) .

Table (3): Post hoc- pairwise comparisons of VHN at the at the different study times within study groups.

Time Point	Compared to	P-value		
		Group I Curodont Repair	Group II GC Tooth Mousse	Group III Whitesmile Mousse
Baseline	After Bleaching	< 0.001*	< 0.001*	< 0.001*
	24-hrs after RM	0.64	0.27	1.00
	1 week after RM	1.00	0.80	0.017*
After Bleaching	24-hrs after RM	< 0.001*	< 0.001*	< 0.001*
	1 week after RM	1.00	0.22	<0.001*
24 hrs. after RM	1 week after RM	0.53	0.003*	<0.001*

*Statistically significant using Bonferroni adjusted significance level

Regarding the difference between the base line versus the recorded increase 24 hours after treatment, no significant difference was recorded in any of the tested groups (p = 0.64 , 0.27 , & 1.00 for groups I,II &III respectively), while the difference between the base line versus 1 week after treatment was significant only in group III (P= 0.017*) ,and not significant in groups I (P= 1.00) or II(P= 0.80).

After bleaching, there was a statistically significant increase of the VHN values 24 hours after treatment versus the recorded post bleaching values in all groups (p= < 0.001*) while after 1week the difference was significant only in group III (p= <0.001*) and not significant in groups I (1.00) or group II (p= 0.22). The difference between 24 hours versus 1week of treatment was significant in groups

II &III (p= 0.003* & <0.001* for both groups respectively) but not significant in group I (P= 0.53).

DISCUSSION

Surface microhardness testing is commonly used to determine whether bleaching agents are destructive to enamel (5 , 16). This is because it is a simple, fast, easy, non-destructive, and dependable test to assess demineralization and remineralization changes affecting bleached enamel. It uses a standard source to determine the material's resistance to plastic deformation. Also, it permits the same specimen to be measured several times reducing the experimental variation (27).

The CDC recommends autoclaving extracted teeth used in enamel microhardness studies because it doesn't influence enamel mineralization, that's why it was chosen in this study. Because of the lower organic component of enamel, it's minimally affected by the temperature and high-pressure produced during the autoclaving process (28). 40% HP chemically activated bleaching gel was used in this study. It was applied over the entire crowns because it's an easier, economic, and more clinically relevant method to enamel blocks (10). Specimens weren't brushed throughout the bleaching procedures to guarantee that any alterations to the enamel surface are only caused by HP without external interferences (29).

During the study period, indentations were made randomly on the flattest points of the enamel surface (guided by the different magnification lenses of the tester to ensure that each indentation is new) with constant weight and time to ensure the accuracy of the measurements **(Figure 1c)**. A minimum distance of 150 µm was ensured between adjacent indentations in order to avoid measurement errors **(Figure 1b)** (30).
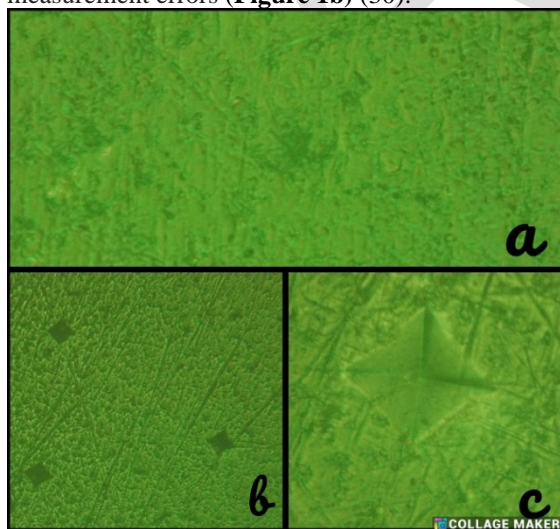

Figure (1): Vickers micro-hardness indentations. a: normal enamel surface, b: three indentations on the enamel surface, c: magnified indentation.

After bleaching, all specimens showed a reduction in SMH. This was consistent with the findings of Pinto et al (31) and Al-Salehi et al (32), who studied the effect of different HP concentrations on microhardness human and bovine enamel samples. They reported a significant decline in microhardness after bleaching and attributed it to the mineral content loss resulting from demineralization caused by oxidation-reduction reactions of bleaching agents. Despite different methodologies many authors reported no (33) or non-significant (31) (34) changes in SMH of enamel specimens stored in artificial saliva for different periods (7,14,15, and 30 days). Therefore, it was chosen as the storage medium throughout this study period.

Overall SMH results among the three study groups showed a statistically significant increase in VHN only 24 hours after application of the different remineralizing agents. While there was no statistically significant difference in VNH at baseline, immediately after bleaching, and 1 week after remineralization. The highest VHN was recorded for Gp I, followed by Gp II, followed by Gp III 24h after remineralization, with the statistical significance only between group I and group III. This was in agreement with the previous work of Soares (16), Where the increase in SMH was attributed to the ability of SAP to induce biomimetic mineralization of the initially demineralized enamel.

Although initial recovery of SMH was shown 24 hrs. after the application of all remineralizing agents, both (SAP- P_{11-4}) and CPP-ACP weren't capable to maintain SMH values 1 week after their application. This was in agreement with the work of Memarpour et al. (35) who studied the remineralization of artificial enamel caries lesions using (SAP- P_{11-4}) associated with different materials at three intervals (baseline, after demineralization, and 28 days after remineralization). They reported that both SAP and CCP-ACP showed a lower VHN than the rest of the study groups 28 days after remineralization.

The justification of these findings is probably related to the remineralizing agents' short-term contact with the enamel. Wierichs et al. (36) reported that 'single application of remineralizing agents is insufficient to provide an adequate supply of minerals lost by demineralization'. Furthermore, it has been claimed that bleaching ingredients released into the dental tissues can last up to two weeks (37). Therefore, remineralizing bleached enamel several times (after each bleaching cycle and 2 weeks after bleaching) is recommended to maintain the benefits achieved 24 hrs. after remineralization (3, 39).

Whitesmile Mousse was added after bleaching process as recommended by manufacturer because of it desensitizing (due to presence of potassium

nitrate), remineralizing (due to presence of sodium fluoride) and anti-cariogenic properties (due to presence of xylitol). Specimens treated with Whitesmile Mousse were different, they showed a statistically significant increase in VHN 24 hours and 1 week after application of Fluoride mousse to each other, and also a statistically significant rise in VHN 1 week following application of Fluoride mousse to baseline data. This is because the newly developed FAP crystals had higher hardness levels when compared to HPA crystals (39).

In summary, this study found that peroxide bleaching decreases enamel's SMH, but to a reversible limit that can be repaired by post bleaching remineralization using agents of different approaches. Thus, the null hypothesis that no difference exists between variable remineralizing approaches on the SMH of bleached tooth enamel was rejected.

CONCLUSIONS

Within the scope of this investigation, the following conclusions were reached; bleaching procedures decrease SMH of dental enamel. Also, that (SAP-P$_{11-4}$) showed the best results over both CCP-ACP and Fluoride regarding initial SMH recovery.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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