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COMPARATIVE EVALUATION OF NANO-HYDROXYAPATITE INCORPORATION BEFORE, DURING AND AFTER CHEMICAL BLEACHING ON HUMAN ANTERIOR TEETH ENAMEL SURFACE AND SUBSURFACE

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ABSTRACT

Objective: Instant effects of bleaching- including surface morphology and chemical structure alteration-induced tooth sensitivity which is the main deterrent for patients to successfully complete their bleaching treatment. Fluoride, calcium, or amorphous calcium phosphate could be added to bleaching gel to reduce bleaching effects. This study aimed at comparing different protocols of Nano-Hydroxyapatite (as a promising re-mineralizing agent) incorporation with bleaching gel effect on surface and subsurface enamel morphologically and chemically.

Methodology: Twenty-eight sound anterior teeth were collected from subjects aged over 30 years. The crowns were divided longitudinally into two halves (mesial and distal). The cut surface of each half was lined with nail polish. **Group I**: teeth were subjected to bleaching agent according to manufacturer instructions. In **Groups II, III & IV** teeth samples were subjected to Nano-hydroxyapatite for 60 min. before, during and after the bleaching treatment respectively. Scanning electron micrographs of the enamel surface and subsurface were obtained and evaluated descriptively. The enamel chemical characterization of the samples was performed by the Scanning Electron Microscope equipped with microanalysis EDXA Element.

Results: All experimental groups showed apparently more morphologically intact surface with variable percentage of surface precipitates. Group III and IV showed positive trials to improve the subsurface enamel structure in which Nano-HA particles binding to enamel structure with no statistically significant change in Ca/P.

Conclusions: the incorporation of Nano-HA particles to the bleaching agent didn't cause relevant chemical alterations of the enamel structure mean while it showed a detectable enamel surface and subsurface morphological preservation.

KEYWORDS: Nanohydroxyapatite, bleaching, enamel, EDXA

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INTRODUCTION

Professional dental bleaching has good long-term influence on the level of oral hygiene and serves as a significant motivation factor for maintaining health of oral cavity.[1] In spite of being validated as effective tooth color lightening method, in-office bleaching causes tooth sensitivity in more than 70% of cases. This is the main deterrent for patients to successfully complete their bleaching treatment.[2] This effect is indeed related to the *insitu* alterations of the surface morphology and chemical properties of dental tissues during bleaching.[3] Long-term effects include rapid recurrent discoloration, [4] due to surface irregularities, with multiple enamel cracks due to decreased enamel mineralization and hardness.[5]

Fluoride[6-8], calcium[9] or amorphous calcium phosphate[11] were incorporated into the bleaching gel to minimize enamel demineralization. Some biomimetic materials such as Nano-hydroxyapatite (Nano-HA) have been introduced as new technique for enamel reconstruction.[10] Owing to its chemical and structural similarity with natural tooth mineral, synthetic Nano-HA is considered an attractive biomaterial that would lead to a considerably superior remineralization.[11-15] Nano-HA has a strong affinity to demineralized surfaces due to its ability to bind to pores created by acid attacks. After adhering, Nano-HA multiplies and organizes into micro clusters forming a uniform apatite layer that may completely overlap interprismatic and prismatic enamel.[16] Nano-HA-enriched whitening gel was produced to overcome enamel demineralization and hypersensitivity. [17]

To the best of our knowledge, [18-20] there are no studies which compare the morphological changes and microstructural modifications of bleaching agent with or without Nano-HA. The present in vitro investigation aimed to evaluate the effects of different Nano-HA application protocols with in-office bleaching agent on the human enamel structure.

MATERIAL AND METHODS

A total of 28 sound anterior teeth were collected. Teeth are surgically extracted for periodontal or orthodontic reasons. This study got ethical committee approval (FDASU-Rec ER112212) by the Faculty of Dentistry of Ain Shams University Research Ethical Committee. After the surgical extraction, teeth were washed with distilled water for 2 min. to remove the blood and biological remains. [21] Teeth exhibited lesions and decays, including hypoplastic defects and cracks were excluded after careful examination with stereomicroscope (SZ-ST Olympus, Japan). Selected teeth were stored in distilled water at 37˚C. Teeth were divided into four groups: seven teeth each. The roots of the teeth were removed 2 mm apically to the cementoenamel junction using diamond discs, and the crowns were divided longitudinally using diamond saw (Buehler Isomet 1000, USA) with copious water irrigation, in buccolingual direction to separate the mesial and distal halves. The cut surface of each half was lined with nail polish. After application of surface treatment to samples according to the group it belonged to, mesial halves of the teeth were used to examine the enamel surface while the distal halves were split horizontally at the middle one third of the sample to examine the subsurface structure of enamel.

Materials: (Table 1)

TABLE (1) Materials used in the experiment.

Material	Chemical Composition	Manufacturer	Application time
Power Whitening YF 40%	hydrogen peroxide 40%, aqua, silica, glycerol, organic amines, polyglycerol, coloring agent.	WHITE smile GmbH, Germany	20 minutes (repeated 3 times) + after mousse material (10 minutes).
Nano-Hydroxyapatite powder*	$Ca_{10}(PO_4)_6(OH)_2$ white powder purity~ 99.9%	Nano Gate	Mixed with the whitening gel.
Nano-Hydroxyapatite gel	$Ca_{10}(PO_4)_6(OH)_2$ 20%w/v	Nano Gate	20 minutes (repeated 3 times) [27] before or after bleaching.

**** Rod-like morphology, width 5–20 nm (typically close to 10 nm) and length < 50 nm (typically between 20 to 40 nm).***

Methodology:

Enamel surface treatment:

- ***Group I (Control Group):*** Power Whitening YF 40%, WHITE smile GmbH, Germany, containing 40% hydrogen peroxide (H_2O_2) was used for in-office bleaching application. Three successive applications as 1-2mm thick layer of the whitening gel on the buccal tooth surface for 20 minutes followed by removal from the tooth surface using suction then water rinsing to have a 60 minutes of total gel application. After-Whitening Mousse was applied for 10 minutes.
- ***Group II:*** Nano-HA gel 20% wt/v was applied to the enamel surface for 20 minutes then washed with distilled water for 30 seconds. This was repeated two more times to have a total gel application of 60 minutes. [22] Then in-office bleaching agent 40% hydrogen peroxide (HP) was applied to the enamel surface following the manufacturer's instructions as in control group.
- ***Group III:*** Nano HA powder was mixed with 40% HP gel in the ratio of 2 g powder to 1 ml gel. [22] The mixture was applied to enamel surface for 20 minutes then removed with suction. This procedure was repeated 2 more times to have a total of 60 minutes of application. The treatment finished off by applying After Whitening Mousse for 10 minutes then samples were rinsed off by water.
- ***Group IV:*** in-office bleaching agent was applied to the enamel surface as per manufacturer's instructions. Then Nano-HA gel 20% wt/v was applied to the enamel surface for 20 minutes then washed with distilled water for 30 seconds. Its application was repeated two more times to have a total gel application of 60 minutes.[22] All samples were kept in fresh water till testing procedures.

Testing procedures

Subgroups containing the mesial halves were used to examine the enamel surface, and the second subgroup samples were split horizontally at the middle third of the enamel, to examine the fractured surface. Splitting is performed using hand tool to divide the tooth structure along a fracture line without sectioning enamel with diamond disc. This is performed to avoid destruction of enamel and to preserve prisms and interprismatic structure either at the surface or subsurface enamel. For Scanning Electron Microscope and Energy Dispersive X-Ray Analysis Spectroscopy (EDXA) (Zeiss Supra 40 field-emission electron microscope), samples were assembled in a sample holder and metallized with vacuum precipitation of a gold film on the enamel surface. SEM operated at 30 kV and at a 12 mm working distance. EDXA was used to examine each enamel surface and subsurface elemental compositional (Ca, P, C, O, F wt.%) changes. The obtained results were analyzed, digitalized.

Statistical analysis: Results were recorded, tabulated, and statistically analyzed.

RESULTS

SEM results

Scanning Electron Microscopy examination of surface enamel of experimental groups showed apparently more intact surface than control group GI with apparent gradual increase of surface precipitates percentage in GII, GIII, and GIV respectively (Fig. 1: A, B, C, and D). Subsurface enamel of GII showed obvious aprismatic layer but mostly no change in the prismatic structure than GI, while GIII represented a superficial artificial enamel layer with detectable subsurface prismatic feature. GIV samples lost the prismatic feature (Fig. 1: E, F, G, and H).

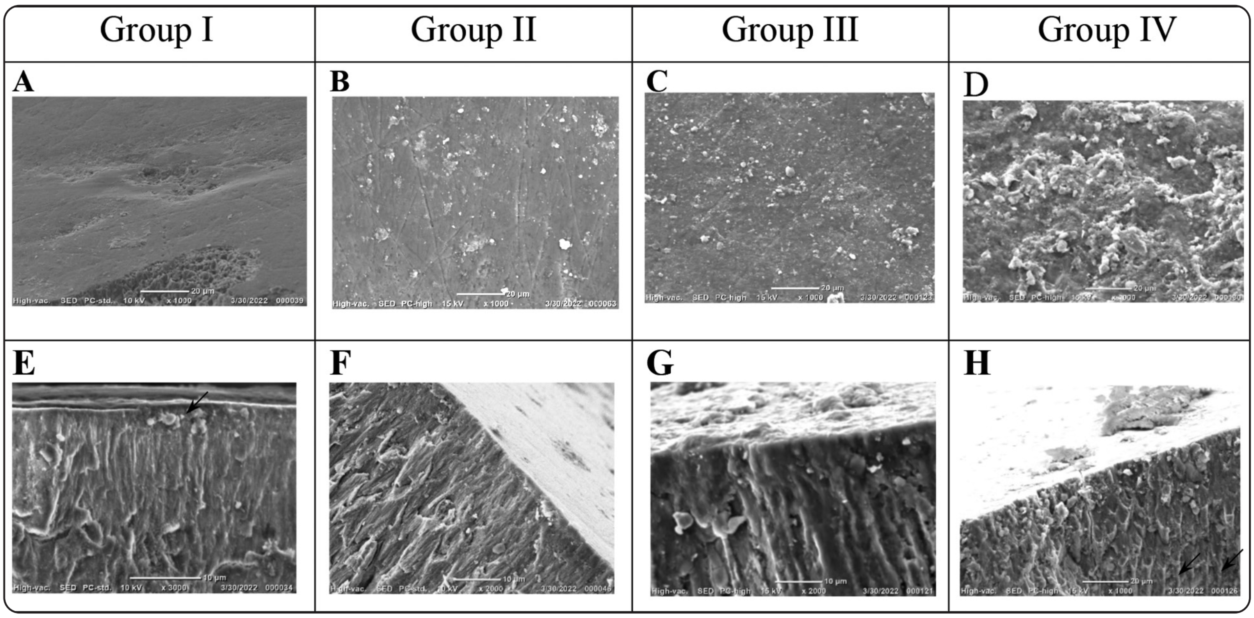

Fig. (1): Enamel surface Scanning electron micrographs of (A) GI: displayed enamel surface with eroded areas, dissolution of aprismatic layer in some areas with visible underneath prismatic layer. (B) GII: showed irregular surface with multiple scratches, pits, and pores. There are areas with slight dissolution of the surface structure with some surface deposits of different sizes. (C) GIII: displayed irregular enamel surface morphology partially masked by multiple deposits. (D) GIV: presented almost total coverage of enamel surface with deposits. (Original Magnification x1000)

Enamel sub-surface Scanning electron micrographs of (E) GI: displayed surface eroded area (arrow), aprismatic layer with different thickness and loss of the prismatic enamel integrity. (F) GII: showed obvious aprismatic layer and a less distinguished pattern of prismatic enamel represented as apparently obvious condensed rod like enamel structure. (G) GIII: showed enamel surface with multiple deposits. Subsurface structure displayed detected aprismatic layer and obvious parallel prismatic structure. (H) GIV: showed surface deposits, ill-defined prisms and inter-prismatic pattern especially near the surface. Deeper areas presented individual prisms (arrows). (Original Magnification x2000)

EDXA Statistical analysis

Recorded data were analyzed using the statistical package for social sciences, version 23.0 (SPSS Inc., Chicago, Illinois, USA). The quantitative data were presented as mean± standard deviation and ranges. The following tests were done: A one-way analysis of variance (ANOVA) when comparing between more than two means. Post Hoc test: Tukey's test was used for multiple comparisons between different variables. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following, P-value <0.05 was considered significant, P-value <0.001 was considered as highly significant & P-value >0.05 was considered insignificant.

For all the surface groups, the chemical composition of dental enamel in terms of relative amounts of Ca, P, O, F and C elements, was analyzed using EDXA (Table 2). The results showed the following alterations in the levels of the chemical constituents:

1. Significant increase of the fluoride content in all groups.
2. Highly significant decrease of P content in GII and GIV.
3. Non-significant decrease of Ca element and increase of C element in all experimental groups compared with GI.
4. Almost no change in O content in all groups except an increase in GII.

For all the subsurface groups, the chemical composition of dental enamel in terms of relative amounts of Ca, P, O, F and C elements, was analyzed using EDXA (Table 3). The results showed only low alterations in the levels of the following chemical constituents:

1. Significant decrease in F content of GIII and GIV, meanwhile it was an increase in GII.
2. Nonsignificant decrease of P and Ca content in all groups except in GIV which showed some increase.
3. Non-significant increase of C and O content in all groups except in GIV group which showed some decrease.

TABLE (2) Showing the mean ± SD values, results of ANOVA and post hoc test for the comparison between different groups regarding weight percentage at enamel surface.

Weight percentage at enamel surface	GI	GII	GIII	GIV	ANOVA	p-value
Ca	56.60±2.95	56.37±12.49	54.53±0.67	55.15±1.53	0.162	0.921
P	29.64±0.78^{A}	26.39±1.56^{B}	29.68±0.70^{A}	28.83±1.20^{A}	13.503	<0.001**
C	5.46±0.72	6.28±1.85	6.51±1.04	7.34±1.14	2.658	0.071
O	8.15±1.88	10.77±10.28	8.90±0.33	8.41±3.18	0.330	0.804
F	0.15±0.01^{B}	0.20±0.14^{B}	0.39±0.00^{A}	0.26±0.21^{AB}	4.665	0.010*
Ca/P ratio	1.91±0.15	2.15±0.53	1.84±0.02	1.91±0.07	1.620	0.211

Significance level p≤0.05, **p-value <0.001 is highly significant; p-value >0.05 is insignificant

Tukey's post hoc: Means sharing the same superscript letter are not significantly different.

TABLE (3) Showing the mean ± SD values, results of ANOVA and post hoc test for the comparison between different groups regarding weight percentage at enamel subsurface.

Weight percentage at enamel subsurface	GI	GII	GIII	GIV	ANOVA	p-value
Ca	51.05±13.85	42.62±10.67	51.05±15.79	56.04±6.17	1.463	0.25
P	24.88±5.02	21.17±7.55	23.58±6.31	28.03±2.32	1.797	0.175
C	13.97±12.38	19.26±12.39	14.45±12.39	7.76±5.70	1.264	0.309
O	9.97±7.54	16.42±6.50	10.83±6.64	8.11±1.74	2.447	0.088
F	0.12±0.11^{B}	0.54±0.34^{A}	0.10±0.06^{B}	0.06±0.01^{B}	10.882	<0.001**
Ca/P ratio	2.06±0.44	2.13±0.48	2.26±0.97	2.01±0.32	0.229	0.875

Significance level p≤0.05, **p-value <0.001 is highly significant; p-value >0.05 is insignificant

Tukey's post hoc: Means sharing the same superscript letter are not significantly different.

DISSCUSION

Hydroxyapatite can be considered as a revolutionary material due to its remarkable remineralizing effects on enamel initial lesions as it performs a protective action against caries and dental erosion [23]. Nano-HA is highly biocompatible and bioactive due to its morphological and structural similarity to the apatite crystal [24-27] and has been incorporated into toothpaste, mouthwashes, and clinical application products to restore the altered enamel integrity [28, 29]. Nano-HA topical application may increase the remineralizing effect since the product is being locally delivered to the required area [27].

The mechanism of Nano-HA contribution in enamel prism remineralization could be explained by: (1) the formation of a thin and homogeneous layer of apatite on demineralized enamel surfaces that seals its small pores [26, 28-30] repairing the altered enamel morphology [31-33] (2) acting as calcium and phosphate reservoir, maintaining a state of supersaturation concerning the enamel minerals, favoring the apatite deposition in the demineralized areas [25, 34, 35]. The effectiveness of Nano-HA towards enamel structure depends on the concentration of the HA used [36], the particles size [37] and the way of its application. The affinity of HA particles toward enamel surface is not uniform, due to the differences in the orientation of the enamel crystallites at the surface [36].

Scanning Electron Microscopy photomicrographs of GII showed apparently less dissolute surface with some attached deposits of different sizes (Fig.B) [38]. The subsurface SEM micrographs showed an obvious aprismatic enamel layer of different thickness which was missed in surface eroded areas, while the prismatic layer represented apparently condensed rod-like enamel structure with less detected prismatic and interprismatic architecture feature (Fig.F) [39].

The non-significant decrease of Ca and P wt.% in GII could be related to the oxidation effect of the peroxide on the applied Nano-HA layer which lacks proteins on the contrary of natural enamel [17]. It is known that high concentrations of HP can modify the Ca/P ratio [40], and reduction in the level of the two elements could lead to an irreversible alteration, preventing the remineralization process from occurring [41]. The protective HA layer could have been dissolved during the bleaching application, leaving the attached deposits, and releasing Ca and P ions which act as buffer system resulting in decreasing the dissolution of the enamel [38], or even a shift of the state from dissolution to homeostasis[42,43] and protecting the enamel subsurface structure from HP penetration [44].

The use of Nano-sized particles in this study promotes its penetration into porosities resulting from the probable mineral loss occurred due to subsequent bleaching [45]. The use of Nano-HA in gel form can also increase the penetration of the crystals through the enamel interprismatic space as the enamel protein located there is capable of capturing mineral solutions and permitting penetration of minerals along the sides of the adjacent crystallites[46-48], and it may result in forming the observable condensed rod like structures appearance in the subsurface micrographs (Fig.F) represented chemically as higher subsurface Ca/P ratio than in the surface and the control group.

Group III displayed irregular enamel surface morphology partially masked by multiple deposits (Fig.C), while enamel subsurface displayed a detected aprismatic layer and underlying obvious parallel prismatic structure (Fig.G). It was concluded that the Nano-HA presence in the bleaching material played a key role in maintaining the structure of the enamel during bleaching [21,49,50]. Meanwhile, Kutuk et al. (2018) [17] did not detect changes in the SEM micrographs; this could be related to the low magnification power that has been used. Adding Nano-HA to the bleaching material increased the amorphous structure of enamel subsurface keeping the prismatic architecture of the enamel structure less effected (Fig.G). [51]

When hydroxyapatite is mixed with HP, it might buffer its acidity and reduce its demineralizing effect [52,53], adhere homogenously to the enamel surface, form a protective layer for the underlying enamel, decrease direct contact of HP with enamel surface, cause supersaturation with enamel apatite[54], and promote its penetration into porosities resulting from the probable mineral loss due to bleaching [45]. The Nanoparticles which penetrated into the interprismatic space could remineralize the subsurface enamel through ion transportation or linking to the interprismatic protein. [47,48]

In case of adding Nano-HA to the bleaching agent, Kutuk et al. (2018) [17] observed an increase in Ca/P ratio that could be related to the use of artificial saliva for 14 days after bleaching with the mixture, While, the Chemical profile of GIII showed that Nano-HA couldn't preserve the chemical surface profile, while the subsurface profile was more mineralized than in control group. This could be related to the buffering effect of Nano-HA which lessens the ability of bleaching material to penetrate the enamel structure [55] to the limit of preserving the morphological and chemical profile of the subsurface enamel structure.

Group IV represented almost a full covering layer of surface deposits (Fig. D) [56,57]. Application of Nano-HA gel after the bleaching treatment permits its binding to the enamel surface tending to fill pores previously created in the surface as a bleaching effect [21]. Indeed, after adhering, Nano-HA particles multiply and organize into micro-clusters to form a uniform apatite layer that may completely overlap interprismatic and prismatic enamel [16], that shown as a full coverage in Fig. D. Nano HA particles were able to pass deeply in the enamel structure through pores and channels formed previously by the bleaching effect [46], and these Nano-HA particles are chemically bonded to enamel in a random arrangement [36], shown as ill-defined prism and interprismatic pattern near the enamel surface, while the prismatic pattern was distinguished deeply (Fig. H).

Group IV represented surface enamel decrease of Ca and P wt.% [56] contre the findings of Misilli et al. (2022) [51]. The relation between Ca and P decrease did not affect the Ca/P ratio. Meanwhile, subsurface enamel structure showed increase of both Ca and P wt.% reflected as a non-significant decrease in Ca/P ratio. It seems that, the chemical effect of bleaching treatment was too massive to be corrected by the Nano-HA gel applied after it; meanwhile the nanoparticles were shown morphologically as surface deposits (Fig. D), and bind randomly to subsurface enamel crystals (Fig. H) as described above, that is reflected as a non-significant increase in Ca, P wt.% but it couldn't reverse the state of demineralization resulted due to the bleaching.

Concerning other elements, the increase of surface F in all experimental groups is probably due to the high affinity of the F contained in the after moss of the bleaching kit to bind with surface Nano-HA particles, which could mechanically improve the failed chemical remineralizing effect with Ca and P ions. The significant decrease of subsurface F in all experimental groups except Group II could be due to the disability of large sized F atom to penetrate the enamel surface [21, 51].

A slight increase in O concentration was found in all samples with Nano-HA incorporation to the bleaching technique except in Group IV in subsurface samples. This fact is probably associated with the action of the peroxide on the mineralized enamel structure, a reaction well mentioned by scientific literature. [41, 58, 59, 60]

Conclusions

All the previous findings suggested that the incorporation of Nano-HA particles to the bleaching agent adopted in this study showed obvious protection and/or correction of the enamel surface morphology with a variable percentage of covering deposits. Mixing of Nano-HA with the bleaching gel preserved the normal feature of subsurface enamel, meanwhile its application before or after the bleaching treatment showed positive trials to improve the subsurface enamel structure in which Nano-HA particles binding to enamel structure.

The Ca/P ratio calculated in this study did not show any statistically significant change in all the experimental groups. [41] In addition to the significant increase of F in all experimental groups, that could lead to improved mechanical properties of the enamel to compensate the negative effects on Ca/P ratio that reflected on enamel demineralization. It is concluded that the incorporation of Nano-HA particles to the bleaching agent adopted in this study was not sufficient to cause relevant chemical alterations of the enamel structure.

RECOMMENDATIONS

Further studies are recommended on the mechanical effects of Nano-HA application before and after bleaching.

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