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Combined Effect of Nanohydroxyapatite and Chitosan on Remineralization of Initial Enamel Lesion in Vitro

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ABSTRACT

Background: The initial clinical manifestation of enamel caries is a white spot lesion (WSL). It is comprised of a porous lesion body and a surface layer that is mostly unaffected. Dissolved calcium and phosphate ions that are somewhat trapped in the adjacent dental plaque biofilm re-precipitate to form it. If remineralization mechanisms are undertaken, it is anticipated that the WSL can be reverted when the process is still at an early stage.

Objective: This study aimed to examine the ex-vivo remineralization effectiveness of chitosan + nanohydroxyapatite combination on artificially induced incipient lesion using Vickers microhardness tester.

Material and Methods: Artificial caries was created chemically by immersing 40 human teeth individually in demineralizing solution for 72 hours. Then the teeth were subjected to a 10-day pH cycle. Samples were assigned to four groups: (1) Control; (2) Chitosan (3) Nanohydroxyapatite and (4) Chitosan/nanohydroxyapatite complex. Surface microhardness measurements were performed prior to lesion formation, after lesion formation and after treatment. Diagnodent was used to assess mineral loss before and after demineralization. **Results:** The study's findings showed that the sample from group 4 (combination of chitosan and nanohydroxyapatite) had the highest level of enamel remineralization, and when compared to the control, there was a statistically significant difference($P<0.05$). The result of this study also showed that DIAGNOdent was unable to detect mineral loss in vitro. **Conclusion:** We can state that the combination of chitosan and nanohydroxyapatite promoted the remineralization of artificially induced incipient caries.

Keywords: Caries, Remineralization, Chitosan, Nanohydroxyapatite, Microhardness. Diagnodent.

INTRODUCTION

The initial clinical manifestation of enamel caries is a white spot lesion (WSL). It is comprised of a porous lesion body and a surface layer that is mostly unaffected. Dissolved calcium and phosphate ions that are somewhat trapped in the adjacent dental plaque biofilm re-precipitate to form it [(1)]. If remineralization mechanisms are undertaken, it is anticipated that the WSL can be reverted when the process is still at an early stage [(2).] The deposit of mineral onto demineralized enamel within the enamel lesion is caused by the movement of calcium and phosphate out of the tooth into the lesion., which is described as remineralization of carious lesions [(3)].

An N-deacetylated chitin derivative substance is chitosan, has gained a great deal of attention. Because of its action in encouraging enamel remineralization due to its readily available nature, bio - compatibility, biodegradability, and nontoxic nature, chitosan has been employed in dental biomaterial [(4,5).] Another compound which has anti carious properties is hydroxyapatite, a substance that is both bioactive and biocompatible and is frequently utilized in dental and medical procedures [(6)]. Nano-hydroxyapatite (NANO-HA) is identical to dental apatite in structure but it is more soluble, more bioactive and has better surface energy than hydroxyapatite [(7)]. According to available reports, NANO-HA may act as a remineralizing agent [(8,9,10)]. NANO-HA can defend teeth by adding a fresh coat of artificial enamel to the tooth's surface, rather than making the current layer harder by adding fluoride [(11)]. This capability of remineralization of early enamel decay under a pH cycle model has been shown in several research using NANO- HA as a biomimetic substance [(9,10)]. If NANO-HA is paired with some other efficient non-fluoride compound that will not affect Nano –HAP, could have a co-operative benefit in promoting remineralization. Then, full remineralization could be expected [(9)].

For our knowledge, no previous studies had been conducted to determine the combined impact of NANO-HA and chitosan. Therefore, the present study was aimed to assess how NANO-HA and chitosan together might affect the ex vivo remineralization of initial lesions, also to determine if the chitosan- NANO-HA mixture is more efficient at enhancing subsurface mineral deposition in WSL under a dynamic pH cycling and then to establish a strong base on which to apply these remineralizing materials in dental treatments. Mechanical characteristics as well as visible surface alterations following remineralization were evaluated using the Vicker surface microhardness as well as the fluorescence technique by DIAGNOdent. The null hypothesis has been stated that chitosan/ NANO-HA mixture has no additional effect on each of the material when applied separately on artificial WSL.

MATERIALS & METHODS

Sample preparation

40 human upper first premolars extracted for orthodontic purposes from individuals between the ages of 12 and 18 years old were chosen. The study excluded any teeth having apparent or identifiable caries, fillings,

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hypoplastic diseases, staining, cracking, and white spot lesions. The teeth were kept in a refrigerator, after debris removal from the surfaces of the teeth, in a solution of 0.1% thymol to avoid growth of bacteria and/or fungi until the beginning of the experimental process. the teeth's buccal surfaces were made flatten and polished by abrasive paper with grit sizes 400, 800, 1000, and 1200successively [12]. Nail polish was used to cover finished surfaces, leaving a workable circular window exposed (approximately 5 millimeters in radius) [13], using sticky tape.

Assignment to a group

Four groups were developed out of the sample by random (n=10) based on the kind of treatment:(1) Control with neither of the remineralizing agent to be examined ;(2) Chitosan group;(3) NANO- HAP group; (4) Chitosan/ NANO -HAP group.

Surface microhardness at baseline

A digitized Vickers microhardness equipment with an indenter diamond in shape was used to take the readings., 500 grams of weight, and 15 seconds of duration, respectively, pointed vertically at the surface of the enamel. The same calibrated apparatus and the same inspector had been used for all of the measurements. The hardness value for each specimen was calculated as the average of three indentations for each sample [14].

Laser fluorescence at baseline

Measurements of the laser fluorescence were made using a DIAGNOdent 2095 (KaVo, Biberach, Germany). The apparatus was validated with accordance to the operation instructions on a small calibrating stone before each test series. The values obtained were categorized by using Lussi and Hellwig classification [15].

Artificial enamel white spot lesion

The demineralization solution was made in accordance with **Ten Cate and Duijster** [16] in order to induce subsurface caries lesion and each sample was placed separately in disposable plastic vials measuring 25 ml, which contains 20 ml of the demineralization solution [12] throughout the 3 days at 37 Cº, after which the samples were rinsed with distilled water for twenty seconds [17].

pH cycle and treatment solution

To immitate the changes in pH that occur in the environment of the mouth, a pH cycling was employed. It consisted of three hours in demineralizing period and twenty-one hours in remineralizing period over the course of 10 days and the temperature was maintained at 37 C◦. According to **Ten Cate and Duijster** [16], a remineralizing solution was produced

Without receiving any treatment, the pH cycles were conducted for three days [18]. Then, throughout the following days (from day 4 to 10), the therapy was administered just after challenges of demineralization.

Group 1, the negative control, received no treatment

All of the teeth in group 2 received a treatment with a solution of 2.5 mg/ml chitosan for one minute with a little brush [19].

For group 3 which is the NANO-HA group, each tooth was soaked in a 10% NANO-HA solution. NANO-HA was prepared according to **Huang *et al*.** [10].

Using a tiny brush, a mixture of chitosan and NANO-HA were delivered to group 4, which is the Chitosan-NANO-HA solution group. It was formed using a modified form of the chitosan-bioglass material preparation method described by **Zhang *et al***. [20], by dispensing 2.5 g of NANO-HA powder into 10 ml of 2.5 mg/ml chitosan solution

After treatment, all teeth were kept in the remineralizing solution for 21 hours after being thoroughly rinsed and dried [16].

Characterization following therapy

The same specimens utilized before demineralization stage testing were characterized by laser fluorescence using DIAGNOdent and by the vicker surface microhardness test, after the development of an enamel white spot lesion and after the end of the pH cycle.

Statistical analysis

Statistical Package for Social Science was used for data interpretation, processing, and representation. (SPSS version -22, Chicago, Illionis, USA). Minimum, maximum, mean and standard deviation (SD), Shapiro-Wilk, Levene test, general linear model, repeated measurement of ANOVA with Bonferroni and Tukey's honestly significant difference (Tukey's HSD) were used. Statistical significance was defined as a P value of ≤ 0.05.

Ethical approval

This study was approved from The Research Ethics Committee, College of Dentistry Baghdad University. Every patient was given his consent form and was informed to participate in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

RESULT

Vickers surface microhardness

Concerning surface microhardness (SMH) at baselines, post demineralization and post treatment for each group there was no statistical significant changes among categories at either the baseline or after demineralization. While, there were statistically significant differences among groups in the remineralization stage. The highest SMH mean value were observed in Chitosan-NANO-HA group (279.850± 59.778) followed by NANO-HA group (245.85 ± 47.375), chitosan group (233.970± 63.623) and control group (166.570± 20.994) (Table 1).

Table (1): Descriptive and statistical test of surface microhardness among stages and groups.

Groups		Baseline	Demin	Remin.	F	P value
Control	Minimum	235.500	124.600	140.000	46.021	**< 0.01**
	Maximum	449.100	170.800	210.200		
	Mean	333.870	148.060	166.570		
	±SD	83.627	14.639	20.994		
Chitosan	Minimum	226.100	51.160	168.500	25.108	**< 0.01**
	Maximum	379.400	283.300	360.000		
	Mean	301.070	139.321	233.970		
	±SD	56.988	79.682	63.623		
NANO-HA	Minimum	201.100	72.450	186.500	21.206	**< 0.01**
	Maximum	360.000	215.700	340.900		
	Mean	276.030	138.845	245.850		
	±SD	51.070	42.640	47.375		
NANO-HA/chitosan	Minimum	241.700	101.500	216.500	40.991	**< 0.01**
	Maximum	468.200	195.700	386.200		
	Mean	344.400	143.643	279.850		
	±SD	67.200	34.017	59.778		
F		2.248	0.078	8.753		
P		0.099	0.972	0.000172		

After demineralization, mean of SMH, were significantly lower than baseline SMH values for all groups. In contrast to the control group, which did not significantly differ, SMH values after remineralization, were significantly higher than demineralization ones. The mean differences between demineralization stage and remineralization stage was the highest in the combination group as shown in table (2).

Table (2): Multiple pairwise comparisons of (SMH) among stages by groups using Bonferroni post hoc test.

Groups	Stages		Mean difference	p value
Control	Baseline	Demineralization	185.810*	**< 0.01**
		Remineralization	167.300*	**< 0.01**
	Demineralization	Remineralization	-18.510	>0.05
Chitosan	Baseline	Demineralization	161.749*	**< 0.01**
		Remineralization	67.100*	**< 0.01**
	Demineralization	Remineralization	-94.649*	**< 0.01**
NANO-HA	Baseline	Demineralization	137.185*	**< 0.01**
		Remineralization	30.180	>0.05
	Demineralization	Remineralization	-107.005*	**< 0.01**
Chitosan+ NANO-HA	Baseline	Demineralization	200.757*	**< 0.01**
		Remineralization	64.550*	**< 0.01**
	Demineralization	Remineralization	-136.207*	**< 0.01**

Regarding SMH between each pair of group analysis, a statistically significant differences were found between chitosan and control group, NANO-HA and control groups and chitosan-NANO-HA and control group. Furthermore, there was no statistically significant difference between various treatment groups as shown in table (3).

Table (3): Multiple pairwise comparisons of surface microhardness among groups in the treatment stage using Tukey's honestly significant difference (Tukey's HSD) post hoc test

Groups		Mean difference	P value
Control	Chitosan	-67.400*	< 0.05
	NANO-HA	-79.280*	< 0.01
	Chitosan+ NANO- HA	-113.280*	< 0.01
Chitosan	NANO- HA	-11.880	1.000
	Chitosan+ NANO- HA	-45.880	0.305
NANO-HA	Chitosan+ NANO- HA	-34.000	0.857

Readings of Laser Fluorescence (DIAGNOdent pen)
Table (4) displays the diagnostic scores for each group. The mean of the readings was (3.3±0.939).

Table (4): Mean and standard deviation of laser fluorescence readings at base line

Groups	mean±SD
Control	3.5±0.971
Chitosan	3.6 ±0.699
NANO-HA	3.2 ±1.135
Chitosan-NANO-HA	3.1 ±0.944

Following the demineralization procedure, the mean of the readings was 3.4 ± 0.933, the scores did not really differ from the initial state data, which is in accordance with the guidelines scores for the DIAGNOdent pen, stay in the sound stage (Table 5).

Table (5): Mean and standard deviation of laser fluorescence readings after demineralization

Groups	mean±SD
Control	3.66 ±0.699
Chitosan	3.6 ±1.173
NANO-HA	3.3 ± 0.948
Chitosan-NANO-HA	3.4 ±0.966

DISCUSSION

Surface microhardness results

A remineralization model of pH cycle was employed, in order to imitate the pH dynamics of the mouth. The pH cycles were repeated for three days without receiving any treatment, to enable the determination of baseline calcium uptake and loss values [18].

As there was standardization in the study (same tooth type and surface investigated, same PH cycling method), a non-statistically significant difference in microhardness readings for sound teeth and after demineralization were identified among different groups. All groups significantly showed drop in microhardness of the enamel surface after three days of demineralization, confirming the development of an artificial subsurface decay, since any reduction in the pH of the surrounding media just below the critical level (5.5) creates an acidic environment which leads the minerals of the tooth, primarily calcium and phosphorous, to migrate outward, creating micro porosity and lowering hardness [21].

This decrease in SMH values is consistent with the findings of **Mehta *et al*** [22].

The SMH was increased in all groups during the remineralization stage when compared to the demineralization stage, this could be explained by the rehardening and remineralizing capabilities of the different agents utilized in the study. MH was increased in the control group than in the demineralization stage, but the change was insignificant. This finding is not surprising since it demonstrates that remineralization may be achieved without remineralizing agent. This could be owing to the available mineral components contained in saliva, which are represented by the remineralizing solution in our study.

For the group treated with NANO-HA, SMH level was increased significantly than the demineralized stage. This came in agreement with **Huang *et al.* [8, 10], Nozari *et al.* [17].**
NANO-HA fills imperfections and pore structure immediately by depositing on the tooth surfaces that is demineralized. As a consequence, there have been reports of fewer holes and imperfections on the surface of enamel. together with enhanced enamel surface hardness [8]. However, in **Huang *et al.* [10]** early studies, testing using polarized microscopy and cross-sectional microhardness analyses, showed that NANO-HA aids mineral deposit in the exterior surface of the lesion instead of their interior. The severely mineralized upper surface may impede mineral ion diffusion into deep parts of a lesion. This might clarify why the application of NANO-HA did not result in full remineralization.

For the group treated with chitosan, statistically significant increase was observed in the remineralization stage than the demineralization one. This could be due to chitosan's positive charge that permits it to attach to surfaces with negative charge, including demineralizing external tooth surface Due to the amino group's chelating ability and its strong adhesion to enamel, chitosan will help serving as an excellent template for calcium(Ca^{2+}) and phosphate ($PO4^{3-}$) ions to rebuild a new tissue like enamel [23]. In this group, minerals were supplied from the remineralizing solution that mimic the composition of saliva.

No previous study had been conducted to study the remineralizing effect of chitosan alone on demineralized enamel, but it had been studied as a substance that prevents the release of essential minerals and hinders with the demineralization of dental enamel [19 ,24]

The group treated with chitosan-NANO-HA complex, when compared to the demineralization stage, the SMH was the greatest of all groups since the NANO-HA operate as an exogenous ion source and promotes remineralization whilst chitosan may act as an agent vehicle [25].

Moreover, since chitosan is a bio-adhesive, it improves the adherence of NANO-HA to demineralized enamel and acts like a reservoir collecting calcium and phosphate ions [26] and in doing so, provide enough ions to the demineralized enamel. There was no previous study to compare the result of the current study for chitosan/NANO-HA group, but many studies had been conducted that apply chitosan as a vehicle to transfer other

remineralizing agent, and proof the synergetic effect of such combination [25, 26].

Laser fluorescence results

Utilizing the DIAGNOdent lasers fluorescence technology, researchers have studied and monitored white-spot lesion [27].

This apparatus produces light wave with length of 655 nm; when activated by the light, changed tooth components glow at various wavelengths. The fluorescence is caused in part by bacteria' metabolic byproducts (porphyrins) [28].

The present study's findings demonstrated that Diagnodent laser fluorescence was not able to identify minerals lost in the study groups during the demineralization process. this may have resulted from the lack of metabolic products from microorganisms (Porphyrins), which are partially responsible for fluorescence. This observation is consistent with the theory put out by Limeback and Azarpazhooh [29] that microorganisms (porphyrins) are partially responsible for the fluorescence. It was stated that Diagnodent is ineffective in identifying artificial caries and in monitoring the reversal of white spot lesions. Our finding is further supported by the hypothesis of Hibst and Paulus [30] that DIAGNOdent measures respond to changes in organic components not the inorganic composition of tooth material.

CONCLUSION

It can be concluded that Chitosan/NANO-HA complex slurry exhibit remineralizing effect on artificial white spot lesion and it has the highest mean difference of microhardness when compared to control. However, there was no statistical difference among treatment groups. In addition, this study showed that DIAGNOdent was unable to detect mineral loss ex-vivo.

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