Original Article

Evaluation of Surface Properties of Two Remineralizing Agents after Modification by Chitosan Nano Particles: An *In vitro* Study

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Abstract

Background: Calcium phosphate-based systems have been introduced as promising bio-mimetic materials due to their close resemblance to the enamel. Chitosan and its derivatives have been an emerging biomaterial due to their additional antibacterial effect and promising re-mineralizing ability. **Aim:** The aim of this study is to evaluate the effect of chitosan nanoparticles on the remineralization of the demineralized enamel surface after being added to nano-hydroxyapatite and nano-calcium phosphate materials. **Setting and Design:** This was *in vitro* study. **Materials and Methods:** Twenty specimens of extracted permanent molars were collected, and then immersed in demineralizing solution, then distributed into four groups according to the remineralizing material. Group 1: Treated with Nano-beta-tricalcium phosphate (Nβ-TCP) gel, Group 2: Treated with Nβ-TCP with chitosan gel, Group 3: Treated with Nanohydroxyapatite (NHA) gel, and Group 4: Treated with NHA with chitosan gel. The surface hardness of the teeth was measured at baseline, after demineralization, and after remineralization. The structural changes in each group were analyzed using the scanning electron microscopy. **Statistical Analysis:** Shapiro–Wilk's test, one-way ANOVA followed by Tukey's *post hoc* test was used. **Results:** In all groups, there was a significant difference in mean Vickers hardness number (VHN) at different intervals, with the highest value found after treatment (301.64–395.65) VHN, followed by the baseline (236.97–276.15) VHN, while the lowest value was detected after demineralization (121.23–124.39) VHN. It was also indicated that baseline treatment, the Hardness percentage change (%) of the nano NHA + Chitosan group showed the highest significant value (55.10%), while the Nβ-TCP group exhibited the lowest significant value (9.56%). **Conclusions:** It can be concluded that NHA and NB-TCP modified by chitosan NPs as remineralizing agents of enamel surface hold promising results.

Keywords: *Calcium phosphates, chitosan, remineralization, scanning electron microscopy*

Introduction

Dental caries is one of the most serious oral health concerns worldwide, clinical data revealed that the permanent teeth are most susceptible to caries in the first 2–4 years of eruption. The teeth are subjected to a continuous cycle of demineralization and remineralization in the oral cavity, where demineralization is losing minerals from the tooth after being subjected to an acidic medium, while remineralization is minerals being restored into the tooth structure. Frequent demineralization and remineralization may lead to the development of initial carious lesions.[1-3]

In recent years, dentistry is directed to a more conservative approach called biomimetic remineralization, which attempts to imitate the natural mineralization process, it has been developed to reduce the enamel demineralization and enhance the remineralizing effect of oral care products, in addition to overcoming the hazards of using conventional fluoride products such as its limited remineralizing capacity, especially on grooves and pits, and the risk of fluoride toxicity.[1,4,5]

Numerous types of remineralizing agents are being used clinically after severally being tested and showing significantly satisfactory results. They have been classified into fluorides and nonfluoride remineralizing agents. Among the recently used nonfluoride, remineralizing agents are calcium phosphate-based nanomaterials, they are used as a source for releasing calcium/phosphate ions and increasing the supersaturation of hydroxyapatite (HA) in demineralized enamel lesions. Many forms of calcium phosphate are available in nature

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such as amorphous calcium phosphate (ACP), tetracalcium phosphate, monocalcium phosphate monohydrate, α-tricalcium phosphate (α-TCP), octacalcium phosphate, beta TCP (β-TCP), and HA. α-TCP is mainly used in bone cement, whereas β-TCP is commonly used in tissue engineering, orthopedics, maxillofacial surgery, and dental clinical applications.[6]

Hydroxyapatite shows the highest stability and the least solubility among calcium phosphate compounds. Its calcium-to-phosphorus ratio is 1:67 with Ca_{10} (PO_4) OH_2 as a molecular formula.[7]

Nano-HA has very close morphology, crystallinity, and structure as a natural enamel apatite, in addition to its good biocompatibility, nontoxicity, higher bioactivity, and mechanical properties compared to larger HA. It acts also as a calcium and phosphorus reservoir. Nano-HA is capable of tremendously increased the amount of remineralization in acidic conditions through the diffusion of more ions in the center of the demineralized area. It has the ability to enamel repairing and prevention of initial lesions progression due to the available adequate size and amount of the Ca_2+ and PO_2− 4 ions.[5]

A systematic review was done in 2022 by Anil *et al*., in 17 studies *in vitro* and 11 *in vivo*, it was reported in many trials that the use of nano-HA has an effective role in enhancing enamel remineralization.[4]

The utilization of natural products is an increasingly common form of alternative therapy which is found to be more and more useful nowadays in the arena of dentistry.

Chitosan is a natural carbohydrate complex of Chitin that is present in the exoskeleton of shrimps, insects, and crustaceans and can be obtained by 70% deacetylation of chitin in a basic solution.[8]

Chitosan has been added to many restorative materials such as glass ionomer, resin composite, and dental adhesives for enhancing their adhesive and antibacterial properties since it is a biocompatible material with low toxicity, antimicrobial, and antioxidant activity.[9]

Recently, the role of chitosan in enamel remineralization has been of great interest. In a study conducted by Arnaud *et al*. on artificial teeth subjected to remineralization-followed by demineralization cycles using chitosan with different concentrations then assessing the amount of diffusion of chitosan into the enamel using optical coherence tomography. It was observed that chitosan concentrations of 2.5 and 5.0 g/mL the dispersion were deep into the dentino-enamel junction and it was proved that chitosan has a very good ability to penetrate deep into the enamel carrying the required ions deeper into the lesion.[10]

Another study was done by Ruan *et al*. on the amelogenin-chitosan hydrogel studying the effect of increasing its viscosity and supersaturation on the alignment and dimensions of synthetic crystals. The results were evaluated by using the scanning electron microscopy and XRD and it was concluded that the orientation of the enamel crystals was significantly enhanced with more supersaturation, and there was a synthesis of larger and irregular enamel-like crystals when the viscosity of chitosan was increased from 1% to 2%.[11]

To the best of our knowledge, the modification of nano-hydroxyapatite and nano-calcium phosphate materials with chitosan nanoparticles has not been reported in studies done on enamel surface remineralization. Accordingly, the current study aimed to assess the effect of chitosan nanoparticles on the remineralization of the demineralized enamel surface of permanent molars when added to nano-hydroxyapatite and nano-calcium phosphate materials.

The null hypothesis is that there is no difference between the effect of nano-hydroxyapatite and nano-calcium phosphate after the addition of chitosan nanoparticles on the remineralization of the demineralized enamel surface of permanent molars.

Materials and Methods

The sample of this study was calculated based on the previous study by Vyavhare *et al*., 2015,[12] the reported microhardness for nano hydroxyapatite and calcium phosphate gel was 163.5 ± 15.4 and 115.0 ± 25.2, respectively. Using power 90% and 5% significance, level 5 samples in each group are required. The sample size was determined using the G*Power program (University of Düsseldorf, Düsseldorf, Germany).

The Research Ethics Committee, Faculty of Dentistry, October University for modern sciences and arts accepted the study protocol in compliance with the Helsinki Declaration, the protocol number: REC-D2109-2, on September 26, 2022.

Materials

The remineralizing materials that have been tested in this study are tabulated in Table 1.

Specimen preparation

Freshly extracted permanent molars with intact enamel surfaces were obtained from the oral surgery clinic of the faculty of dentistry, October University for Modern Sciences and Arts, Cairo, Egypt. Informed consent was obtained from the patients for the use of the extracted molars for this experimental study and for protecting patients' confidentiality. The teeth were examined carefully; the inclusion criteria were the permanent molars that were intact and free from cracks, spots, and other dental defects.

The selected teeth were cleaned from adherent tissues and remnants and then kept in distilled water for no longer than 2 months.

Table 1: Patch number, composition, and manufacturer of the remineralizing materials have been tested in this study

Material	Patch number	Composition	Manufacturer
Hydroxyapatite gel	HP202102	10% hydroxyapatite NPs + 5% carboxymethyl cellulose + 85% DH_2O	Nano Gate, Cairo, Egypt
Nano hydroxyapatite + chitosan NPs gel	HPCS202101	10% hydroxyapatite NPs + 2% chitosan NPs + 5% carboxymethyl cellulose + 83% DH_2O	
Nano β-tricalcium phosphate gel	CP202101	10% nano β-tricalcium phosphate NPs + 5% carboxymethyl cellulose + 85% DH_2O	
Nano β-tricalcium phosphate + chitosan NPs gel	CPCS202101	10% nano β-tricalcium phosphate NPs + 2% chitosan NPs + 5% carboxymethyl cellulose + 83% DH_2O	

DH_2O: Distilled water, NPs: Nanoparticles

Therefore, the tooth root was removed by diamond disks. Two rectangular specimens (3 mm × 3 mm) were obtained from each crown under water-cooling. A total of twenty specimens were collected, and each specimen was embedded into epoxy resin blocks.

Preparation of demineralized enamel lesions

Each specimen was immersed in 10 mL of demineralizing solution, the solution was renewed every 48 h for a total period of 1 week to develop enamel lesions.[13] The composition of the demineralizing solution was 50 mM acetic acid, 2.2 mM CaCl2, and 2.2 mM NaH2PO4 at pH 4.4.[14] The samples were submerged in distilled water for 1 min following the demineralization process, and then sonicated for 5 min to remove any debris and stored in distilled water.

Remineralization of the demineralized enamel lesions with testing materials

The demineralized specimens were carefully wiped to eliminate the excess water, then the specimens were allocated to four groups according to the remineralizing testing materials: Group 1 (n = 5): Treated with Nano β-TCP (Nβ-TCP) gel, Group 2 (n = 5): Treated with Nβ-TCP with chitosan gel, Group 3 (n = 5): Treated with Nano-Hydroxy-apatite (NHA) gel, and Group 4 (n = 5): Treated with NHA with chitosan gel.

Each remineralizing gel was applied with a copious amount covering the specimen surface by using a micro-brush, and after 6 h, the specimens were washed with distilled water to remove the gel and left to dry.

The remineralization procedure was repeated seven times for a total remineralization period of 42 h. Six hours of remineralization were corresponding to remineralization in the oral cavity during the night.[15]

Surface hardness testing

Digital Vickers hardness tester (NEXUS 4000 TM, INNOVATEST, model no. 4503, Netherlands) was used for surface hardness testing at the following intervals: Baseline, after the development of demineralized enamel, and after remineralization. The surface hardness testing was performed with a load of 200 g for 15 s; three points were made on the surface of each specimen. The impression diagonal length was measured, and the hardness was calculated by using the following equation: Vickers hardness number (VHN) $=1.854P/d^2$, where P represents the indentation load and d is the impression diagonal length.

Scanning electron microscope

After surface hardness testing, a representative specimen from each group was examined using Scanning electron microscope (SEM) Quanta FEG 250; a baseline specimen, demineralized enamel specimen, and a specimen from each remineralized group "Nβ-TCP, Nβ-TCP with Chitosan, NHA, and NHA with Chitosan."

Statistical analysis

The numerical data were expressed as mean and standard deviation values. Data normality was investigated using the Shapiro–Wilk's test and by displaying data distribution. Data revealed that parametric distribution was analyzed using the one-way ANOVA followed by Tukey's *post hoc* test. In all tests, the significance level was set at <0.05.

The statistical analysis was accomplished with R statistical analysis software version 4.1.3 for windows (R Core Team 2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.)

Results

Microhardness data showed a parametric distribution within the group's results. Hardness values of inter and intra-group comparisons are presented in Table 2, at baseline and after treatment, there was a significant difference between the values of different groups respectively. At baseline, *post hoc* pair-wise comparisons showed Nβ-TCP to have a significantly higher value than NHA. While after treatment, they showed all groups to have significantly different values from each other.

Within all groups, as presented in Table 2, there was a significant difference at different intervals between the values measured with the highest value found after treatment, followed by baseline, while the lowest value was measured after demineralization. All *post hoc* pairwise comparisons were statistically significant.

Results of intergroup comparisons for hardness percentage change (%) are presented in Table 3, for demineralization treatment and baseline treatment intervals, there was a significant difference between different groups. While for the difference between baseline and demineralization, the difference was not statistically significant.

For demineralization-treatment *post hoc* pair-wise comparisons were all statistically significant. While for baseline treatment, they showed the nano NHA + Chitosan group to have a significantly higher value than other groups. In addition, they showed the Nβ-TCP group to have a significantly lower value than other groups.

SEM micrographs demonstrated that enamel has a normal prismatic surface with a fish scale appearance as in Figure 1a. After demineralization, the micrographs as in Figure 1b show many surface defects, roughness with occasional cracks, and pores resulting from the dissolution of the organic-inorganic elements. After treatment with the remineralizing agents, the micrographs as in Figure 1c-f showed a noticeably smoother surface, the irregularities are repaired, and it is more likely to resemble the natural enamel surface before treatment.

Discussion

Enamel demineralization is a slowly progressing process, accordingly, its early detection and proper management play an important role in the possibility of its reversal.[5]

Different systems delivering calcium-phosphate have been postulated, these systems comprise either crystalline or ACPs in addition to fluoride-based formulations.[16]

Calcium phosphate-based systems have been introduced as promising bio-mimetic materials due to their close resemblance to enamel.[17] β-TCP is an alluring calcium phosphate-based complex due to its bioactivity and excellent compatibility with all biological systems. B-TCP is soluble at pH <6, so it shows a high concentration of calcium in the saliva which will be later utilized during the remineralization process.[17]

Another type of calcium phosphate as a biomimetic remineralizing agent is the nano-hydroxyapatite, they are

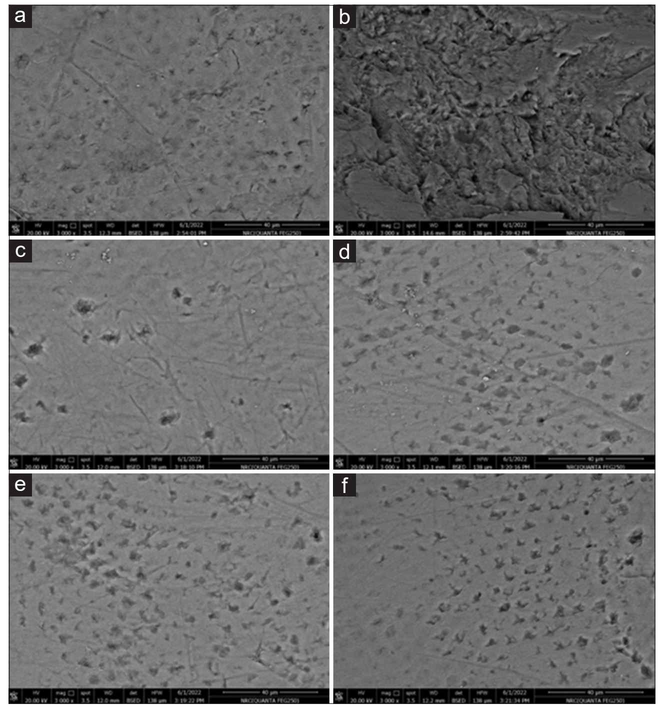

Figure 1: (a) SEM micrograph of baseline group representative, (b) SEM micrograph of demineralized group representative, (c) SEM micrograph of Nano Hydroxy apatite (NHA) + Chitosan group representative, (d) SEM micrograph of NHA group representative, (e) SEM micrograph of nano-beta-Tricalcium phosphate (Nβ-TCP) group representative, (f) SEM micrograph of Nβ-TCP + Chitosan group representative

Table 2: Inter and intragroup comparisons for hardness values

Measurement	Hardness, mean±SD				*F*	*P*
	Nβ-TCP	Nβ-TCP + chitosan	NHA	NHA + chitosan		
Baseline	276.15±9.00[A,b]	262.94±16.24[A,B,b]	236.97±4.59[B,b]	256.73±22.46[A,B,b]	6.11	0.006*
After demineralization	123.89±1.09[A,c]	123.60±7.15[A,c]	121.23±3.71[A,c]	124.39±6.31[A,c]	0.37	0.774
After treatment	301.64±3.90[D,a]	361.69±4.49[B,a]	331.91±1.58[C,a]	395.65±2.51[A,a]	734.33	<0.001*
F	1833.89	720.16	4467.57	501.22		
P	<0.001*	<0.001*	<0.001*	<0.001*		

Means with different upper and lowercase superscript letters within the same horizontal row and vertical column respectively are significantly different (*) significant (*P*<0.05). SD: Standard deviation, NHA: Nano hydroxy apatite, Nβ-TCP: Nano beta-tricalcium phosphate

Table 3: Intergroup comparison for hardness percentage change (%)

Measurement	Hardness percentage change (%) (mean±SD)				*F*	*P*
	Nβ-TCP	Nβ-TCP + chitosan	NHA	NHA + chitosan		
Baseline - demineralization (decrease)	55.09±1.71[A]	52.87±3.69[A]	48.19±0.47[A]	51.14±6.16[A]	3.09	0.057
Demineralization - treatment (increase)	143.50±4.35[D]	195.34±4.50[B]	174.01±9.11[C]	218.65±14.48[A]	61.51	<0.001*
Baseline - treatment (increase)	9.56±0.72[C]	40.57±4.07[B]	40.11±2.95[B]	55.10±14.21[A]	32.24	<0.001*

Means with different superscript letters within the same horizontal row are significantly different (*) significant (*P*<0.05). SD: Standard deviation, NHA: Nano hydroxyapatite, Nβ-TCP: Nano beta-tricalcium phosphate characterized by having a chemical and crystal structure like that of enamel.[18,19]

Prophylactic approaches would show more effectiveness in caries prevention if combined with antibacterial principles. From this aspect, remineralization would show efficient results by combining the remineralization systems with natural antibacterial materials.[5,18]

Chitosan and its derivatives have been an emerging biomaterial.[20] The use of chitosan in its nano form showed better absorption and adhesion properties in contrast to chitosan solution.[21] The use of hydrogel increases the contact time between the active ingredients and the enamel surface.[18] Following these principles, hydrogels containing nano chitosan were introduced for enamel remineralization, stabilizing CP clusters and guiding their arrangement.[22]

On the other hand, it was postulated that chitosan's impact on remineralization is minute; however, its effect on anticariogenic activity is noticeable. Consequently, chitosan was suggested to be used as a substrate to immobilize the Nano-building CP units, thus amorphous to poorly crystalline nonstoichiometric HP is formed.[5]

The hybridized Chitosan Calcium Phosphate microgel as a remineralizing agent exhibits several advantages; the fact that chitosan is bio-adhesive ensures better adhesion between the hybridized chitosan calcium phosphate and enamel with demineralized defects, it is also considered a reservoir for calcium and phosphates ions thus supplies the demineralized enamel surface by the sufficient number of ions needed for its biomimetic mineralization.[22]

Microhardness assessment is interconnected to the enamel strength arising from the unity of minerals adhered within the enamel structure.[5]

Dental caries plays a critical role in reducing enamel microhardness.[23] Therefore, enamel mineral content can be quantified indirectly by measuring the surface microhardness indicating any enamel surface alterations.[21]

Accordingly, this study aimed to determine the effect of chitosan nanoparticles in the remineralization of the enamel surface when added to NHA and NB-TCP materials by assessing their microhardness and by SEM examination.

The null hypothesis was rejected as the addition of chitosan nanoparticles had a significant effect on the remineralization of the demineralized enamel surface; at baseline treatment, the nano NHA + Chitosan group had the highest significant value while the Nβ-TCP group exhibited the lowest significant value.

In the current study, the VHN was used to determine the surface microhardness, it is characterized by its high accuracy and the possibility of applying forces with different sizes and repeated measurements.[24-26]

Results of the microhardness test revealed that the NHA group showed a higher mean value than the Nβ-TCP group, the higher effect of NHA might be attributed to the elevation of calcium concentration, which could be explained by the ability of NHA in assisting ions to diffuse in the middle of the demineralized area, thus increasing the enamel renovation. Nanosized particles can block any tiny porosity in the demineralized area and aid in the creation of a new apatite layer by its scaffolding action through captivating calcium and phosphate ions from surrounding saliva to the enamel surface. This was in accordance with Juntavee *et al*., 2021 and Juntavee *et al*., 2021.[5,27]

Moreover, Sari *et al*., 2021, stated that NHA particles because of their tiny size, fill up small pores and depressions on the demineralized enamel surface thus repairing it.[18]

Results of the micro-hardness test also showed that NHA + Chitosan group possessed significantly the highest value followed by NB-TCP + Chitosan then the NHA group, while the NB-TCP group had significantly the lowest value. Thus, the addition of chitosan had a significant effect on the enamel microhardness.

These findings might be explained by the action of chitosan as a template in the biomineralization process and its control of the mineral crystallites through its molecular interaction with the minerals.[22] In acidic media, the chitosan macromolecule becomes positively charged due to the protonation of its amino groups. Accordingly, the negatively charged surfaces as the enamel becomes attractive to the positively charged chitosan, thus adhesion between the chitosan calcium phosphates and the demineralized enamel is enhanced, where chitosan acts as a reservoir for calcium and phosphates ions, providing the demineralized enamel by the number of ions required for its biomimetic mineralization.[22]

Also, Yilmaz *et al*., 2020 and Yan *et al*., 2021,[28,29] stated that the broad-spectrum antimicrobial activity of chitosan results in its interaction with gram-positive and gram-negative bacteria, forming a film that acts as an effective antibacterial barrier on the tooth surface, which helps to significantly increase the surface microhardness.

Moreover, chitosan molecules transport mineral ions into carious lesions inhibiting enamel demineralization.

The size of chitosan nanoparticles allows them to invade the surface layer into the deep carious defects and chemical bonds formed between the amino groups of chitosan and the metal ions hydroxyapatite of demineralized enamel prisms. This was supported by Magalhães *et al*., 2021.[21]

Our results were in accordance with Song *et al*. in 2021,[30] they prepared carboxy-methyl chitosan and lysozyme nano gels encapsulating ACP and assessed its ability to form a prismless enamel-like layer on the surface of demineralized enamel. Their results indicated that the incorporation of chitosan had a significant effect in the formation of an aprismatic enamel-like layer, concluding that this material might be promising for the treatment of early enamel caries.

These results were confirmed by the descriptive characteristics of the remineralized enamel surface revealed by the SEM analysis as shown in Figure 1c-f. All groups treated with the remineralizing agents exhibited smooth and homogeneous surfaces with no porosity, resembling that of natural enamel. This was in accordance with Juntavee *et al*., 2021.[5]

Limitation (s) and future prospective

Since this study was conducted in a laboratory environment, it was not conceivable to mimic the *in vivo* characteristics, speediness, and flow rate of saliva. Accordingly, supplementary clinical studies are further required.

Conclusions

Within the limitations of this study, it can be concluded that NHA and NB-TCP materials modified by chitosan NPs as remineralizing agents of enamel surface hold promising results. Additional clinical trials are required to verify the remineralization prospective of these novel materials.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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