Original Article

The Impact of Chitosan and Ozonated Water on the Remineralization of White Spot Lesion *In Vitro*

Fatima Saeed J. Al-Ward, Nada Jafer M. H. Radhi

Department of Pediatric and Preventive Dentistry, College of Dentistry, University of Baghdad, Baghdad, Iraq

Abstract

Background: Dental caries is a complex, dynamical process that is controlled and initiated by a variety of variables. **Objective:** This study aimed to examine the ex-vivo remineralization impact of chitosan pretreated with ozonated water on artificially-induced incipient lesion. **Materials and Methods:** Artificial white spot caries was produced chemically by immersing 44 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) Ozonated water and (4) Chitosan application after treatment with 0.1 mg/ml ozonated water. Surface microhardness measurements were performed prior and after lesion formation then after treatment. Vickers microhardness tester and scanning electron microscope for representative teeth from the study sample were performed to assess enamel structure quantitatively and qualitatively. **Results:** The study's findings showed that the sample from group 4 (chitosan after ozonated water treatment) had the highest level of enamel remineralization, and when compared to the control, there was a statistically significant difference($P<0.05$). **Conclusion:** It could be stated that the combination of treatment of chitosan and ozone promoted the remineralization of artificially induced white spot lesion.

Keywords: Caries, chitosan, microhardness, ozonated water, remineralization

Introduction

Dental caries is a complex, dynamical process that is controlled and initiated by a variety of variables.[1] Even while investigation has improved our understanding of tooth caries and reduced its prevalence, certain studies have found that dental caries is still a significant tooth illness.[2] In the oral cavity there are two natural events, demineralization and remineralization. Diet, tooth care, and bacterial growth could all encourage demineralization to prevail. Saliva's buffering properties promote phosphate and calcium to deposit on teeth and produce newer mineral, facilitating remineralization.[3] As a result, modulating the demineralisation-remineralisation equilibrium is essential for preventing dental cavities.[4]

For all carious teeth, the standard course of therapy is the removal of the caries and its replacement with a restoration.[5] Nevertheless, many years of research have resulted in the "minimally invasive" strategy, which includes finding and treating these spots earlier, focusing prevention over the old surgical technique.[3]

Following the development of minimal interventions dental treatment for treatment dental decay, ozone had been presented as an innovative technique to support in reversing early carious lesion. It is a powerful oxidant that is extremely bactericidal against *Streptococcus mutans*[6] and has been used in the management of initial cemental decay,[7] caries in dentin hypersensitivity,[8] and has powerful antimicrobial effects on anaerobic microorganism isolated from patients with periodontitis.[9,10] Ozone's remineralizing impact on early enamel caries is continuously being questioned. Huth *et al.*[11] stated that using ozone in individuals with a high caries risk helped

Address for correspondence: Dr. Fatima Saeed J. Al-Ward, Department of Pediatric and Preventive Dentistry, College of Dentistry, University of Baghdad, Health Directorate, Baghdad, Iraq.
E-mail: fatima.yahya1901@codental.uobaghdad.edu.iq

Submission: 01-Nov-2022 **Accepted:** 13-Nov-2022 **Published:** 27-Apr-2023

How to cite this article: J. Al-Ward FS, M. H. Radhi NJ. The impact of chitosan and ozonated water on the remineralization of white spot lesion *in vitro*. Med J Babylon 2023;20:33-40.

Access this article online

Quick Response Code:

Website: www.medjbabylon.org

DOI: 10.4103/MJBL.MJBL_261_22

to recover early fissure caries over a three-month period. Ozone can be delivered onto the tooth surface using either gas or water.[12] Ozone could be applied also as gel.[13]

Another promising agent used in medicin and dentistry is chitosan, an N-deacetylated chitin derivative that is biocompatible and non-toxicic.for this reason, chitosan has found widespread use in biomedical disciplines such as medication administration, wound treatment, tissue substitution, and so on.[14]

Chitosan had been used as a remineralizing therapy for white spot lesion in combination with other materials like amelogenin,[15] phosphorylated chitosan,[16] chitosan after pretreatment with bioglass[17] and calcium phosphate[18] and had been proven to be an efficient combination.

This study was carried out to detect the impact of chitosan, ozonated water, and the resultant impact of both on the remineralization of early enamel lesions in *ex vivo* over a dynamic pH cycle subsequently to lay a powerful basis for the use of these non-fluoride anti-caries compounds in dental setting.

Materials and Methods

This study included 45 permanents, sound, human upper first premolars extracted for orthodontic purposes between the ages of 12 and18 years.

The study excluded teeth with cracks, discoloration, hypoplastic abnormalities, obvious caries, restorations, and white spot lesions. To avoid bacterial and/or fungal infection, the teeth were kept in a solution that contains 0.1% thymol and preserved in the refrigerator. Each tooth's buccal surface has become flattened and smooth using abrasive paper with grit sizes 400, 800, 1000, and 1200, respectively.[4] A usable circular window with a radius of about 5 millimeters was left visible after finished surfaces were covered with nail polish using adhesive tape.[19]

46 teeth made up the entire sample. A scanning electron microscope was used to inspect two teeth, one for healthy and one for demineralized enamel after creation of white spot lesion). The remaining 44 teeth were assigned randomly into four treatment groups n = 11), as follows: 1) Control using neither of the remineralizing agents;2) Chitosan group;3) ozonated water group;4) ozonated water then chitosan group. One tooth from each group was chosen to be examined under a scanning electron microscope SEM), and the remaining ten were utilized for a test of the surface microhardness.

According to Ten Cate and Duijsters,[20] the demineralization solution was made, and each sample was placed separately in disposable plastic vials measuring 25 ml that contained 20 ml of the demineralizing solution.[5] Throughout three-day period at 37 C, after which the samples were gently rinsed with distilled water for 20 seconds.[21]

To imitate the pH dynamic behavior of the oral cavity media, a remineralization pH cycle model was used, which include three hours in demineralizing solution and twenty-one hours in remineralizing solution and the temperature was maintained at 37C°.[20] Without the test agent, the pH cycles are carried out for three days to establish starting point for calcium absorption and loss.[22] For the days that follow days 4 through 10), the therapy is administered once daily for a certain amount of time, depending on the agent, immediately after the demineralization challenge.

For group 1 which is the control negative no treatment was performed.

For group 2 which is the chitosan group application a solution of 2.5 mg\ml chitosan was applied by means of small brush for 1 minute.[23]

For group 3 which is the ozone group, all teeth were rinsed by 0.1 ppm ozonated water for one minute.[12]

For group 4, the teeth were washed with ozonated water of 0.1 ppm for 1 minutes and dried then chitosan solution 2.5 mg\ml is applied by small brush for 1 minute.

Ozonated water was prepared using corona discharge ozone generator which installed in accordance with the owner's manual and the bubbling stone of the device was placed into the bottom of 1000 ml plastic graduated cylinder that contained iced distilled water.

To be sure that the concentration of ozone in water is correct, and keep it constant during the work, a chemical diagnostic test by specialized kit CHECKIT® Comparator) was used according to the operating instructions. Throughout the course of the experiment, the demineralizing and remineralizing solutions were newly prepared each third day, whereas the treatment solutions have been made every day.

Three measurements of surface microhardness were taken: once at the baseline, once following the development of a white spot lesion, and once after the pH cycle was finished. The tests were taken using a digital Vickers microhardness tester with an indenter diamond in shape that was pointed vertically at the enamel surface and had a load and time of 500 gram and 15 seconds, respectively. The same calibrated equipment and examiner were used for all of the measurements. The hardness value for each specimen was determined by calculating the mean of three indentations for every reading.[24]

Surface morphology was qualitatively analyzed using a scanning electron microscope. The specimens were then placed inside a coating machine with a vacuum system, allowed to air dry, and then were coated with gold. Then the samples were ready to be scanned by SEM at ×10000 and 20000 magnifications[25]

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 utilized. Statistical significance was defined as a P value of ≤ 0.05.

Ethical approval

The Declaration of Helsinki, the ethical code of the World Medical Association, was followed when conducting this research. This study was approved from The Research Ethics Committee, College of Dentistry Baghdad University. A local ethics committee evaluated and approved the study protocol as well as the consent form and subject information according to the document number 335321 in 4-4-2021 by College of Dentistry/ University of Baghdad to get this approval.

Results

Vickers surface microhardness

[Figure 1] displays the mean of all study groups at baseline, following demineralization, and after therapy. The mean, standard deviations, and percentages of surface recovery for the microhardness at baseline, after demineralization and following treatment are displayed in [Table 1]. No statistical significant changes were found among groups at either the baseline or after demineralization. While groups' differences in the remineralization stage were statistically significant. The chitosan group pretreated with ozonated water had the highest SMH mean value, followed by the chitosan group, ozonated water group, and control group. Additionally, there is a notable reduction and increase at various phases within various study groups.

For all groups, the mean SMH levels following demineralization were significantly less than the baseline SMH values, as presented in [Table 2]. SMH values after remineralization were statistically considerably greater than those after demineralization, with the exception of the control group, which showed no statistically significant change.

The same table reveals that the combination group chitosan pretreated with ozonated water) had the highest mean differences between the demineralization and remineralization stages p<0.01).

Between each pair of group analysis which is demonstrated in [Table 3], there were statistically significant differences between the control group and the chitosan group., chitosan pretreated with ozonated water and control groups with the highest mean difference was in the combination group, while ozone alone shows no statistical difference in comparison to the control group in the remineralization stage $P > 0.05$).

Scanning electron microscope

The goal of the SEM investigation was to assess the surfaces' topographic alterations. of one representative sample from each study group in addition to one sound tooth and one tooth after demineralization.

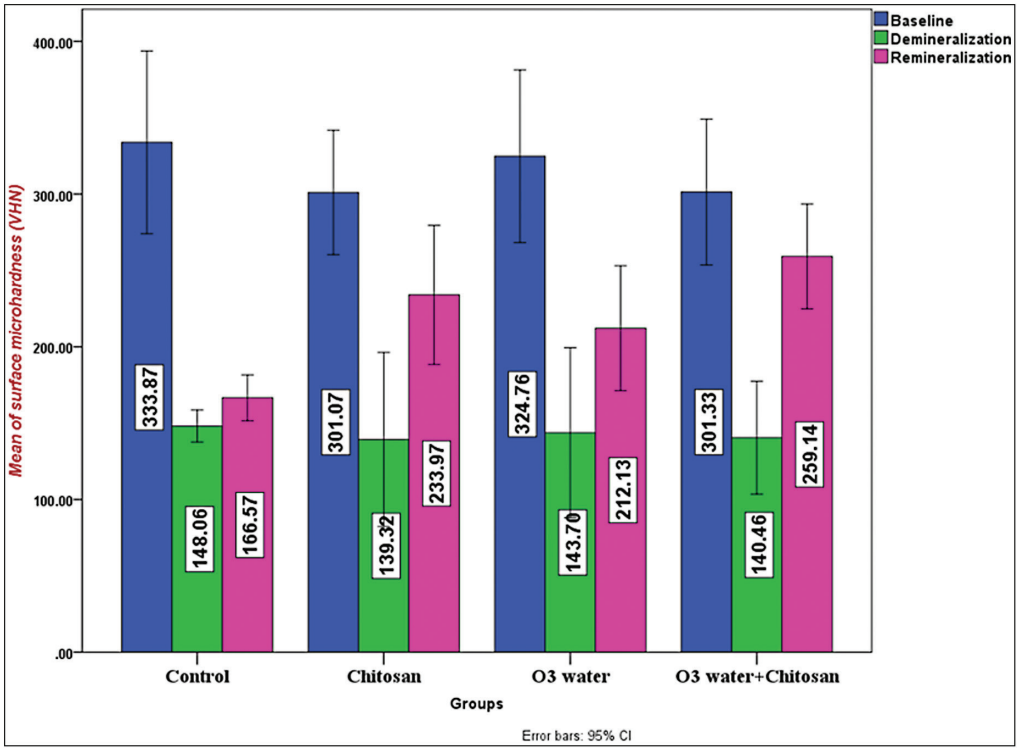

Figure 1: Mean of enamel microhardness at baseline, after demineralization, and after remineralization

Table 1: Descriptive and statistical of surface microhardness among stages and groups

Groups		Baseline	Demin	Remin.	F	P value
Control	Minimum	235.500	124.600	140.000	36.481	0.000
	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	22.401	0.000
	Maximum	379.400	283.300	360.000		
	Mean	301.070	139.321	233.970		
	±SD	56.988	79.682	63.623		
O3 water	Minimum	231.300	51.200	110.800	26.235	0.000
	Maximum	475.500	300.000	310.000		
	Mean	324.760	143.698	212.130		
	±SD	78.988	77.866	57.181		
O3 water +Chitosan	Minimum	236.900	73.200	185.200	26.709	0.000
	Maximum	450.300	214.700	342.000		
	Mean	301.330	140.464	259.140		
	±SD	66.730	51.638	47.951		
F		0.530	0.040	6.134		
P		0.666	0.989	0.002		

Table 2: Multiple Pairwise Comparisons of surface microhardness among stages by groups using Bonferroni post hoc test

Groups	Stages		Mean difference	p value
Control	Baseline	Demineralization	185.810	**0.000**
		Remineralization	167.300	**0.000**
	Demineralization	Remineralization	-18.510	0.899
Chitosan	Baseline	Demineralization	161.749	**0.000**
		Remineralization	67.100	**0.006**
	Demineralization	Remineralization	-94.649	**0.000**
O3 water	Baseline	Demineralization	181.062	**0.000**
		Remineralization	112.630	**0.000**
	Demineralization	Remineralization	-68.432	**0.001**
O3 water+Chitosan	Baseline	Demineralization	160.866	**0.000**
		Remineralization	42.190	0.125
	Demineralization	Remineralization	-118.676	**0.000**

Table 3: Multiple Pairwise Comparisons of surface microhardness among groups in the treatment stage using Tukey's Honestly Significant difffrence

Groups		Mean difference	P value
Control	Chitosan	-67.400	**0.029**
	O3 water	-45.560	0.298
	O3 water +Chitosan	-92.570	**0.001**
Chitosan	O3 water	21.840	0.999
	O3 water +Chitosan	-25.170	0.999
O3 water	O3 water +Chitosan	-47.010	0.259

SEM analysis revealed the smooth and homogeneity in the pattern of the sound enamel surface that appear smooth with undamaged enamel surface structure, devoided from any alteration as shown in [Figure 2].

The dense enamel structure, however, was lost in SEM investigations of demineralized etched enamel surfaces having a large number of interspaces, showing a noticeable roughness with small pores, and cavitations in the whole surface of the demineralized enamel as shown in [Figure3].

For control group, SEM still show the porosities and surface irregularities; [Figure 4].

[Figure 5] demonstrates partial covering of the porosity in enamel with recovered mineralized tissue after SEM analysis of remineralized enamel restored with chitosan solution.

For group treated with ozonated water show surface porosities and roughness, [Figure 6].

[Figure 7] show enamel surface treated with chitosan after pretreatment with ozonated water. A notable characteristic of this group was a remarkable reduction in surface imperfections and porosities. A uniform and dense layer of mineralized tissue was created by the regenerated crystals on the enamel surface.

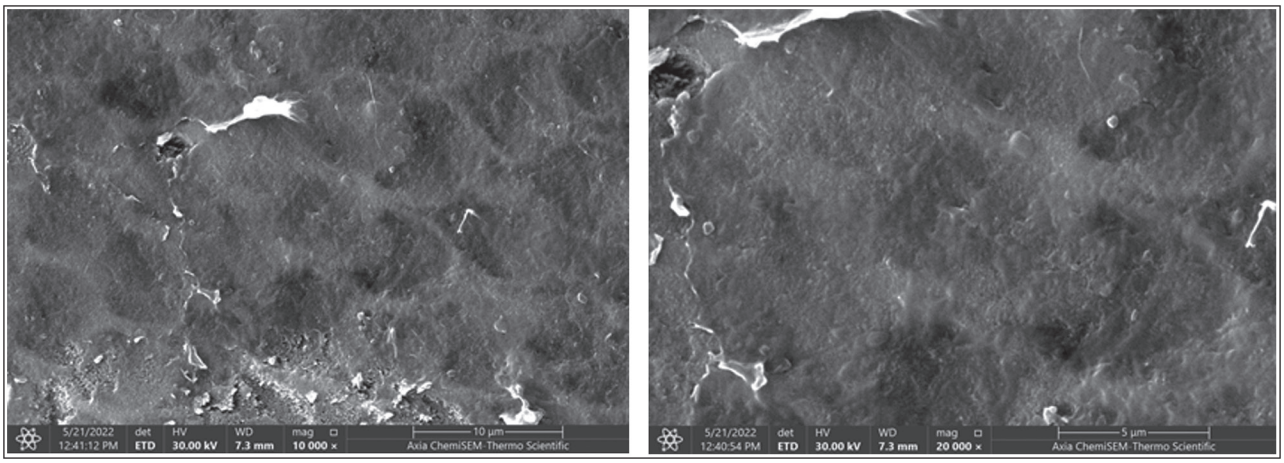

Figure 2: Enamel surface of the sound tooth. A 10000 x B20000 x

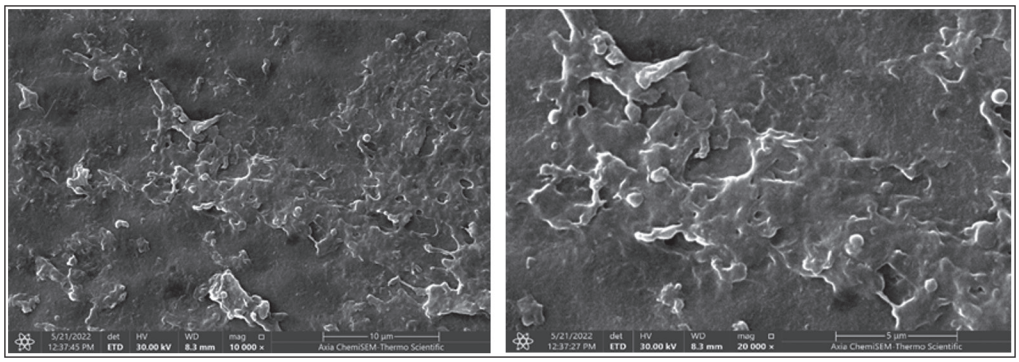

Figure 3: Enamel after demineralization. A 10000 x B20000 x

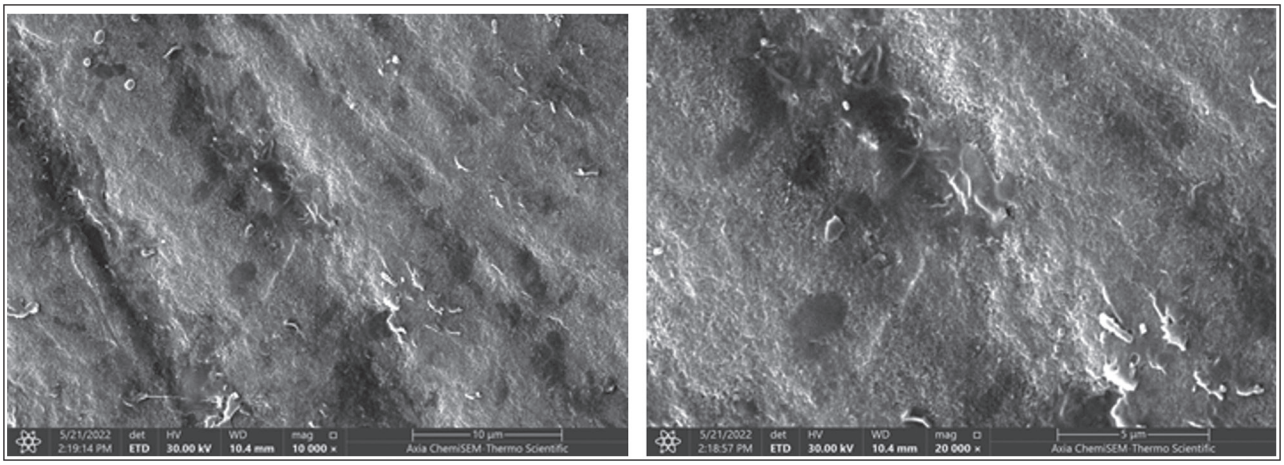

Figure 4: Enamel surface in the control group. A 10000 x B 20000 x

Discussion

Evidence for the efficacy of ozone therapy in remineralizing white spot lesions is inconclusive.[26] On the other hand, Chitosan has been utilized as a substance that suppresses the demineralization of dental enamel, rather than a remineralizing agent.[23,27,28] Moreover, chitosan's ability to remineralize demineralized enamel has previously been tested in conjunction with other remineralizing agents.[15-18,29] The purpose of the current investigation was to determine the impact of chitosan, ozonated water, and chitosan following ozonated water pretreatment on the *in vitro* remineralization of white spot lesions.

Microhardness measurements for healthy teeth and after lesion development showed statistically insignificant variations between the groups, as the investigation was standardized (same kind of teeth were utilized, the

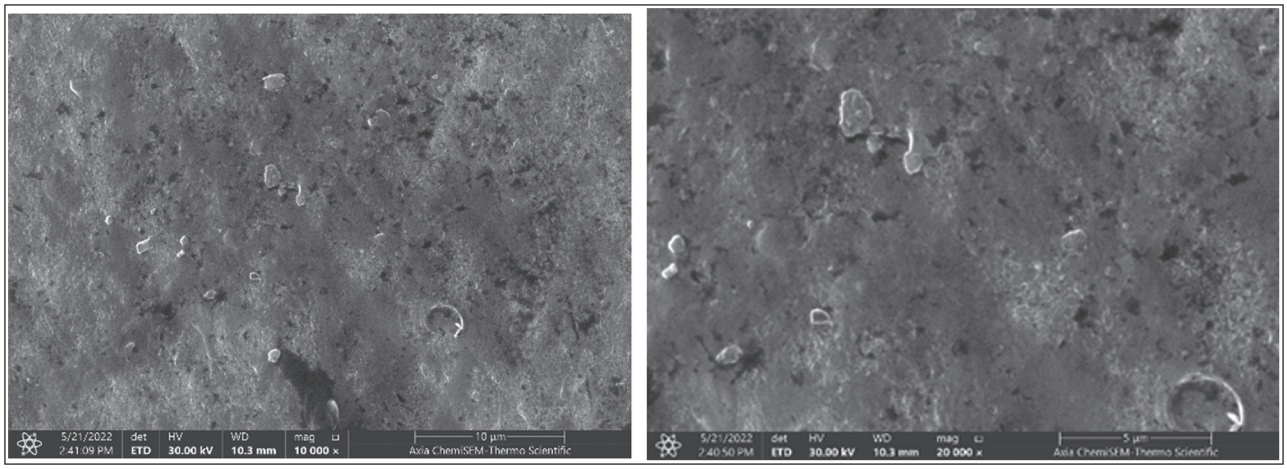

Figure 5: Enamel surface after treatment with chitosan. A 10000 x B 20000 x

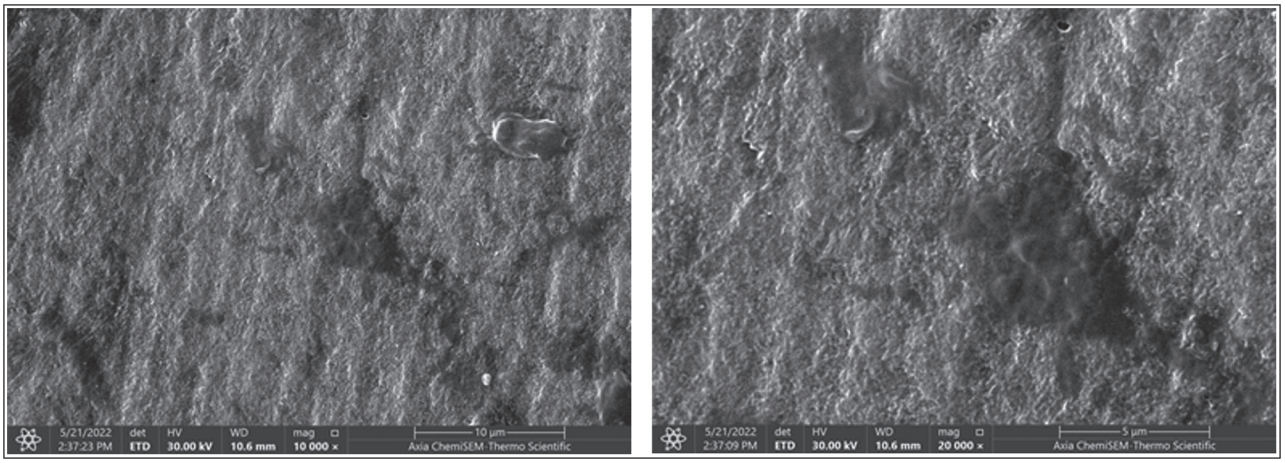

Figure 6: Enamel surface after treatment with ozonated water A 10000 x, B 20000 x

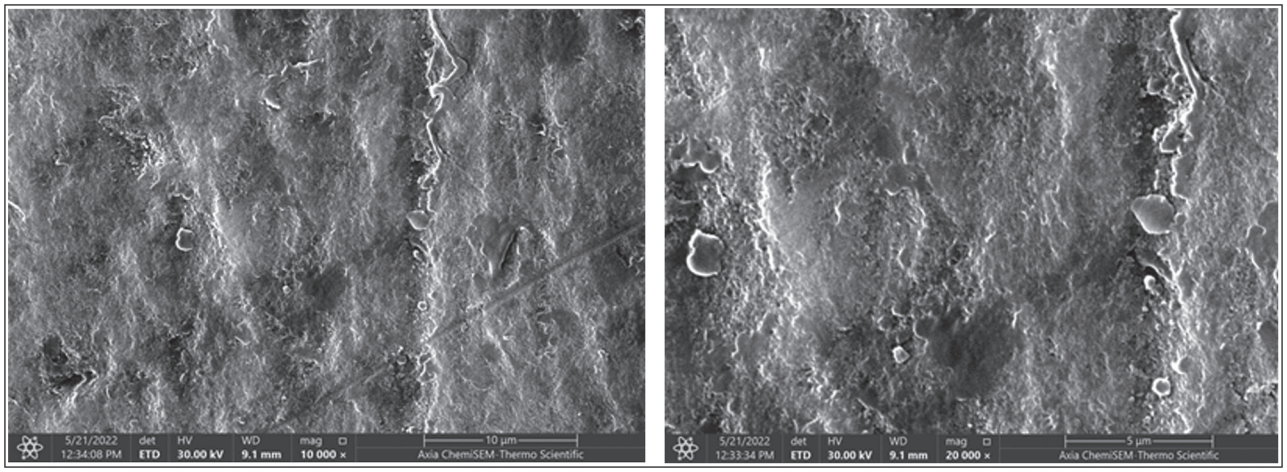

Figure 7: Enamel surface after treatment with chitosan after pretreatment with ozonated water A 10000 x, B 20000 x

same tooth surface was inspected, and the same pH cycle method was employed). After three days in the demineralization stage for all groups, a statistically highly significant decline in the enamel surface microhardness was discovered as a sign of the onset of an artificial white spot lesion, because any drop in the pH of the environment below the critical pH 5.5) creates an acidic environment that encourages the tooth minerals to migrate outward, resulting in micropores and reducing hardness).[30] This decrease in SMH values was similar to the findings of Mehta *et al.*,Li *et al.*,Zhang *et al.*, Lata *et al.*[2,31-33]

The rehardening and remineralizing capacities of the various agents used in the investigation may account for the SMH increasing in all study groups during the remineralization stage compared to the demineralization stage.

Because chitosan's positive charge permits it to attach firmly to negative charges, like demineralizing enamel, a statistically significant increase was seen in the remineralization stage in the group treated with chitosan compared to the demineralization stage. The amino group's chelating activity and strong adsorption to enamel make chitosan can function as a useful template for calcium Ca2+) and phosphate PO4-) ions to reconstruct more recent mineralized tissues that resemble enamel.[16] In this case, minerals are received from the remineralizing solution that closely resemble the minerals found in saliva

No prior research had been done to study the remineralizing effect of chitosan alone on demineralized enamel, but it had been studied as an agent that hinders the release of mineral elements during the demineralization process of dental enamel.[23,27,28]

A statistically significant increase was seen in the remineralization stage for the group that received ozonated water compared to the demineralization stage and this came in accordance with Samuel *et al.,*2016.[16] The mean difference between the two stages was less than that of chitosan, while the group treated with chitosan after treatment with ozonated water was the highest microhardness after remineralization with the highest mean difference between the two stages. The microhardness finding was confirmed by SEM micrograph which show decrease in porosities and irregularities. The current study's findings, which are consistent with prior investigations, showed that ozone alone had an effect, but that this effect was boosted when chitosan solution was employed following pretreatment with ozonated water. These studies apply fluoride as an adjunct therapy[26] or nanohydroxyapatite.[16,30,34]

It is possible that the oxidizing properties of ozone can eliminate the proteins out from demineralized areas, facilitating the transport of calcium and phosphate thus remineralizing the lesions.[35,36]

Conclusion

Ozone therapy has been shown to have an effect on demineralized enamel, this effect was superior when additionally treated with chitosan.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Sivapathasundharam B, Raghu AR. Dental Caries. In: Rajendran B, Sivapathasundharam B, editor. Shafer's textbook of oral pathology. 5th ed. Amsterdam: Elsevier publishers; 2006. p. 567-658.
2. Mehta AB, Kumari V, Jose R, Izadikhah V. Remineralization potential of bioactive glass and casein phosphopeptide-amorphous calcium phosphate on initial carious lesion: An in-vitro ph-cycling study. J Conserv Dent 2014;17:3-7.
3. Madan N, Madan N, Sharma V, Pardal D, Madan N. Tooth remineralization using bio-active glass - A novel approach. J Academy Adv Dental Research 2011;2:45-50.
4. Soares R, De Ataide IN, Fernandes M, Lambor R. Assessment of enamel remineralisation after treatment with four different remineralising agents: A scanning electron microscopy (SEM) study. J Clin Diagn Res 2017;11:ZC136-41.
5. Rahiotis C, Vougiouklakis G. Effect of a CPP-ACP agent on the demineralization and remineralization of dentine in vitro. J Dent 2007;35:695-8.
6. HabeeB, H M, AL-Mizraqchi A S Ibraheem A F. Effect of ozonated water on adherent Mutans Streptococci (In vitro study). Journal of Baghdad College of Dentistry 2009;21:18-23.3.
7. Baysan A, Whiley RA, Lynch E. Antimicrobial effect of a novel ozone- generating device on micro-organisms associated with primary root carious lesions in vitro. Caries Res 2000;34:498-501.
8. Azarpazhooh A, Limeback H, Lawrence HP, Fillery ED. Evaluating the effect of an ozone delivery system on the reversal of dentin hypersensitivity: A randomized, double-blinded clinical trial. J Endod 2009;35:1-9.
9. Ahmmad BZ, Ibraheem LM. Anti-microbial effect of different time exposure of ozonized gas and ozonized water on periodontal pathogens (In Vitro Study). J Bagh Coll Dent 2017;29:78-82.
10. Ahmed BZ, Ibrahim LM. The effect of exposure times of gaseous ozone on bacterial growth of actinobacillus actinomycetemcomitans and porphyromonas gingivalis isolated from sub gingival plaque patients with chronic periodontitis. Int J Oral Health Med Res 2018;5:1-4.
11. Huth KC, Paschos E, Brand K, Hickel R. Effect of ozone on non-cavitated fissure carious lesions in permanent molars. A controlled prospective clinical study. Am J Dent 2005;18:223-8.
12. Samuel SR, Dorai S, Khatri SG, Patil ST. Effect of ozone to remineralize initial enamel caries: In situ study. Clin Oral Investig 2016;20:1109-13.
13. Al-Chalabi MZ, Mohammed AN, Ahmed BZ. Comparison of ozonated gel and chlorhexidine treatments on gingival crevicular fluid volume associated with plaque-induced gingivitis. Biochem Cell Arch 2021;21: 649-1653.
14. Jay S, Dutta PK. Preparation, antibacterial and physicochemical behavior of chitosan/ofloxacin complexes. Int J Polym Mater 2010;59:793-807.
15. Ruan Q, Liberman D, Bapat R, Chandrababu KB, Phark JH, Moradian-Oldak J. Efficacy of amelogenin-chitosan hydrogel in biomimetic repair of human enamel in ph-cycling systems. J Biomed Eng Inform 2016;2:119-28.
16. Zhang X, Li Y, Sun X, Kishen A, Deng X, Yang X, *et al*. Biomimetic remineralization of demineralized enamel with nano-complexes of phosphorylated chitosan and amorphous calcium phosphate. J Mater Sci Mater Med 2014;25:2619-28.
17. Zhang J, Lynch RJM, Watson TF, Banerjee A. Remineralisation of enamel white spot lesions pre-treated with chitosan in the presence of salivary pellicle. J Dent 2018;72:21-8.
18. Simeonov M, Gussiyska A, Mironova J, Nikolova D, Apostolov A, Sezanova K, *et al*. Novel hybrid chitosan/calcium phosphates microgels for remineralization of demineralized enamel–A model study. European Polymer Journal 2019;119:14-21.
19. Bartlett DW, Coward PY. Comparison of the erosive potential of gastric juice and a carbonated drink in vitro. J Oral Rehabil 2001;28:1045-7.
20. ten Cate JM, Duijsters PP. Alternating demineralization and remineralization of artificial enamel lesions. Caries Res 1982;16:201-10.

21. Nozari A, Ajami S, Rafiei A, Niazi E. Impact of nano hydroxyapatite, nano silver fluoride and sodium fluoride varnish on primary teeth enamel remineralization: An in vitro study. Journal of clinical and diagnostic research: JCDR 2017;119:ZC97.
22. ten Cate JM, Exterkate RA, Buijs MJ. The relative efficacy of fluoride toothpastes assessed with ph cycling. Caries Res 2006;40:136-41.
23. Arnaud TM, de Barros Neto B, Diniz FB. Chitosan effect on dental enamel de-remineralization: An in vitro evaluation. J Dent 2010;38:848-52.
24. Chuenarrom C, Benjakul P Daosodsai P. Effect of indentation load and time on knoop and vickers microhardness tests for enamel and dentin. Materials Research 2009;12: 473-6.
25. Hegde I, Pretorius E. Traditional coating techniques in scanning electron microscopy compared to uncoated charge compensator technology. Microsc Res Tech 2012;744:343-6.
26. Duggal MS, Nikolopoulou A, Tahmassebi JF. The additional effect of ozone in combination with adjunct remineralisation products on inhibition of demineralisation of the dental hard tissues in situ. J Dent 2012;40:934-40.
27. Visveswaraiah P M, Prasad D, Johnson S. Chitosan a novel way to intervene in enamel demineralization - An in vitro study. Int J Curr Microbiol Appl Sci 2014;3: 617-27.
28. Irfani NF, Gunawan HA Amir LR. Effect of chitosan application on the decreased enamel demineralization process in vitro surface damage test. In Journal of Physics: Conference Series 2018;1073:052006. IOP Publishing.
29. Zhang J, Lynch RJM, Watson TF, Banerjee A. Chitosan-bioglass complexes promote subsurface remineralisation of incipient human carious enamel lesions. J Dent 2019;84:67-75.
30. Srinivasan S, Prabhu V, Chandra S, Koshy S, Acharya S, Mahato KK. Does ozone enhance the remineralizing potential of nanohydroxyapatite on artificially demineralized enamel? A laser induced fluorescence study. In Lasers in Dentistry SPIE XX 2014;8929:18-25.
31. Li X, Wang J. Junior A Chang J. The remineralization of enamel: A review of literature. Journal of Dentistry 2014;3042:12-20.
32. Zhang Q, Zou J, Yang R, Zhou X. Remineralization effects of casein phosphopeptide-amorphous calcium phosphate crème on artificial early enamel lesions of primary teeth. Int J Paediatr Dent 2011;21:374-81.
33. Lata S, Varghese NO, Varughese JM. Remineralization potential of fluoride and amorphous calcium phosphate-casein phospho peptide on enamel lesions: An in vitro comparative evaluation. J Conserv Dent 2010;13:42-6.
34. Mowafy MM, Ghorab SM, Hammad SM. Effect of ozone with or without nano-hydroxyapatite paste on chemically induced intact carious lesions around orthodontic brackets in Vitro Study). J Dent Med Sci 2019;18:70-75.
35. Atabek D, Oztas N. Effectiveness of ozone with or without the additional use of remineralizing solution on non-cavitated fissure carious lesions in permanent molars. Eur J Dent 2011;5:393-9.
36. Baysan A, Lynch E. Management of root caries using ozone. KaVo Dental Ltd website. URL: http://www.kavo.com/forum/ upfiles/4/ Db84539.pdf. (2002) [Last accessed on 11 Nov 2015].