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Effect of ozone with or without nano-hydroxyapatite paste on chemically induced intact carious lesions around orthodontic brackets (In Vitro Study)

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Abstract
Introduction: *To evaluate the effect of ozonated water with and without nano-hydroxyapatite (NHA) paste on remineralizing artificially created initial enamel caries around orthodontic brackets using polarized light microscopy and Vickers microhardness tester*
Material and Methods: *A total of 120 extracted human premolar sound teeth were collected and coded from 1 to 120. Metallic brackets were bonded to the buccal surface of the teeth, then specimens were divided into 4 groups following different remineralization regimen: ozonated water, ozonated water + 10 % NHA paste, 10 % NHA paste alone, and control (saliva). The surface microhardness (SMH) was measured using HVS-50 Digital Display Vickers microhardness tester at baseline. The specimens were subjected to demineralizing solution to create initial enamel caries and enamel SMH was recorded. The specimens were subjected to pH cycling model then enamel SMH was recorded. The results were validated qualitatively using polarized light microscope (PLM). The results were analyzed using repeated measures, one-way ANOVA with post hoc multiple comparisons at α =0.05 level.*
Results: *A statistically significant difference was found between all treatment groups and control one (P<0.001). The greatest depth of remineralization was found in ozone-treated group then ozone + NHA then NHA alone. Ozone-treated group exhibited maximum remineralization depth under the PLM.*
Conclusion: *Ozonated water remineralization was the most effective of all. The remineralizing ability of ozone is seen with and without an adjunct remineralizing agent. Additionally, ozonated water enhanced the remineralizing potential of NHA.*
Keywords: *demineralization, hardness, polarized light microscopy, tooth remineralization, ozone*

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I. Introduction

The earliest detectable sign of enamel demineralization is the appearance of white spot lesions (WSLs). Numerous precedent studies have revealed that patients treated with orthodontic brackets increase the risk of developing WSLs as far as 85%.[1,2] In such cases, further procedures after completion of orthodontic therapy are required to cover or eliminate these spots. Hence, the means for removal of such incipient enamel caries form an important role in caries prevention, leading to evident removal of the lesion.

Ozone has been introduced as a recent approach to help in reversing of initial carious lesions in response to advancement of minimal intervention dentistry to treat caries. This is attributed to ozone's marked antimicrobial and oxidizing properties. It has the ability to oxidize pyruvic acid which is produced by the cariogenic bacteria, into acetate and carbon dioxide.[3]

The remineralizing action of ozone on initial enamel caries is still under debate. Huth et al.[4] claimed that application of ozone, in patients with high caries risk, relatively recovered initial fissure caries along a 3 months period. Moreover, a study investigating ozone's potential to kill cariogenic bacteria (S. *mutans* and L. *acidophilus*) resulted in complete prevention of such strains to grow.[5] On the other hand, a recent in vitro study investigated by Tahmassebi et al.[6] showed that ozone treatment alone had no effect on protecting enamel against demineralization or promoting remineralization. Thus,it is greatly beneficial to include ozone in the protocol of non-invasive treatment of initial dental caries for further assurance of its remineralizing effect.

Likewise, the application of nano-hydroxyapatite (NHA) to deceptively repair defective enamel has been of great concern in recent dental research. This owes to its structural and chemical similarity to enamel

structure.[7]Ebadifar et al.[8] reported that toothpaste containing NHA showed a higher remineralizing effect than toothpaste containing fluoride.

Yet, there are inadequate studies in the literature about the reversing action of ozone on incipient caries with intact enamel surface. Considering that, the purpose of our study was to assess the effect of ozonated water, with and without NHA addition, on reversing initial enamel caries around orthodontic brackets.

II. Materials And Methods

The calculated sample size of the study was 120 specimens at 5% level of significance and 80 % power. A total of 120 caries-free human premolars extracted for orthodontic purposes were used in this in-vitro study. Teeth with caries, cracks, pitting, hypo-mineralization, fluorosis or previous treatment with chemical agents were excluded. Teeth were evaluated under dental microscope with x40 magnification (M320 F12, Leica Microsystems, Wetzlar, Germany) for gross irregularities, cracks or hypoplastic areas. All teeth were polished with fluoride-free prophypaste (i-Faste, i-dental, Lithuania) and rubber cup, washed with tap water and stored in distilled water at room temperature until time of use. The root portion of each tooth was embedded in a cylindrical block of acrylic resin using specific molds to facilitate their utilization. Metallic Roth brackets (Morelli Ltd, SP, Brazil) with 0.022 inch slot were bonded to the buccal surface of the teeth using Transbond XT (3 M Unitek, Monrovia, Calif, USA) according to manufacturer's instructions.

Blocks were numbered randomly from 1 to 120. Then, they were allocated into 4 equal groups (n=30) based on their remineralization protocol: Group 1- ozonated water, Group 2- ozonated water and finger-brushed with tooth paste containing 10% NHA, Group 3- finger-brushed with tooth paste[9] containing 10% NHA only, Group 4- saliva (control group). Three of these groups were experimental (groups 1-3) and one of them was a control group.

The specimen surfaces were dried and the surface microhardess (SMH) at baseline was measured using Vickers micro-hardness tester prior to any acid exposure.

All the specimens were immersed in demineralizing solution (2mM $CaCl_2$, 2mM NaH_2PO_4, 50 mM CH_3COOH at pH 4.4)[10] for 4 days to resemble an active area of demineralization leading to formation of artificial caries.[11]

The specimen surfaces were dried and the SMH was re-measured using Vickers microhardness tester after initial caries induction.

pH cycling model:

The pH cycling model was settled upon to mimic the oral cavity regarding the progressive course of alternating demineralization and remineralization.[11] All specimens in the experimental groups were exposed to the pH cycling model for a 28 day period. Each of the specimens was immersed in the demineralizing solution for a 3-hour period daily. Then, each specimen was treated with its corresponding remineralizing agent for a 1-minute period. Next, all the specimens were immersed in the remineralizing solution (1.5mM $CaCl_2$, 0.9mM NaH_2PO_4, 0.15M KClin 0.1 m Tris buffer at pH 7)[10] for a period of 21 hours. This was followed-up with treatment of the samples again with the corresponding remineralizing agent for 1 min. The remineralizing solution was replenished every 48 hours and the demineralizing agent replenished every 5 days. Ozonated water delivered 2 minutes daily, divided, to the corresponding group. NHA paste was applied onto the specimen using the index finger twice daily. On daily basis, the teeth in the control group were subjected to the demineralizing solution for 3 hours followed up by immersion in artificial saliva that was replaced every 2 days.

To prevent the compromise of the solutions reaching the saturation threshold, fresh solutions were added according to the guidelines set by Featherstone and Zero (1992).[12]

After the execution of the pH cycling steps, all the specimens' SMH were re-assessed using Vickers hardness test.

Microhardness test procedure

The specimens' SMH were assessed using Digital Display Vickers Micro-hardness Tester (Model HVS-50, LaizhouHuayin Testing Instrument Co., Ltd. China). A 200g load was applied with a Vickers diamond indenter to the surface of the specimen for 20 seconds. Three indentations were placed on the surface of each specimen at a distance of at least 0.5 mm away from the adjacent indentations. With the help of a 20x objective lens, the lengths of the indentations' diagonals were measured by built-in scaled microscope. Then, SMH values were calculated from Vickers values using the following equation:

$HV=1.854\ P/d^2$

where, HV is Vickers hardness value in Kgf/mm^2, P is the load in Kgf and d is the length of the indentation's diagonals in mm.[13]

Polarizing Light Microscope

Considering the evaluation of the depth of remineralization, representative specimens' sections from all the four groups were longitudinally sectioned down to an approximate thickness of 150µm. Firstly, the specimen was sectioned to a thickness of 300 to 400µm with a flexible diamond disc (DFS-Diamon, Germany). The sections were further ground to the required thickness with descending grits of carborundum (600-1100 grit).Each section was cleaned with deionized water and air-dried and then was aligned longitudinally and fixed on the slide using DPX mounting medium. Each section obtained was analyzed for depth of the lesion under PLM (Orthoplan, Leitz, Wetzlar, Germany) with digital camera (Leica MC190 HD, Germany). The lesion depth was measured from the surface of the tooth to the maximum depth using the Leica Application Suite software (Leica LAS EZ v3.0).

III. Statistical Analysis

The collected data were coded, processed and analyzed using Statistical Package of Social Science (SPSS) program for windows (version 16). Quantitative continuous data were presented in mean and standard deviation (SD).

One way Analysis of variance (ANOVA) test was used for testing significance of means between different groups while repeated measures ANOVA test was used for testing means within each group (at least one pairwise difference). If the p-value < 0.05, we reject null hypothesis (H0) and conclude that there is a significant difference between at least one pair of means. Bonferroni post hoc test was performed to test where the pairwise differences are.

IV. Results

The descriptive statistics of the SMH of the studied groups at baseline, after demineralization and after remineralization by the corresponding remineralizing regimen are presented in Table I. According to the ANOVA, there were significant differences between the SMH values in-between groups comparison after remineralization ($p<0.05$). The highest mean SMH was found to be the group treated with ozonated water. The within comparison showed significant difference between the different phases in each treated group ($p<0.001$). In the control group, receiving no treatment but saliva showed no significant difference between baseline and final SMH ($p>0.05$).

These findings were further verified by the PLM evaluation revealing that the ozone-treated group had the greatest depth of remineralization followed by group treated with ozone and NHA, then NHA alone and saliva, respectively, as shown in Fig 1-2.

Within group comparisons

Groups	Treatment (n=30)	Enamel hardness Mean ± SD	P value [a]	Post hoc comparison [b]
Ozone	Baseline Demineralization Remineralization	261.13 ± 18.3 241.15 ± 17.3 293.20 ± 26.8	<0.001*	(Baseline <remineralization) > demineralization
Ozone +NHA	Baseline Demineralization Remineralization	259.30 ± 23.1 235.51 ± 16.2 288.81 ± 18.7	<0.001*	(Baseline <remineralization) > demineralization
NHA	Baseline Demineralization Remineralization	255.94 ± 18.1 236.85 ± 18.5 280.52 ± 20.7	<0.001*	(Baseline <remineralization) > demineralization
Saliva (control)	Baseline Demineralization Remineralization	253.23 ± 21.3 234.94 ± 20.0 269.01 ± 30.7	<0.001*	(Baseline = remineralization) > demineralization

Between groups comparisons

Treatment (n=30)	Groups	Enamel hardness Mean ± SD	P value [c]	Post hoc comparison [b]
Baseline	Ozone Ozone + NHA NHA Saliva (control)	261.13 ± 18.3 259.30 ± 23.1 255.94 ± 18.1 253.23 ± 21.3	NS	----
Demineralization	Ozone Ozone + NHA NHA Saliva (control)	241.15 ± 17.3 235.51 ± 16.2 236.85 ± 18.5 234.94 ± 20.0	NS	----
Remineralization	Ozone Ozone + NHA NHA Saliva (control)	293.20 ± 26.8 288.81 ± 18.7 280.52 ± 20.7 269.01 ± 30.7	0.001*	Group 3 = (group 1 = group 2) > group 4

*P < 0.05 is considered significant

a Repeated measures ANOVA
b Post hoc comparison
c One-way ANOVA

Table 1 The descriptive statistics of the SMH of the studied groups at different levels of mineralization

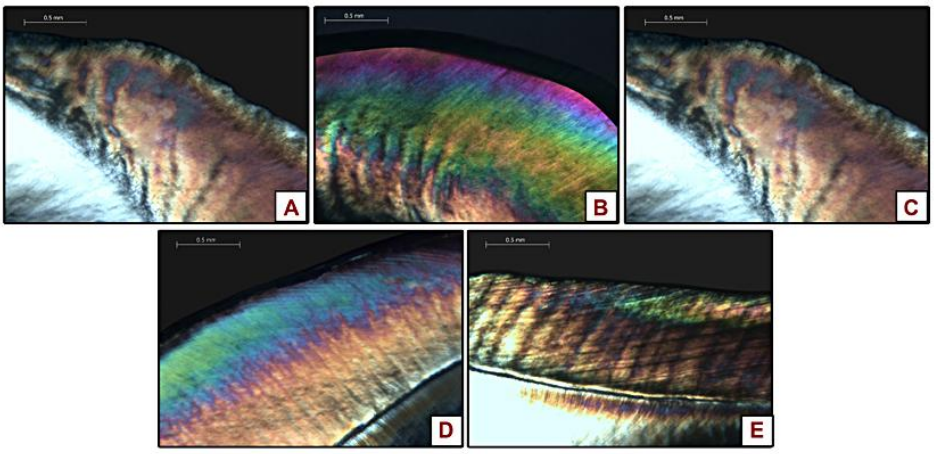

Figure 1 The photomicrographs taken under PLM (A), demineralized enamel (B), treated with ozone alone (C), treated with ozone+ NHA (D), treated with NHA alone (E), saliva(control)

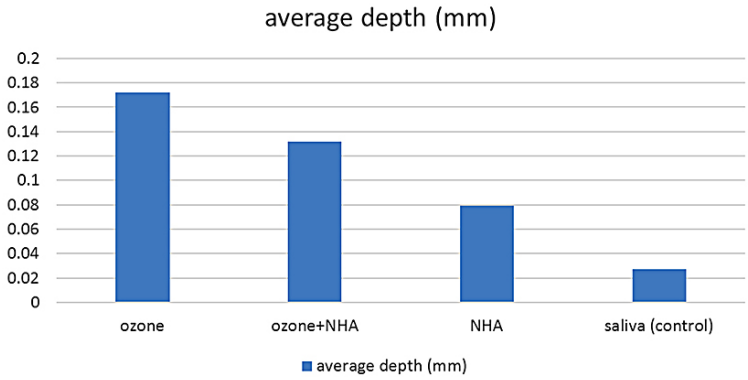

Figure 2 Graph representing the average depth of remineralization of the representative specimen of each group as seen under PLM

V. Discussion

Enamel demineralization can form rapidly because of the high and continuous challenge of plaque when associated with orthodontic appliances. Demineralization is a reversible process; hence, the partially demineralized HA crystals in teeth can grow to their original size if they are exposed to favorable remineralizing conditions.[14]

The aim of this study was to evaluate the potential remineralization effect of ozonated water against demineralized enamel around orthodontic brackets. In the same manner, the combined treatment of ozonated water and NHA was put to investigation. The enamel surface changes were assessed using Vickers microhardness tester and PLM.

Ozone therapy has a wide range of dental applications owing to its exceptional properties such as anti-microbial, anti-inflammatory, immune-stimulating, anti-hypoxic, detoxicating and biosynthetic actions.[15,16] Nonetheless it has gained its popularity in dental practice for non-invasive, effective dental care meeting the demands of the patients.[17]

The results of this study indicated that all of the treatment procedures used in this study showed significant remineralization of artificially created enamel caries. The specimens in all the experimental groups had experienced a decrease in microhardness after demineralization, excluding any disparity, and had rehardened after remineralization.

The specimens treated with ozonated water have shown the highest SMH values with a mean of 293.20±26.8 Kgf/mm^2. This can be interpreted in various ways such as the bactericidal effect of ozone on

cariogenic bacteria[18] or its oxidizing ability to eliminate the proteins in demineralized enamel promoting lesion remineralization through facilitating the diffusion of remineralizing agents.[19]

In accordance with our results, Yamayoshi and Tatsumi[20] revealed that ozone was a strong oxidizing agent to the microorganisms' cell wall and cytoplasmic membranes. Moreover, Baysan et al.[21] have assessed the antimicrobial effect of ozone on oral microorganisms in vitro giving significant reduction in the count of microorganism cells. The results of Sadatullah et al.[18] have rationalized our study revealing that the exposure to the ozonated water for 30 sec reduced the total bacterial population of the 24-hour plaque by 45.3%.

On the other hand, Baysan and Beighton[22] examined ozone ability to kill microorganisms related to non-cavitatedocclusal caries. Their results were contradictory to the current study's results, where Baysan and Beighton's results revealed failure to significantly reduce bacterial count in infected dentine found under demineralized enamel. More recently, Tahmassebi et al.[6] investigated the effect of ozone on the progress of induced caries-like lesions on enamel showing that ozone treatment alone had no effect on protecting enamel against demineralization or promoting remineralization.

Atabek and Oztas[19] have revealed that ozone application, whether applied alone or with a remineralizing solution, is an effective method for caries reversal. He B et al.[23] and Huang S et al.[24] undergone studies that showed significant improvement in SMH post-treatment with NHA toothpaste. Increase in mineral content after treatment with NHA paste was evident in the study of Tschoppe et al [25] while no significance was found to be in the study of Itthagarun et al.[26]

The significant increase in SMH in the NHA-treated group establishes that this material re-hardens the softened enamel by gradual deposition of the mineral that precipitates and nucleates in the dark zone of demineralization thereby offering complete biomimetic regeneration of the lost enamel crystallites.[27] Moreover, Enan and Hammad have revealed that there is a direct proportionality between NHA concentration and the rate of enamel remineralization, up to a certain limit.[28]

Surprisingly, the enamel SMH of the control group has been restored beyond the baseline level but insignificantly ($p>0.05$). This result is in harmony with the opinion of Featherstone[29] confirming that with good oral hygiene remineralization may be established. This may be due to the bio-available mineral components readily found in saliva.

The dark zone representing the remineralization depth of each representative specimen may be interpreted by possible filling of the micro-pores on demineralized enamel as revealed by Hannig et al.[30]and Roveri et al.[31] This would enhance the remineralizing effect and boost the mechanical properties of the tooth assuring the results of micro-hardness revealed by this study.

Withal, the investigators should apprehend the constraints of this method which cannot completely simulate the intraoral conditions, thus the proposal of the in vitro results for clinical use should be within limits. Moreover, the pH cycling model may not thoroughly simulate the intraoral settings because some changes exist in pH variations based on the patient's hygienic means, nutritional habits, fluoride consumption and salivary composition and quality.[32]

To conclude, ozone therapy is found to be an effective method in reversing demineralized enamel. Moreover, ozone significantly enhanced the remineralizing ability of NHA compared to NHA paste alone. In spite of the promising results of our study, clinical research is needed to substantiate the mechanism of action of ozone. These results should be confirmed clinically to counteract any limitations of our in vitro study.

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