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The Effect of Ozone on Progression or Regression of Artificial Caries-like enamel Lesions in vitro

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Abstract

Ozone therapy combines the use of ozone gas and adjunct products called Reductant and the patient kit, which all contain fluoride. This study investigated the effect of Ozone on the progression or regression of artificial caries-like lesions on enamel following pH cycling conditions *in vitro*. A randomised, single blind, four legs design was used. 20 full thickness enamel slabs were allocated to each of the four groups which were: Fluoride free toothpaste (control); Ozone alone; Reductant/Patient Kit alone and a combination of both Ozone/Reductant/Patient Kit. Artificial lesions were created and subjected to the pH cycling regime for a 14 days period. Assessments were carried out before and after the pH cycling on the slabs using the Microhardness testing and Quantitative Light-induced Fluorescence (QLF). Statistical significant difference were found in the percentage change of enamel microhardness before and after pH cycling between Ozone/Reductant/Patient Kit group and all the other three groups of the study, as well as between Reductant/Patient Kit group and control There was a statistical significant difference in the change of size and severity of the lesion (ΔQ) between all the three regimes tested and the control with a trend favouring Ozone/Reductant/Patient Kit group. In our model, it appeared that Ozone treatment alone is not effective in protecting the enamel against demineralisation or promoting remineralisation, unless combined with the reductant/patient kit, which contain high levels of fluoride.

Introduction

Ozone (O_3) is a very powerful oxidizing compound whose antimicrobial effects have been known for many years. Direct application of Ozone gas to the coronal or root tooth surface is claimed to have a sterilising effect by disrupting the cell walls of microorganisms within seconds, leading to immediate functional cessation.[1 and 2] It is consequently alleged to be able to reverse arrest or slow down the progression of dental caries. It is also maintained that Ozone is useful for reducing the microbial flora in cavitated lesions, before fillings are inserted.

HealOzone is a certified Medical Device [Conformité Europèene (CE) marked] for the management of occlusal pit and fissure caries, and root caries. According to the manufacturer, HealOzone devices are currently in use in dental practices in the UK and more than one million people have already received HealOzone treatment.[2] The HealOzone technology has not yet received Food and Drug Administration (FDA) approval in the USA.

There are a few published studies concerning the clinical effectiveness of Ozone treatment. One of the earliest published studies in this field was by Baysan et al[3], who reported that Ozone application for either 10 or 20 seconds was effective to kill the great majority of micro-organisms in primary root carious lesions (PRCLs) *in vitro*. In a later study Baysan and Lynch[4], showed that after nine months 45% of PRCLs reversed from severity index 2 to 0 in the Ozone only group whilst none of the lesions became hard in the control group with similar findings reported by.[1] Also Abu-Salem[5], reported statistically significant effect of treatment upon clinical severity scores with time. However in contrast to the above studies, Abu-Naba'a[6] reported no significant difference between the two intervention groups - Ozone plus Reductant group versus air treatment plus Reductant only group at 12 months follow up. Similarly Baysan and Beighton[7] found Ozone treatment of non-cavitated occlusal lesions for 40 seconds failed to significantly reduce the numbers of viable bacteria in infected dentine beneath the demineralised enamel.

A recent in situ study by Duggal et al[8] investigated the effect of Ozone on inhibition of mineral loss from human enamel and dentine under a cariogenic challenge (14 days study regime) using the microhardness testing. The investigators concluded that Ozone

has no additional effect on the inhibition of dental hard tissue demineralisation as compared with the use of Reductant and Patient Kit.

Cochrane review concluded that given the high risk of bias in the available studies and lack of consistency between different outcome measures, there was no reliable evidence that application of Ozone to the surface of carious teeth stops or reverses the carious process.[9]They concluded that there is not enough high quality evidence to support the use of Ozone gas in a primary care setting. There is insufficient evidence on the effectiveness of HealOzone treatment for this technology to be recommended, except as part of well-designed RCTs.

Similarly, Brazelli et al [10] in a systematic review that aimed to assess the effectiveness and cost-effectiveness of HealOzone for the management of pit and fissure caries, and root caries, concluded that the current evidence on HealOzone is insufficient to conclude that it is a cost-effective addition to the management and treatment of occlusal and root caries. They also stated that in order to make a decision on whether HealOzone is a cost-effective alternative to current preventive methods for the management of dental caries, further research into its clinical effectiveness is required.

There are limited studies in the literature about the effects of Ozone on enamel and its potential to inhibit demineralisation and enhance remineralisation *in vitro*. Furthermore, as the use of ozone is always accompanied by the use of a Reductant and a Patient Kit for home use, both of which contain high concentrations of fluoride, it is possible that the benefit reported in some studies on caries is a result of these fluoride containing products rather than ozone alone.

From the preceding literature review it seems that the effect of Ozone on the progression of dental caries remains controversial. Therefore, it would be important to study the effect of Ozone and the fluoride containing adjunct therapies that accompany its use on early carious lesion *in vitro.*

Material and Methods

Ethical approval was not required as this was an *in vitro* study. Approval was obtained from the Leeds Dental Institute Tissue Bank where all the teeth used in the study were collected. Statistical advice was sought and the sample size was calculated by making estimations using data from similar study carried out by Al-Mullahi.[11] Using the Stata/ SE 10.1 software it was found that for 90% power and significance level of 0.05 the sample size of 2 slabs per group was required.

Inclusion and exclusion criteria

Intact first or/and second, upper and lower premolars extracted for orthodontic reason under general or local anaesthesia at Leeds Dental Institute were selected. Teeth with signs of caries, trauma, erosion, restorations or any malformation were excluded from the study.

Enamel Slab Preparation

Enamel slabs that were used in the study were from human premolars extracted for orthodontic reasons and stored in a solution of distilled water and 0.1% thymol (Sigma Aldrich) at room temperature. The teeth were carefully screened by transillumination and transmitted light using low–power microscopy for the detection of cracks (Leitz, Wetzlar®, Germany), caries or any malformations. Suitable teeth were selected and lightly abraded with fine paper to remove the outermost enamel and any remnants of pellicle. A Well Diamond Wire Saw, water cooled, cutting machine for sectioning was used (Well® Walter EBNER, CH-2400 Le Loche). The buccal and lingual surface of each tooth was separated and was then cut into two slabs each, according to the relevant standard operating procedures (about 2mm wide, 4mm length, and 2mm depth). Each slab was used for one of the four legs, in order to standardise slabs with the same origin throughout the entire study. After cutting, the slabs were polished whilst wet using fine grit abrasive paper (P1000 Weodry paper, 3M) in combination with 5μm and 1μm aluminia paste to remove the outermost enamel and dentine and any remnants of the pellicle and to achieve a flat surface. Care was taken not to fully abrade the enamel.

Once the enamel slabs had been prepared, they were kept moist in water with thymol, in micro-centrifuge tubes sealed with Parafilm to prevent leakage of the thymol solution and dehydration of the enamel. They were then sent to the Department of Immunology

of the University of Liverpool, where they were exposed to gamma irradiation (4080Gy). This level of exposure provides sterilisation without altering the structural integrity of the enamel.

Surface Microhardness measurements

Baseline Surface Microhardness measurements were performed prior to any acid exposure. Microhardness was assessed using a computer-aided Duramin Indenter Machine (Struers A/S, DK 26-10, Denmark) and expressed as the length of the indent (μm). The indentations were made using a Knoop diamond under 100 g load for 15 seconds. The depth of indenter penetration was measured by means of an image analysis system. Five indentations, spaced 50 μm apart, were made for each slab and the mean was estimated. The same procedure was followed after the creation of the acid lesions (before the pH cycling) and then at the end of the 14^{th} day..[12] In total, there were 3 measurements of microhardness for each slab during the study.

Creation of artificial caries-like lesions and QLF measurement

For creation of caries-like lesions in the enamel slabs, a rectangular window (approximately 2 mm × 3 mm) was marked on each enamel slab using a carbon pencil. The rest of the enamel slab apart from the marked area was coated with two coats of transparent nail varnish (Max Factor "Infinity") and was placed in plastic containers with an acidified lactic acid homogenous gel in room temperature.

The slabs were left in this gel for 48 hours in room temperature in total, after which a white spot was visible on each enamel slab.

Enamel slabs were assessed after the creation of white spot lesions and at the end of the 14 day test period using the QLF. All the slabs were dried for 5 seconds with compressed air prior to imaging, and were examined in a dark room.

Enamel slabs were exposed to approximately 10 mW/cm^2 violet-blue light (wavelength: 290-450 nm). The images were acquired using a miniature CCD camera kept inside the hand piece through a 520 nm high-pass filter, transmitting only light at wavelengths over 520 nm. The QLF camera hand piece was fixed at a position that provided optimum illumination of the enamel block surface. A patch was drawn around the carious lesion site by the study examiner with its borders on sound enamel. Inside this patch, the fluorescence levels of sound tissue were reconstructed by using the

fluorescence radiance of the surrounding sound enamel. The percentage difference between the reconstructed and the original fluorescence levels was calculated.

Data were collected, stored and analysed by custom-made software (Inspektor Research Systems BV, Amsterdam, The Netherlands). Demineralised areas appeared as dark spots. Fluorescence radiance levels less than 95% of reconstructed sound fluorescence radiance levels were considered to be artificial early caries lesions. Three metrics were obtained: ΔF (average change in fluorescence, in %), lesion area (mm^2), and ΔQ (multiplication of ΔF and area).

To ensure that images of the enamel slab were always captured in the same camera positions and from the same angles, the software uses video-repositioning techniques. The video-repositioning technique displays baseline and live images simultaneously and computes their correlation based on similar geometry of the fluorescence intensities.

The enamel slabs were coded using block randomisation based on a random table of numbers, according to computer programme of random allocation (S-Plus, insightful corporation, Seattle WA) and were randomly allocated to each of the four study groups. Each of the four slabs of each premolar was allocated to one of the four study groups.

The code was kept with another member of staff. As the products used in the study were not indistinguishable the study investigator could not be blinded during the treatment period. However, the investigator was blinded while obtaining the final measurements using the microhardness testing and QLF at the end of the 14 days pH cycling period.

Protocol for the study

The study was carried out in accordance with principles of Good Clinical Practice (GCP/ICH) and Good Lab Practice.

A prospective randomised, single blind, four legs design was used. Twenty full thickness enamel slabs were randomly allocated to each of the four groups which were:

1. Fluoride free toothpaste (control)
2. Ozone alone
3. Reductant/Patient Kit alone
4. Combination of Ozone/Reductant/Patient Kit groups

PH Cycling Regime

In each leg of the study the enamel specimens were dipped in 50 ml of demineralisation solution (acetic acid buffer) for 2 minutes.[13] After the demineralisation challenge the enamel specimens were rinsed with distilled water for 1 minute and then placed in 50 ml of day-time artificial saliva for 1 hour. Using this process enamel specimens were subjected to 5 demineralisation challenges daily. For the control group, after the first and last demineralisation challenge the enamel slabs were dipped for 5 minutes in fluoride free toothpaste, rinsed with distilled water and following that they were dipped in slurry 1:3 (F free toothpaste/ day-time artificial saliva) for 10 minutes. During the night the enamel specimens were kept in night-time artificial saliva. In all instances when the enamel slabs were taken off from the saliva, demineralisation solution and treatment regimens were rinsed with distilled water. The enamel slabs were kept in an incubator at $37^{o}C$ in between dipping and overnight. Artificial saliva (day/night-time) was changed daily to prevent any contamination or bacteria growth. Also a new volume of acetic acid was used for each demineralisation challenge. All the solutions were prepared on daily basis.

The daily cycling protocol for the three treatment groups as in control group, the only difference being that for Ozone group, at day 1, 4, 8 and 11, Ozone was applied for 60 seconds on each enamel slab. In Reductant plus Patient Kit group at day 1, 4, 8 and 11, Reductant was dropped manually on the enamel lesions using a small vehicle (supplied by the manufacture), the slabs were then dipped in F^- toothpaste (from Patient Kit) and in slurry made from 1 part of F^- toothpaste (from Patient Kit) and 3 parts day-time artificial saliva. Also, after the 2^{nd}, 3^{rd} and 4^{th} daily demineralisation challenge the enamel slabs were dipped for 2 minutes in F^- rinse which was provided in the Patient Kit. Finally in the Ozone plus Reductant plus Patient Kit group Ozone was applied for 60 seconds on each enamel slab at day 1, 4, 8 and 11, Then Reductant was dropped manually on the enamel lesions using a small vehicle (supplied by the manufacture). The daily cycling protocol was as in Reductant plus Patient Kit group. The summary of the study protocol is shown in Figure 1.

Statistical analysis

The SPSS statistical software package for Windows version 15.0 (SPSS Inc. Illinois) was used for data analysis, calculation of 'P' values and confidence intervals. A significance level of $\alpha<0.05$ was adopted. The data were initially tested using the Kolmogorov-Smirnov test in order to check their normality.

In cases that the One Way ANOVA test showed statistical significant difference between the groups, then we conducted multiple comparisons in order to find out where the differences lay. However as the sample in this study was small in order to prevent false positive result the Bonferroni adjustment was used.

Using the Kolmogorov-Smirnov test it was found that the data for the difference in white spot lesion area and the difference in ΔQ before and after pH cycling were not normally distributed; therefore non-parametric tests (Kruskal-Wallis and Mann-Whitney U tests) were used. The Bland-Altman plots were used in order to assess the intra-examiner reproducibility.[14]

Results

Results for Microhardness

The primary parameters were the degree of progression or regression of artificial caries-like lesions on enamel which were defined as the changes in microhardness and Quantitative Light-induced Fluorescence (QLF) measurements from the start of treatment period (baseline value). Irrespective of the sequence of treatment, microhardness measurements showed that Ozone/Reductant/Patient Kit, and Reductant/Patient Kit groups showed similar enamel and dentine softening. Fluoride free group showed the most softening of the enamel and dentine compared to baseline (Figure 2).

The boxplot for the distribution of the percentage (%) difference (change) in enamel microhardness showed that the Control group had the smallest median value followed by Ozone group. The Ozone plus Reductant plus Patient Kit group had the highest median (2.44%). Also it was obvious that there was a wider variation in the percentage differences in enamel microhardness in Ozone plus Reductant plus Patient Kit group compared with the other three groups.

It was found that the data were normally distributed according to Kolmogorov-Smirnov test (P=0.3). The One Way ANOVA test showed that there was a statistically significant difference (P<0.05) in the mean % differences (changes) in enamel microhardness between the groups. The Bonferroni method was then applied and it revealed no statistical significant difference in the mean % changes (differences) in enamel microhardness between Control group and Ozone group, and between Ozone group and Reductant plus Patient Kit group. However, there was a clear trend in less reduction in enamel microhardness after the pH cycling in the Reductant plus Patient Kit group compared with Ozone alone group.

Results for QLF

For the QLF, the three main parameter that were statistically analysed were the difference in white spot lesion area, the difference in ΔF and the difference in ΔQ.

Difference in white spot lesion area

The comparison between the groups revealed that the Control group showed the most profound increase of white spot lesion area after pH cycling compared with baseline, whereas the Ozone plus Reductant plus Patient Kit group showed the most significant decrease of white spot lesion area compared with baseline.

The boxplot for the distribution of the difference in white spot lesion area showed that there was a wider variation in differences in area in Control group and Ozone group compared to Reductant plus Patient Kit group and Ozone plus Reductant plus Patient Kit group. Also the median values for the Ozone group and the Ozone plus Reductant plus Patient Kit group were almost the same (Figure 3). In these data 3 outliers were present in Control group, 2 in Ozone group, 2 in Reductant plus Patient Kit group and 4 in Ozone plus Reductant plus Patient Kit group.

The data were not normally distributed according to Kolmogorov-Smirnov test (P=0.004). The Kruskal-Wallis test showed that there was a statistically significant difference (P=0.019) in the changes in area between groups. So, the Mann-Whitney U Test was used to compare between groups, and it was found statistical significant difference in the area changes (differences) between Control and Ozone group (P=0.043) and between Control group and Reductant plus Patient Kit group (P= 0.004),

as well as between Control and Ozone plus Reductant plus Patient Kit group (P=0.009). No statistical significant difference was found between the other groups (P>0.05).

Difference in ΔF

The ΔF difference (change) (%) was measured using the following formula:

|ΔF before pH cycling|- |ΔF after pH cycling|

The comparison between the groups revealed that the Control group showed the most profound increase in loss in fluorescence between the demineralised area and the sound enamel in the end of pH cycling compared to baseline, whereas the Reductant plus Patient Kit group showed the most significant decrease in fluorescence loss between the white spot lesion area and the sound enamel in the end of pH cycling compared to baseline.

The data were normally distributed after testing using the Kolmogorov-Smirnov test (P=0.28). The One Way ANOVA test showed that there was *not* a statistically significant difference (P=0.13) in the mean differences (changes) in ΔF between the groups. Since One Way ANOVA test indicated no significant difference, no pairwise comparisons of groups were conducted.

Difference in ΔQ

The ΔQ difference (change) (%mm^2) was measured using the following formula:
|ΔQ before pH cycling|- |ΔQ after pH cycling|

The comparison between the groups revealed that the Control group showed the most profound increase in the volume of the lesion in the end of pH cycling compared to baseline, whereas the Ozone plus Reductant plus Patient Kit group showed the most significant decrease in the volume of the lesion in the end of pH cycling compared to baseline measurements (Table 1).

The data were not normally distributed according to Kolmogorov-Smirnov test (P=0.007). The Kruskal-Wallis test showed that there was a statistically significant difference in the differences in ΔQ between groups (P=0.025). So, the Mann-Whitney U Test was used to compare between groups, and it was found statistical significant difference in the ΔQ changes (differences) between Control group and Ozone group (P=0.049) and between Control group and Reductant plus Patient Kit group (P= 0.005),

as well as between Control and Ozone plus Reductant plus Patient Kit group (P=0.017). No statistical significant difference was found between the other groups.

Intra-examiner reproducibility

The intra-examiner reproducibility was tested using the Bland-Altman plot. The study investigator (NC) randomly retested 15% of the enamel slabs at the end of the study (after the pH cycling) for microhardness, white spot lesion, ΔF and ΔQ measurements.

For the enamel microhardness measurements the bias, which indicates the level of agreement on 'average', was -1.57 KHN. This value was very close to 0 indicating that on average there was a good level of agreement. For the white spot area measurements the bias, which indicates the level of agreement on 'average', was -0.01 mm^2. This value was very close to 0 indicating that on average there was a good level of agreement. For the ΔF measurements the bias, which indicates the level of agreement on 'average', was -0.14%. This value was very close to 0 indicating that on average there was a good level of agreement. For the ΔQ measurements the bias, which indicates the level of agreement on 'average', was 0.03%mm^2. This value was very close to 0 indicating again that on average there was a good level of agreement.

Correlation between Microhardness testing and QLF

The Pearson correlation coefficient was used to measure the correlation between the percentage difference in enamel microhardness (%) and difference in ΔQ (%mm^2) before and after pH cycling. A positive correlation between the two variables existed (P=0.026). Nevertheless, the correlation between the values of two variables was weak (r=0.25) (Figure 4).

Discussion

There is conflicting evidence regarding the clinical effectiveness of Ozone treatment. A recent Cochrane review[9] identified only three RCT's.[4 and 5] The remaining articles were excluded for reasons such as lack of blinding, randomisation or controls, less than 6 months follow-up, or lack of investigation of extracted teeth. Furthermore the included studies were judged to be at high risk of bias. In addition, given the statistically significant effectiveness of fluoride treatment reported in the Cochrane review, [14-16] it is

imperative that the effects of ozone are evaluated in the way its clinical use is prescribed, which is always alongside the fluoride containing products, i.e Reductant and patient kit. Therefore the present study aimed to investigate additional effect of Ozone, in combination with the fluoride containing adjuncts used with it on the progression or regression of artificial caries-like lesions on enamel following pH cycling conditions *in vitro*. This was achieved by using demineralised enamel slabs, where artificial lesions had already been created *in vitro*. This has the benefit of allowing both demineralisation and remineralisation to be studied. In order to create the artificial lesions we used an acidified hydroxyethyl cellulose gel. It has been proposed that it is preferable to use hydroxyethyl cellulose in order to create artificial lesions as it is easier to be produced, the formation of lesions is consistent, there are uniform areas of demineralisation and the produced lesions are more rigid, compared with the lesions produced with acetic acid buffer.[17] To avoid excessive loss of minerals during the lesions formation and in order for the enamel slabs to be able to withstand further demineralisation challenge during the pH cycling, an acidified hydroxyethyl cellulose gel was used for two days in this study.

The method of pH cycling simulates the pH changes taking place in the plaque by subjecting the enamel to a series of de- and remineralisation challenges.[18] A 14 days period was chosen in this study in order to have sufficient time to produce changes in the pre-demineralised enamel slabs. The same pH cycling period has been used successfully in many previous studies in order to assess de- and remineralisatio.[18-20]

In most of the previous studies Ozone has been applied for the duration of 10 to 120 seconds per tooth. In this study Ozone was delivered for 60 seconds on each enamel slab per application. This was carried out four times in the 14 days pH cycling period. The number of Ozone applications was increased in the current study as this was an *in vitro* study and Ozone could exhibit only its oxidizing properties against acids, and no antimicrobial effect could be demonstrated in an *in vitro* model such as the one we used.

After the pH cycling in order to monitor the progression or regression of the carious lesions, a second image with the QLF was obtained. It is critical that the new image obtained to be superimposed as accurately as possible over the original image of the demineralised area. In this study we found some difficulties to superimpose the second image over the first. To overcome this problem the camera's position and angle from the slabs should have been better standardized during the measurements. Nevertheless,

it is unlikely that this had a major effect on the accuracy of the results as the QLF software uses video repositioning techniques and the new image is recorded only if its correlation with the initial image is higher than 0.98.

To our knowledge no other studies in the literature have used Ozone alone to assess its effect on de- and remineralisation of enamel. Our results indicated that when used alone, ozone did not have a significant effect on preventing further demineralisation or promoting remineralisation as seem from the results of rthe microhardness and QLF respectively. . In addition to other well published mechanisms of proposed action of ozone, it is known that Ozone has a severely disruptive effect on the bacterial population in the carious lesion and obliterates the cariogenic bacteria, thereby swinging the equilibrium in favour of remineralisation. No more acid can be produced within the lesion when the acid-producing bacteria are eliminated. As this was an *in vitro* study the effect of Ozone on elimination of bacteria in the lesion could not be studied. On the other hand, it is possible that Ozone because of its powerful oxidizing properties decreased carbohydrates and acids within the lesions and so enhanced remineralisation. Also, Ozone has the ability to remove organic material in the carious lesions and enables calcium and phosphate ions to diffuse through the lesions, which might account for the small differences comparted to control group in our study

Although in the present study we failed to prove a statistically significant difference between Reductant plus Patient Kit group and Ozone group, there was a very clear trend in less reduction in enamel microhardness after the pH cycling in favour of the Reductant plus Patient Kit group. The beneficial effect of fluoride is showed in many reports from previous studies, where the resistance of enamel to acid demineralisation and reduction of mineral loss and lesion depth in the presence of fluoride in solution was evident.[21 and 22] It is possible that these differences may have been statistically evident if the sample size had been even larger.

The significant remineralisation in Ozone plus Reductant plus Patient Kit group shows the synergistic effect of Ozone and fluoride. As it is already mentioned, it has been theorized that Ozone can oxidize organic material within the carious lesion. This reportedly opens up “channels” within the dental tissue to allow the penetration of calcium, phosphate, and fluoride ions to allow remineralisation of the surface. These remineralised surfaces are more resistant to subsequent decay and acidic challenges.[23]

Generally, conflicting data exist about the parameter "lesion severity" as a determinant for lesion repair. Strang et al[24] showed mineral deposition during *in situ* remineralisation to be (positively) correlated to the amount of mineral lost during the formation of the lesions. Also, *in situ* studies have shown that smaller lesions were more prone to demineralisation than larger ones.[25 and 26] White et al, [27] on the contrary, demonstrated *in vitro* that deeper lesions remineralised less rapidly.

The results of the current study are in accordance with ten Cate et al[22] who found that when shallow ('early') lesions were studied the predominant effect of fluoride was to inhibit demineralisation up to 50% in the 'classical' fluoride range 0–1,500 ppm, and this increased to 70% when 3,000 ppm F^- pastes were given.

The synergistic effect of Ozone and fluoride may be explained by the suggestion that Ozone can inhibit hypermineralisation. Baysan and Lynch[4] demonstrated that it is possible to remineralise or arrest primary root carious lesions and that a dentifrice containing 5,000 ppm F^- was significantly more effective than that containing 1,100 ppm F^-. On the other hand, it has been suggested that the remineralisation of primary root carious lesions following the use of a dentifrice with high fluoride content may result in the hypermineralisation on the surface of the lesions and this would prevent further remineralisation occurring through the lesion depth.

The results of the current study indicated that ozone alone has a minimal effect but this was enhanced when fluoride containing products were used in combination with use of ozone and this is in agreement with recently published study that also disputed the efficacy of ozone without its constituent adjunct therapies. [8]

Figure 1. The flow chart of the study protocol

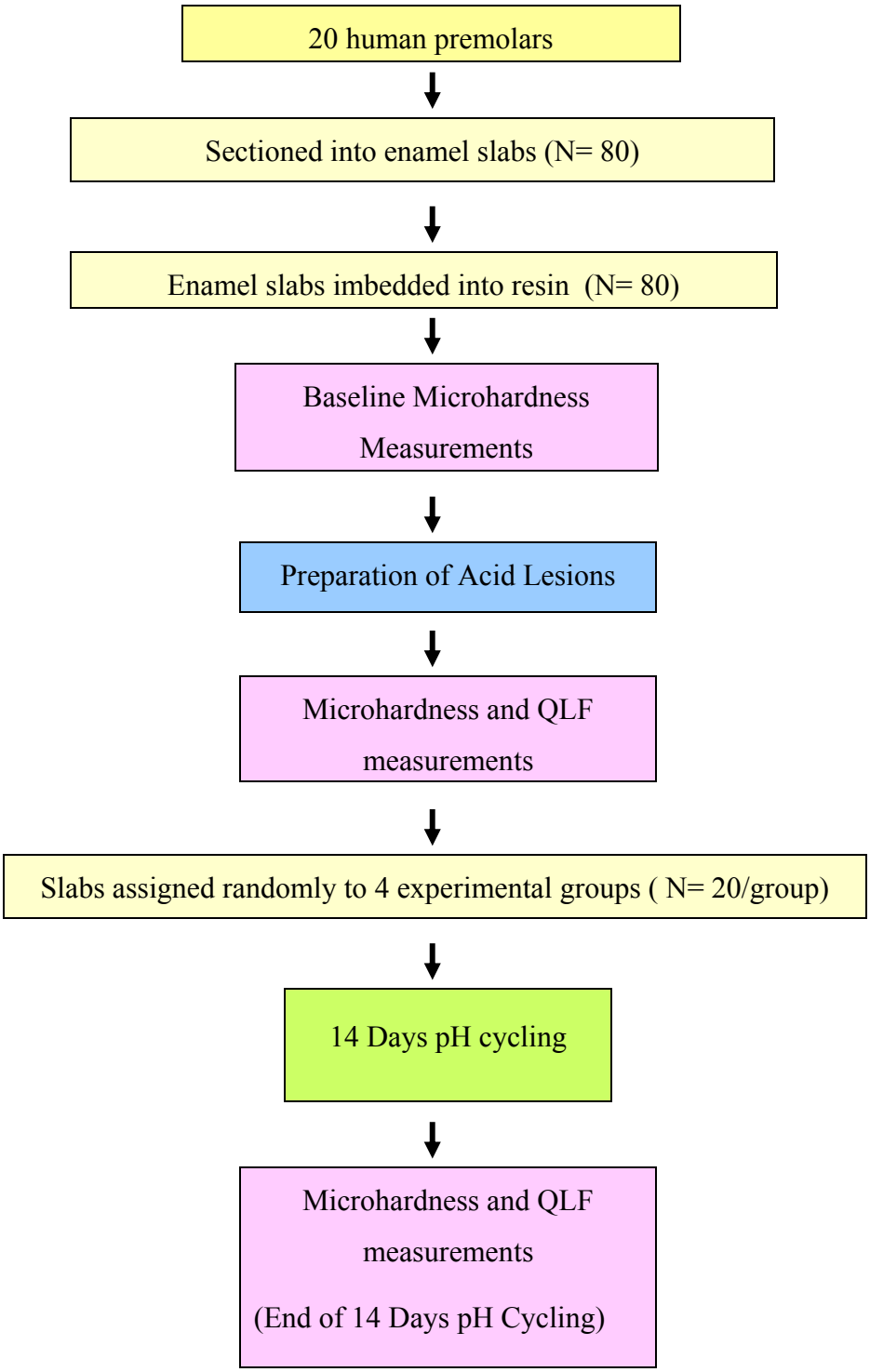


Figure 2: Boxplot for the distribution of the percentage (%) difference (change) in enamel microhardness in all groups (error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data).

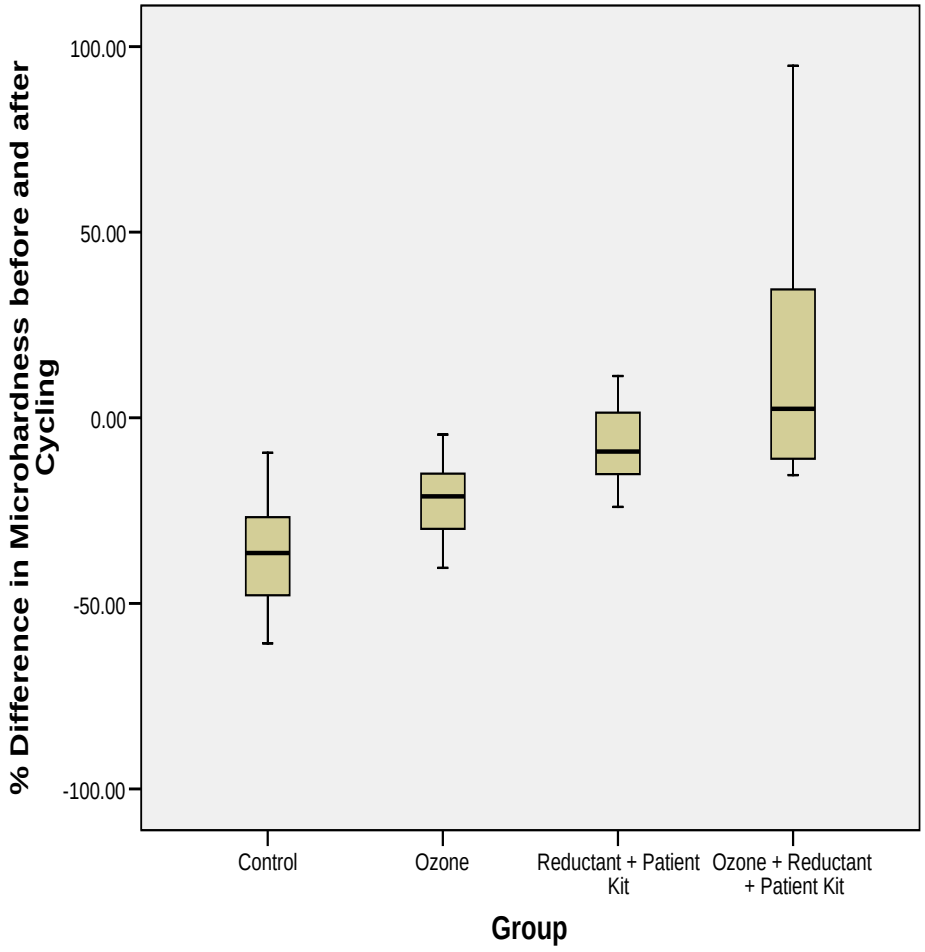


Figure 3: Boxplot for the distribution of the difference in white spot lesion area (mm^2) in all treatment groups (error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data).

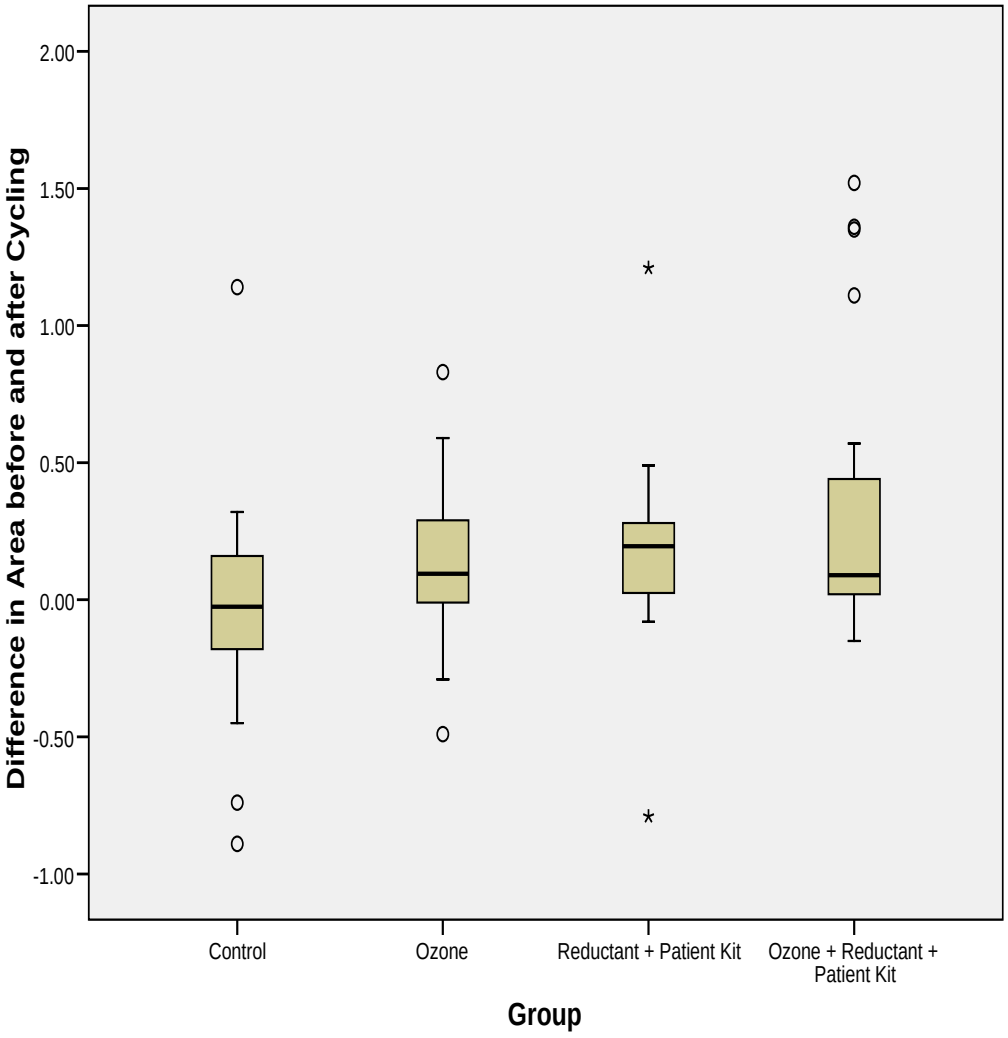
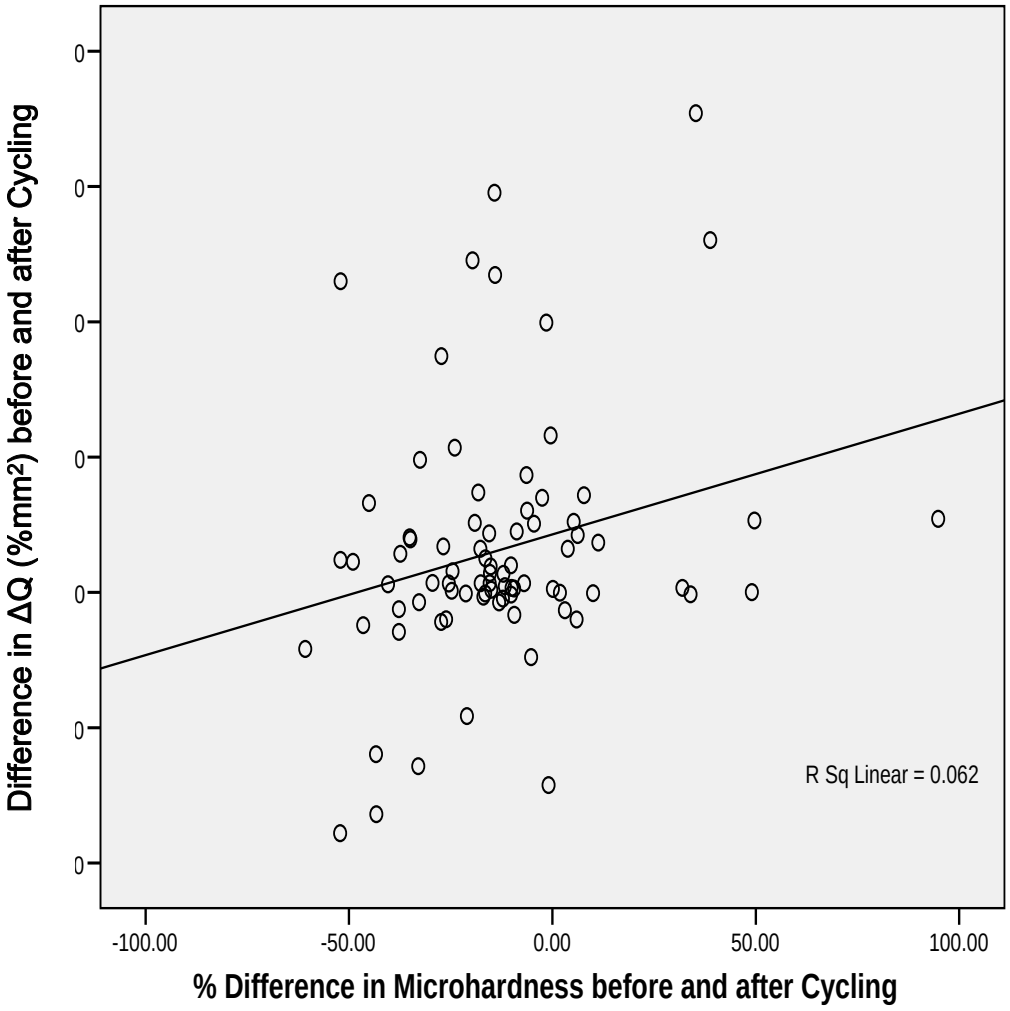


Table 1: The mean difference in the volume of lesion (ΔQ) before and after pH cycling in all groups.

Group	Mean ΔQ before pH cycling ± SD (%mm^2)	Mean ΔQ after pH cycling ± SD (%mm^2)	Mean Difference in ΔQ (%mm^2) before and after cycling ± SD
Control Group	-4.19 ± 6.50	-4.93 ± 6.87	-0.75 ± 4.43
Ozone Group	-3.68 ± 5.36	-2.04 ± 2.41	1.64 ± 3.72
Reductant + Patient Kit Group	-5.04 ± 5.30	-3.00 ± 3.51	2.04 ± 3.94
Ozone + Reductant + Patient Kit Group	-4.94 ± 6.73	-1.63 ± 2.14	3.31 ± 5.41

Figure 4: Plot of Difference in ΔQ (%mm^2) related to percentage difference in enamel microhardness (%) before and after pH cycling.



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