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ORIGINAL RESEARCH

# Evaluation of the Efficacy of a Single Subgingival Irrigation with Ozonated Water on Gingival Inflammation, Lactate Dehydrogenase Enzyme, and Alkaline Phosphatase Enzyme Activity in GCF in the Subjects undergoing Fixed Orthodontic Appliance Therapy: A Clinical and Biochemical Study

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## ABSTRACT

**Introduction:** The aqueous form of ozone is a potential antiseptic and anti-inflammatory agent, shows less cytotoxicity than gaseous ozone or established antimicrobials, and thus fulfills optimal cell biological characteristics in terms of biocompatibility for oral application.

**Aim:** To evaluate the efficacy of a single subgingival irrigation with ozonated water on gingival inflammation and lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activity in the gingival crevicular fluid (GCF) in patients undergoing fixed orthodontic therapy.

**Materials and methods:** A total of 15 participants having full mouth fixed orthodontic appliances for at least past 3 months were enrolled in the study. The study was conducted for 28 days during which GCF was collected for analysis of LDH and ALP activity; plaque index (PI), gingival index (GI), gingival bleeding index (GBI), and probing pocket depth (PPD) were recorded and scaling and root planing (SRP) was performed at baseline,14, 21, and 28 days. Subgingival irrigation with aqueous ozone was performed after SRP at baseline. Wilcoxon signed rank test and Spearman's ranked correlation and nonparametric methods were used for statistical analysis. For all the tests, a p-value of 0.05 or less was considered for statistical significance.

**Results:** Subgingival ozone irrigation is an effective method that can be performed during monthly visits on orthodontic patients to reduce the gingival inflammation which is caused due to the plaque-retentive nature of orthodontic appliances.


**Keywords:** Gingival crevicular fluid, Gingivitis, Orthodontics, Ozone.

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## INTRODUCTION

Patients undergoing fixed orthodontic appliance therapy often develop gingivitis, with the response characterized by gingival inflammation and hyperplasia since adequate levels of plaque control are difficult to achieve in these patients.[1] Various chemical agents, such as chlorhexidine, sanguinarine, and saline in the form of subgingival irrigation, have been shown as beneficial adjuncts to normal manual toothbrushing for plaque removal in orthodontic patients.[2] In order to control the disease, the goal of treatment of gingivitis should be based on biofilm destruction. An alternative approach to suppress subgingival bacteria is to inhibit their growth by changing the subgingival environment, which has been shown to be highly anaerobic with a prevailing low oxygen tension.[3] Various agents, such as molecular oxygen,[4] hyperbaric oxygenation,[5] and hydrogen peroxide[6] have been applied for modification of oxygen tension. It has been shown that repeated subgingival oxygen irrigation in previously untreated deep periodontal pockets resulted in a significant clinical improvement in the periodontal baseline conditions.[7]

Ozone is one of the most powerful antimicrobial agents available for use in medicine and dentistry. In the 1920s, Dr Edwin Parr, a Swiss dentist, started to use $O_3$ as a part of his disinfection system.[8] Nagayoshi et al[9] observed that Gram-negative bacteria, such as *Porphyromonas endodontalis* and *Porphyromonas gingivalis* were substantially more sensitive to ozonated water (0.5–4 $mL^{-1}$) than Gram-positive oral Streptococci and

*Candida albicans* in pure culture. Huth et al[10] in their *in vitro* study found that the aqueous form of ozone (1.25–20 lg $mL^{-1}$), as a potential antiseptic agent, showed less cytotoxicity than gaseous ozone (4·× $10^6$ lg $mL^{-3}$) or established antimicrobials, such as chlorhexidine digluconate (0.12 and 0.2%), sodium hypochlorite (5.25 and 2.25%), and hydrogen peroxide (3%) and thus concluded that aqueous ozone fulfils optimal cell biological characteristics in terms of biocompatibility for oral application. Nuclear factor-kappa β (NF-kβ) activity in oral cells in periodontal ligament tissue from root surfaces of periodontally damaged teeth was inhibited following incubation with aqueous ozone (20 lg $mL^{-1}$), suggesting that it has an anti-inflammatory capacity.[11] A recent systematic review also concluded that there is good evidence of ozone biocompatibility with human oral epithelial cells, gingival fibroblast, and periodontal cells in *in vitro* conditions.[12]

Lactate dehydrogenase is an enzyme that is normally limited to the cytoplasm of cells, and it is only released extracellularly after cell death. The activity of LDH in GCF is significantly correlated with gingival inflammation and tissue destruction consequent to periodontitis in humans.[13-15] Also, it has been demonstrated that LDH activity in GCF increases during the course of orthodontic treatment and that its levels are significantly greater in dental sites undergoing compression stress. Moreover, this enzyme activity may increase in teeth wearing orthodontic appliances even if they do not undergo orthodontic movement, potentially as a consequence of gingival inflammation produced by the presence of the plaque-retentive appliances.[16] Alkaline phosphatase is a calcium- and phosphate-binding protein and a phosphorhydrolytic enzyme. It is a membrane-bound glycoprotein produced by many cells, such as polymorphonuclear leukocytes, osteoblasts, macrophages, and fibroblasts within the area of the periodontium and gingival crevice.[17] The ALP is considered to be an important indicator of bone formation and is a phenotypic marker for osteoblast cells. The presence of ALP in the saliva and GCF is usually indicative of inflammation and/or destruction of periodontal tissues. The level of ALP is positively correlated with the severity of the periodontal disease.[18]

The anti-inflammatory effects of ozone seen in the periodontal field need to apply in the control of gingival inflammation and accompanying increased LDH and ALP activity in GCF of orthodontic patients. Hence, the purpose of this pilot study was to evaluate the clinical effects of a single subgingival irrigation with ozonated water on gingivitis in subjects undergoing fixed orthodontic appliances and also to correlate the clinical effects with the LDH and ALP activity in GCF.

## STUDY POPULATION AND METHODOLOGY

### Subjects

This single-center, single-blind, clinical study included subjects currently undergoing orthodontic treatment with full-mouth brackets and archwires. The subjects were recruited from the Department of Orthodontics and Orofacial Orthopedics, Mahatma Gandhi Mission's Dental College and Hospital, Kamothe, Navi Mumbai, Maharashtra, India. From the 65 orthodontic patients examined, 15 subjects (7 females and 8 males) met the inclusion criteria and agreed to participate in the study.

Subjects with good general health, at least 24 natural teeth in the mouth excluding the third molars, and full-mouth fixed orthodontic appliances for a minimum of 3 months were included in the study. After screening, each subject needed to have at least 50% bleeding sites and 50% dichotomous plaque score. Oral and written information regarding the study was given to each enrolled subject. Written consent was signed by subjects willing to participate in the study. The study protocol, consent form, and patient information sheet were approved by the Ethical Committee, Mahatma Gandhi Mission's Dental College and Hospital, Kamothe, Navi Mumbai, Maharashtra, India, and the study was conducted based on the principles outlined in the Declaration of Helsinki of 1975, as revised in 2008, on experimentation involving human subjects.

The following subjects were excluded from the study: (i) medical history of any liver, heart, kidney, and muscle diseases that are known to affect LDH levels, (ii) medical history of heart murmur, rheumatic heart disease, rheumatic fever, mitral valve prolapse, or history of any condition that might put them at risk if a bacteremia were to occur, (iii) pregnant or planning a pregnancy within the next 3 months, or if they were taking antibiotics, (iv) current history of medications likely to affect gingival health, (v) advanced periodontitis or rampant dental caries based on a noninvasive examination, (vi) removable oral prostheses or removable orthodontic appliances, (vii) requirement of premedication with antibiotics for dental appointments, (viii) those who had received any surgical or nonsurgical therapy 6 months prior to the start of the study, (ix) those who had received any chemotherapeutic mouth rinses and oral irrigation during the past 6 months, and (x) smokers.

### Methodology

The study was carried out in the Department of Periodontics, Mahatma Gandhi Mission's Dental College and Hospital, Kamothe, Navi Mumbai, Maharashtra, India. The study period of 28 days was divided into three time intervals, namely baseline, 14th, and 28th day.

At baseline, the clinical parameters, PI,[19] GI,[20] and GBI,[21] were recorded at distofacial, facial, mesiofacial, and entire lingual gingival marginal surfaces of all the teeth present. Probing pocket depth was accomplished using six-point probing in all the teeth with a UNC-15 periodontal probe and was recorded to the nearest millimeter demarcation followed by full-mouth scaling and collection of GCF for biochemical analysis. All the recordings of clinical parameters were made by same examiner, who was blinded as to the treatment condition and who did not review the data sheets from previous visits while recording clinical parameters and GCF collection.

Following this, the subjects underwent full-mouth subgingival irrigation with ozonated water that was released from an irrigation device, "Alfa-Omega Dental Jet." The device released a single pulsating stream of ozonated water from the nozzle that could be adjusted for different speeds and pressures ranging from 66 to 130 kPa and an ozone output of 0.1 ppm, at a noise output of <70 dB and water outflow of 280 to 420 mL. To facilitate subgingival ozone irrigation, a 20-gauge blunt needle was attached to the tip of the nozzle of the ozone dental jet holder. A stop clock was used to set the irrigation time to 15 seconds at each tooth, after which the irrigation was stopped.

After irrigation, the patients were instructed to brush twice daily for minimum 2 minutes, using a standard tooth brush (Colgate Super Flexible with medium consistency bristles) and tooth paste (Colgate dental cream) provided to them. The subjects were recalled on the 14th and 28th day from the baseline for recording the clinical parameters and collection of GCF for biochemical analysis.

## Gingival Crevicular Fluid Sampling Procedure

The subjects were seated in an upright position in the dental chair. To avoid contamination with saliva, sites were chosen from maxillary anterior teeth. Supra gingival plaque and calculus were removed with hand instrumentation prior to the collection of GCF. Care was taken not to touch the gingival margin to prevent stimulation and bleeding. The calibrated microcapillary tubes (0–5 µL) were placed extracrevicularly at the mesiofacial, distofacial, or midfacial surface of the tooth. A standardized volume of 5 µL of GCF was collected each for ALP and LDH assay. Microcapillary tubes contaminated with blood were discarded. The collected GCF was immediately transferred to the sterilized microcentrifuge/Eppendorf tubes that contained 45 µL of normal saline for ALP and 100 µL freshly prepared phosphate buffer saline. The tubes were then transported for analysis which was done within two hours of collection of the crevicular fluid.

## Determination of LDH Activity

A diagnostic kit (Infinite by Accurex Biomedical Pvt. Ltd) was used for the estimation of LDH activity based on UV kinetic method.

The kit consisted of two reagents:

- Reagent 1
  - Tris buffer, pH 6.8
  - Ethylenediaminetetraacetic acid
  - Nicotinamide adenine dinucleotide (NADH)
  - Sodium pyruvate
  - Sodium chloride
- Reagent 2

### *Aqua-4 Distilled Water*

Working solution was prepared by mixing reagents 1 and 2 in 4:1 ratio.

Then, 100 µL of the prepared reagent was taken in an Eppendorf tube and 10 µL of the sample was added to it. The enzyme activity was recorded using an automated analyzer and the result was obtained in the form of a digital readout.

### *Principle of the Reaction*

The LDH catalyzes the reduction of pyruvate by NADH to form lactate and $NAD^+$. Catalytic concentration is determined from the rate of decrease of NADH.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

## Determination ALP Activity

A diagnostic kit (Autozyme by Accurex Biomedical Pvt. Ltd) was used for the estimation of ALP based on kinetic method.

The kit consisted of two reagents:

- Reagent 1
  - p-Nitrophenyl phosphate
  - Magnesium ion (Mg[2]+)
  - Tris/carbonate buffer (pH 10.2 ± 0.2 at 25ºC)
- Reagent 2

### *Aqua-4 Distilled Water*

The GCF (5 µL) was diluted 10 times with 45 µL of normal saline, to make 50 µL of sample. The diluted GCF was then centrifuged for 3 to 5 minutes at 3500 to 4500 rpm in a microcentrifuge; 10 µL of the clear supernatant was utilized for the analysis of ALP activity. The working agent was prepared by adding 2.2 mL of distilled water to reagent 1. Then, 500 µL of the prepared reagent was taken in an Eppendorf tube and 10 µL of the sample was added to it. The enzyme activity was recorded using an automated analyzer and the result was obtained in the form of a digital readout.

**Table 1:** Normality tests showing the distribution of data

| *Clinical and biochemical parameters at 14th and 28th days from baseline* | *Kolmogorov–Smirnov*[a] | | | *Shapiro–Wilk* | | | |
|---|---|---|---|---|---|---|---|
| | *Statistic* | *df* | *p-value* | *Statistic* | *df* | *p-value* | *Interpretation* |
| PI-14 | 0.143 | 15 | 0.200* | 0.933 | 15 | 0.305 | Normal |
| PI-28 | 0.149 | 15 | 0.200* | 0.944 | 15 | 0.433 | Normal |
| GI-14 | 0.226 | 15 | 0.039 | 0.826 | 15 | 0.008 | Non-normal |
| GI-28 | 0.237 | 15 | 0.023 | 0.899 | 15 | 0.090 | Non-normal |
| GBI-14 | 0.117 | 15 | 0.200* | 0.975 | 15 | 0.929 | Normal |
| GBI-28 | 0.099 | 15 | 0.200* | 0.987 | 15 | 0.997 | Normal |
| PPD-14 | 0.345 | 15 | 0.000 | 0.709 | 15 | 0.000 | Non-normal |
| PPD-28 | 0.345 | 15 | 0.000 | 0.709 | 15 | 0.000 | Non-normal |
| LDH-14 | 0.160 | 15 | 0.200* | 0.934 | 15 | 0.309 | Normal |
| LDH-28 | 0.177 | 15 | 0.200* | 0.934 | 15 | 0.312 | Normal |
| ALP-14 | 0.202 | 15 | 0.101 | 0.893 | 15 | 0.075 | Normal |
| ALP-28 | 0.116 | 15 | 0.200* | 0.962 | 15 | 0.726 | Normal |

[a]Lilliefors significance correction; *This is a lower bound of the true significance; df: Degree of freedom

**Table 2:** Mean and standard deviation at three time intervals, i.e., at baseline, 14th day, and 28th day

| *Time interval (days)* | *PI* | | *GI* | | *GBI* | | *PPD* | | *LDH* | | *ALP* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Mean* | *±SD* | *Mean* | *±SD* | *Mean* | *±SD* | *Mean* | *±SD* | *Mean* | *±SD* | *Mean* | *±SD* |
| 0 | 1.30 | 0.24 | 1.84 | 0.32 | 1.38 | 0.28 | 4.07 | 0.88 | 106.83 | 19.80 | 50.04 | 18.91 |
| 14 | 0.85 | 0.30 | 1.16 | 0.33 | 0.85 | 0.31 | 3.47 | 0.64 | 73.05 | 12.97 | 49.51 | 14.42 |
| 28 | 0.82 | 0.30 | 1.07 | 0.27 | 0.84 | 0.34 | 3.47 | 0.64 | 70.62 | 11.76 | 51.11 | 15.74 |

*Principle of the Reaction*

$$\text{p- p-Nitrophenyl phosphate} + H_2O \xrightarrow[Mg^{2+}]{ALP} \text{p-Nitrophenol} + \text{phosphate}$$

## Statistical Analysis

Data analysis was done using Statistical Package for the Social Sciences version 2.0. All variables were expressed as mean with standard deviation. The mean values were tested for normality using Kolmogorov–Smirnov with Lilliefors significance correction and Shapiro–Wilk test. Paired t-test was used to find which pair differed significantly with respect to time. For non-normal data, Wilcoxon paired sample test was used. For all the variables, $p \leq 0.05$ was considered significant.

## RESULTS

The test of normality showed that the PI, GBI, LDH, and ALP activity values were normally distributed, whereas the GI and PPD values were non-normal (Table 1). The data were expressed in the form of mean and standard deviation (Table 2). The mean difference of values at 14 and 28 days from the baseline was considered for analysis. The paired t-test for PI, GBI, and LDH enzyme indicated a significant decrease ($p \leq 0.05$) with respect to time, whereas the ALP activity did not show any significant difference ($p \geq 0.05$) (Table 3). Wilcoxon signed rank test for both GI and PPD showed significant decrease ($p \leq 0.05$) with respect to time intervals (Table 4).

**Table 3:** Paired t test showing mean difference in PI, GBI, LDH, and ALP activity on 14th and 28th days from the baseline

| *Clinical and biochemical parameters at 14th and 28th days from baseline* | *Mean difference* | *t-test* | *df* | *p-value* |
|---|---|---|---|---|
| PI-0–PI-14 | 0.44580 | 5.494 | 14 | 0.000* |
| PI-0–PI-28 | 0.47800 | 6.044 | 14 | 0.000* |
| GBI-0–GBI-14 | 0.53267 | 8.394 | 14 | 0.000* |
| GBI-0–GBI-28 | 0.53987 | 8.867 | 14 | 0.000* |
| LDH-0–LDH-14 | 33.78533 | 12.497 | 14 | 0.000* |
| LDH-0–LDH-28 | 36.21600 | 12.122 | 14 | 0.000* |
| ALP-0–ALP-14 | 0.53133 | 0.257 | 14 | 0.801 |
| ALP-0–ALP-28 | −1.06333 | −0.567 | 14 | 0.580 |

$p \leq 0.05$ *was considered significant; df: Degree of freedom

**Table 4:** Wilcoxon's signed rank test showing mean difference in PI, GBI, LDH, and ALP activity on 14th and 28th days from the baseline

| *Gingival index* | *GI-0–GI-14* | *GINGI-0–GINGI-28* |
|---|---|---|
| Z value | −3.408[a] | −3.408[a] |
| p-value | 0.001* | 0.001* |
| PPD | PPD-0–PPD-14 | PPD-0–PPD-28 |
| Z value | −2.264[a] | −2.264[a] |
| p-value | 0.024* | 0.024* |

[a]Based on positive ranks; Wilcoxon signed rank test; $p \leq 0.05$* was considered significant

## DISCUSSION

A short-term, single-center, single-blind, prospective clinical and biochemical study was carried out to evaluate the clinical effects of a single subgingival irrigation with 0.1 ppm ozonated water on gingival inflammation LDH

and ALP activity in subjects undergoing fixed orthodontic appliance therapy.

Results of the present study showed that subgingival irrigation with 0.1 ppm ozonated water caused significant reduction in values for all the clinical parameters PI, GI, GBI, and PPD from baseline to 14th day and from baseline to 28th day. The results were in accordance with the reduction in PI, GI, and GBI seen in the study by Kshitish and Laxman[22] which evaluated the effect of oral irrigation with 0.1 ppm ozonated water and 0.2% chlorhexidine in moderately deep periodontal pockets on the clinical parameters of chronic and aggressive periodontitis patients.

The possible mechanism for reduction of gingival inflammation could be associated with antibacterial effect of ozone on the plaque microorganisms or by a disruption of subgingival plaque.[7] The antiinflammatory effects of ozonated water (aqueous ozone) observed in the present clinical study are also supported by an *in vitro* study by Huth et al[10] which showed that NF-kβ activity in oral cells in periodontal ligament tissue from root surfaces of periodontally damaged teeth was inhibited following incubation with aqueous ozone.

In addition to the effects of subgingival ozonated water irrigation, the improvement seen in PI and GI may also be attributed to the subject's improved oral hygiene as a response to anticipation of forthcoming oral examination during study intervals, i.e., Hawthorne effect.[23]

There was a significant reduction in GBI scores at 14th and 28th days. A prolonged observation period will allow a better estimation of extinction of the effects.

A significant reduction in PPD seen at 14th and 28th days could have resulted from reduction in gingival inflammation.

In the present study, there was a significant reduction after ozone irrigation in the total LDH enzyme activity from baseline to 14th and 28th day. This significant decrease is in accordance with the results of Serra et al[24] who attributed this increased LDH levels in the GCF to the tissue resorption in both the compressed and tensional sites, or even secondary to a possible cell necrosis in the periodontal ligament during the orthodontic treatment and also in a cross-sectional study by Wolff et al.[15] Also, this enzyme activity may increase in teeth wearing orthodontic appliances even if they do not undergo orthodontic movement, potentially as a consequence of gingival inflammation produced by the presence of the plaque-retentive appliances.

Previous studies[16,24] have shown an increased LDH enzyme activity in orthodontic patients, which needs to be controlled along with gingival inflammation to facilitate orthodontic tooth movement. The results of this pilot study showed that a single subgingival irrigation of ozonated water can effectively reduce the gingival inflammation in orthodontic patients, which is also reflected in the reduction of total LDH unit activity levels. The effect of reduced inflammation is maintained over a 28-day (4-week) period, which frequently coincides with scheduled orthodontic appointments during active treatment. In addition, no adverse effects were observed with the use of subgingival irrigation with 0.1 ozonated water during the 1-month study trial period.

The ALP activity did not show any changes at the 14th day and slight increase in the mean ALP activity was seen at the 28th day from the baseline. It has been reported that ALP activity is at higher levels in periodontal ligament than in connective tissues and, if mechanically stressed, can release ALP.[25] Increased ALP activity was observed from the 7th day onward with significant peaks on the 14th and 28th days in the mesial site of the distalized canine in a study conducted by Dhar et al.[26] Thus, constant osteoblastic and osteoclastic activity due to orthodontic forces could be the reason for the unchanged or increased levels of ALP activity during the study.

In comparison with classical periodontal treatment modalities, such as systemic and local antimicrobials, ozone therapy is quite inexpensive. However, further research is needed to standardize the indications and treatment procedures of ozone therapy. Further randomized control trials with larger sample size comparing the efficacy of subgingival ozonated water irrigation with positive control, such as 0.2% chlorhexidine and negative control, such as saline are required to confirm the effects of 0.1 ppm ozonated water.

## CONCLUSION

At the end of 1 month, a single subgingival irrigation of 0.1 ppm ozonated water could effectively reduce the gingival inflammation in orthodontic patients. Thus, subgingival ozone irrigation can be an effective method that can be performed during monthly visits on orthodontic patients to reduce the gingival inflammation because of plaque-retentive orthodontic appliances. However, further randomized controlled trials are required to validate the use of ozone irrigation in orthodontic patients for plaque control measures.

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