DOI: 10.7860/JCDR/2017/26708.10129

Original Article

Dentistry Section

Antibacterial Activity of Freshly Prepared Ozonated Water and Chlorhexidine on *Mutans Streptococcus* When Used as an Oral Rinse – A Randomised Clinical Study

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ABSTRACT

Introduction: Dental caries is one of the most common causes of morbidity of the tooth. Attempts have been made to reduce the pathogen population size i.e., *Mutans Streptococci* (MS) to demote the incidence of caries and increase the resistance of the tooth to cariogenic attack.

Aim: To evaluate the antibacterial efficacy of freshly prepared ozonated water, in proposing it as an alternative mouth rinse on MS in comparison to Chlorhexidine (CHX).

Materials and Methods: Subjects with high caries incidence and MS counts more than 10^5 Colony Forming Unit (CFU) were selected and divided by block randomization into two groups of 23 subjects each. The subjects were advised to use the respective mouth rinses under the operator surveillance, consecutively for 14 days. Stimulated salivary samples were collected from the subjects on the first day, 7th and 14th day to analyse the changes in MS counts during the course of use of oral rinses. The obtained data was tabulated and statistically analysed.

Results: Freshly prepared ozonated water showed a statistically significant reduction in MS counts after an interval of 7 days and 14 days when compared to CHX.

Conclusion: Ozonated water when consecutively used as a mouth rinse resulted in a significant reduction of MS counts. Hence, it can be used as an alternative to chlorhexidine.

Keywords: Aqueous ozone, Dental caries, High caries risk, Mouth rinse

INTRODUCTION

Dental caries still remains to be one of the most common diseases and a global health problem that affects about 60%-90% of the population [1-3]. The bacterial colonization of *Mutans Streptococci* (MS) on the tooth, makes it more vulnerable for the development of dental caries [4,5]. At the outset, caries diagnosis and treatment were restricted and primarily aimed at detection and restoration of cavitated lesions, thus futile in dealing with the underlying aetiologic factors. Now, as a part of the medical model, preventive treatment for caries is mainly based on reducing the pathogen population size and increasing the resistance of the tooth to cariogenic attack. Many chemotherapeutic agents or antimicrobial agents are available to reduce the MS counts, amongst which CHX (0.12%) is the most commonly used [6,7]. CHX is bacteriostatic at low concentrations and bactericidal at high concentrations. It has a broad antimicrobial spectrum (i.e., against Gram positive/negative bacteria and fungi), thus making it effective against many oral bacterial species. But the long term use of CHX has few disadvantages such as teeth staining [8] and altered taste sensation [9].

Ozone (O_3) is the triatomic form of oxygen (O_2). It is oxygen in its most active state and is an extremely potent oxidant that has been shown to possess broad spectrum antimicrobial activity. The oxygen radicals cause cellular lysis by penetrating the microbial cell membrane in the presence of water, thus affecting its osmotic stability [10]. Ozone, in either gaseous or aqueous phases, is widely used as a disinfectant due its wide range of microbiologic and metabolic activity [11]. The efficacy of ozonated water as mouth rinse on MS has not been reported earlier. In proposing ozonated water as another potential antimicrobial mouth rinse, it is important to compare it with other agents. Thus, the objective of the study was to assess and compare the effect of ozonated water and 0.2% CHX on the level of MS in the saliva when used as a mouth rinse.

MATERIALS AND METHODS

This open label randomized two arm parallel group's clinical study was conducted in RIMS government dental college, Kadapa, Andhra Pradesh, India, for over a period of two months from September 2010 to October 2010. The study was reviewed and approved by the Institutional Ethical Committee. A total of 46 healthy young adults, who volunteered, were explained about the study and written informed consent was obtained from them. Both males and females in the age group of 16 and 30 years, having DMFT index to be more than 3, missing teeth due to past caries experience, MS counts >10^5CFU/ml (tested at their initial visit) and those who were neither using antibiotics nor any mouthwash from past six months were enrolled. Subjects with missing teeth due to reason other than caries and/or who have undergone radiation therapy or having any dysfunction of the salivary glands, and/or who have undergone any surgical or non-surgical therapy in the past six months were excluded. The subjects who fulfilled the inclusion criteria were recalled after two days, and were then randomly allocated by block randomization to two groups. Any carious lesion open to the oral cavity was excavated and restored prior to the participants' allocation to treatments. The random selection of the blocks was done using a list of computer generated random numbers. The variables used for sample size calculation was the reduction in bacterial counts (Efficiency). The power of the study was 80%. A study of independent two treatment study in with 1:1 ratio was planned. Prior data indicated that the reduction in bacterial counts rate with ozone (gas) is 0.5. For experimental subjects, it is 0.9. Therefore, study included 23 experimental subjects and 23 control subjects to be able to reject the null hypothesis such that the success rates for experimental and control subjects are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05.

Thus, Group A, comprising of 23 participants, were instructed to use undiluted 0.2% CHX solution (clohex, Dr. Reddy's Laboratories Limited) for 45 to 60 seconds once daily, for 14 days. Group B, included another 23 participants, who were given freshly prepared ozonated water to use as a mouth rinse once daily for 14 days [Table/Fig-1]. Ozonated water was prepared by using a tabletop "ozone generating device" (V-Can Industries, Chennai), by bubbling ozone gas into the distilled water.

All the participants were asked to chew a piece of paraffin wax; the stimulated saliva of about 1 ml-2 ml was collected and tested for the baseline MS counts. They were instructed to use 10 ml of the respective mouth rinses and thoroughly rinse for 45 to 60 seconds. The participants were asked to visit the department for the next 14 consecutive days and use of mouth rinses was done under the observation of the observer. The Group B participants were given freshly prepared ozonated water every day. At the end of the 7th day and 14th day, saliva sample was again collected from both the groups. As all the participants were selected from the dental college, there was a good compliance.

All the saliva samples (baseline, 7th day and 14th day samples) were serially diluted in 0.05 M potassium phosphate buffer (pH 7.1) and inoculated on the MSB–Mitis Salivarius Bacitracin agar, which is a selective media for *S.mutans.* A sterile inoculating loop with 3.26 mm internal diameter which holds about 0.01 ml saliva was streaked over the surface of culture plates. Later they were incubated at 37^{0}C in a candle jar environment for 48 hours. After two days the number of MS CFU per 1 ml of saliva were estimated by the formula, CFU/ml = (no. of colonies x dilution factor)/volume of culture plate. Identification of the *S.mutans* was based upon colony morphology and also standard biochemical characteristics. The obtained data was tabulated and subjected to statistical analysis. The inhibition of the bacterial counts to around 50% of the baseline after two weeks was considered as the cut off point.

STATISTICAL ANALYSIS

Data was entered, and tabulated using excel spread sheet. Repeated measures ANOVA and unpaired t-tests were used appropriately. A p-value less than 0.05 was considered statistically significant. Chi-Square test was used to compare percentage Inhibition in bacterial counts at day-14 between patients using CHX and ozonated water.

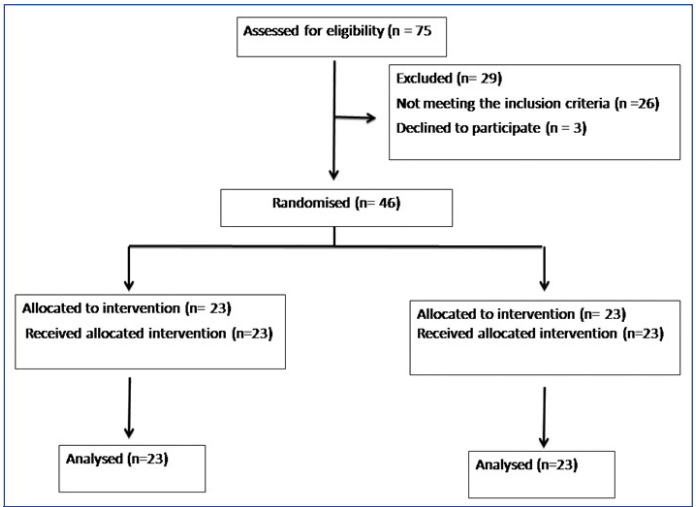
RESULTS

Out of 75 participants who were eligible, 29 were excluded from the study, as 26 of them did not meet the inclusion criteria and 3 of them declined to participate. Thus, 46 participants were selected, randomised and analysed with power and sample size calculator version 3.1.2. All the participants showed good compliance [Table/Fig-1]. Both the groups showed a statistical significant difference in the reduction of the MS counts after 7 and 14 days. [Table/Fig-2] shows the comparison between ozonated water group and CHX group. Ozonated water group showed a high significance (p-value < 0.001) indicating a better anti bacterial property when compared to CHX. The percentage inhibition of the bacterial counts at day-14 between subjects using CHX and ozonated water was compared which also showed a high significance [Table/Fig-3].

DISCUSSION

Despite the recent declining trends, dental caries still is a common disease. Due to the multifactorial nature of the disease, there is no single marker or method that can accurately assess the patient's caries risk [12,13]. One of the most popular caries risk assessments promoted by Krasse B is to measure salivary samples for levels of MS [14].

Studies have reported a positive correlation between dental caries and the degree of infection with MS [15]. There is also evidence



[Table/Fig-1]: Flow diagram of the study.

Groups	Baseline	Day-7	Day-14	p-value<0.001
Chlorhexidine (Group A)	196.52±48.86	163.91±43.25	109.13±37.04	B vs. Day-7
				B vs. Day-14
Ozonated Water (Group B)	202.17±59.23	122.61±44.54	68.70±32.24	B vs. Day-7
				B vs. Day- 14
Total	199.35±53.77	143.26±48.17	88.91±39.96	B vs. Day-7
				B vs. Day- 14
Absolute Change from baseline				
Chlorhexidine (Group A)	-	32.61±12.51	87.39±19.82	p<0.001
Ozonated Water (B)	-	79.57±28.04	133.48±38.09	p<0.001
Grand Total	-	56.09±32.01	110.43±38.00	p<0.001

[Table/Fig-2]: Comparison of chlorhexidine and ozonated water on bacterial counts at Baseline (B), 7 and 14 days (unpaired t-tests).

Percentage inhibition in bacterial counts	Chlorhexidine	Ozonated water	p-value
Day – 14			
<50%	16	0	p<0.001
≥50%	7	23	

[Table/Fig-3]: Comparison of percentage inhibition in bacterial counts at day-14 between patients using chlorhexidine and ozonated water (Chi-square test).

that an increased level of *S.mutans* may precede the development of caries lesions [16]. Thus, subjects' past caries experience was taken as a main criteria to include them in the study as it still remains the most powerful single predictor of future caries increment [17].

To prevent or reduce the prevalence of dental caries, antimicrobial control of the dental plaque flora i.e., MS in particular may prove beneficial in adjunct to other methods [18]. Many antimicrobial agents (antibiotics) [19], cationic detergents [20], halogens [21], phenolic compounds [22], plant extracts [23], and salts of metal ions [24] have been tried, amongst which fluoride represents one of the most successful agents. Bisbiguanide Chlorhexidine is a potent chemotherapeutic agent against MS [7], which has been studied extensively and thus considered gold standard. The cationic bisbiguanide binds to the negatively-charged sites on the cell wall, destabilizes it and results in the leakage of intracellular products. It maintains its antibacterial activity for a long period of time by its ability to bind to variety of substrates [7].

Ozone was discovered by CF Schonbein in 1839 [25]. It was first suggested as a disinfectant for drinking water in view of its powerful ability to inactivate microorganisms (against bacteria, fungi, protozoa, and viruses). The aqueous form is easy to handle and is a potent microbicidal solution suitable as a soaking solution for medical and dental instruments. Nagayoshi M concluded in his

study that ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl, when used as an irrigant especially when combined with sonication, and showed a low level of toxicity against cultured cells [26].

Cardoso MG evaluated ozonated water as an irrigating agent during endodontictreatment and proved its efficacy on the microbial suspension [10]. Sadatullah S et al., measured the supragingival plaque micro organisms (total CFU) before and after rinsing once with 0.1 ppm of ozonated water and unveiled a reduction in the microbial load including *Streptococcus mutans* [27]. The antibacterial activity of ozonated water was further substantiated when used as a subgingival irrigant where not only did it curtail the periodontal pathogens but also the healing period [28,29]. Ebensberger U et al., documented that when the avulsed teeth were irrigated with nonisotonic ozonated water for around 2 minutes before replantation, it provided both mechanical cleansing and decontamination of the root surface without any adverse effect on periodontal cells [30]. Białoszewski D et al., designed a prototype device to prepare freshly prepared ozonated water with ozone concentration in the range of 1.5 to 3 µg/ml and proved its efficiency to kill almost all cells of the bacterial and yeast test strains after a very short exposure of 30 seconds [31].

In this study, the concentration of the gas displaced from table top ozone generator was analysed to be 2.4 mg/l (>2 ppm O_3) similar to Katti SS et al., [28]. The participants who used ozonated water were more willingful as it did not differ much from the normal water either in taste or appearance. Ozone in the gaseous form is a selective oxidant and affects only certain compounds but whereas the aqueous form is highly unstable and rapidly decomposes through a complex series of chain reactions resulting in the formation of hydroxyl (OH) radicals which are among the most reactive oxidizing species.

The principle advantage of MSB agar is that it allows the isolation of the species even when present in the low numbers relative to the total population. There was a significant decline of MS counts in ozonated water group when compared to the participants who used CHX mouth rinse, thus proving better antibacterial property of ozonated water, which could be the result of oxidation potential of ozonated water that destructs the cell wall and cytoplasmic membranes of bacteria and fungi. Ozone when dissociates into oxygen, creates an oxygen-rich environment, thus disturbing the normal ecosystem of the plaque. The enzymatic control system of the cell is blocked as ozone inhibits glycoproteins, glycolipids, and other amino acids the outcome of which is the functional cessation and death of the microorganism [32]. The percentage inhibition of the bacterial counts at day-14 between subjects using CHX and ozonated water when compared noticeably signified that all the participants in the ozonated water had more than 50% diminution of the baseline bacterial count [Table/Fig-3]. Ozonated water has also been proposed to promote remineralisation of incipient carious lesion by causing decarboxylation of the pyruvic acid produced by acidogenic bacteria to acetic acid during cariogenesis [33]. An in vitro study conducted on agar wells manifested that ozonated water showed complete inhibition of *S.mutans* and *E.feacalis* [34].

In the present study, CHX mouth rinse when used for a week showed an appreciable decrease in MS counts, similar to Persson RE et al who stated that subjects with high *S.mutans* counts ($\geq 10^6$) reduced the count to almost zero after rinsing daily for six weeks [35]. The main advantage of CHX is its antibacterial activity and substantivity but has short comings such as mucosal desquamation, impaired wound healing and fibroblast attachment to the tooth surfaces [36]. Ozonated water satisfies most of the ideal requirements of a mouth rinse. The only shortcoming is that O_3 molecule is unstable and ozonated water should be prepared immediately before use. Its oxidizing property gradually decreases with time. Within an hour after preparation, half the mixture is transformed into oxygen while only the other half of the mixture is still ozone.

Thus, aqueous ozone (freshly prepared ozonated water) is a possible alternative oral antiseptic agent with high antibacterial activity against oral pathogens without resistance development [37] and fulfils optimal cell biological characteristics in terms of biocompatibility for oral application [38].

LIMITATION

One of the limitations of this study could be a small population that is tested. In future, ozonated water can be further tested for its efficacy on the smear layer both in tooth preparation and root canal procedures.

CONCLUSION

Within the limitations of the study, the use of ozonated water as a mouth rinse resulted in considerable declension of MS counts. Due to the easy availability of economical table top ozone generator, ozonated water can be recommended as an alternative daily mouth rinse to all the patients who are at high risk to caries and also as a preventive measure to all the other patients.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Jan 11, 2017**
Date of Peer Review: **Feb 04, 2017**
Date of Acceptance: **Jun 10, 2017**
Date of Publishing: **Jul 01, 2017**