

Anti-Microbial Effect Of Different Time's exposure of ozonized Gas And Ozonized Water On periodontal pathogens (*In Vitro* Study)

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ABSTRACT

Background: the oral cavity is consider to be an open ecosystem, with the balance between the microorganism's entrance and the defenses of the host. The initiation of periodontitis has been associated with restricted kinds of anaerobic bacteria, such as *Aggregatibacter actinomycetemcomitans* (A.a) and *Porphyromonas gingivalis* (P.g) in plaque subgingivally. Ozone has a biological effects on bacteria due to oxidation of bio-molecules and its toxins. The aim is to determine and compare the antimicrobial effect of gaseous ozone and ozonized water on the growth of isolated anaerobic bacteria (A.a and P.g) when exposed to different time intervals.

Materials and methods: This experiment is done by ozone generator OLYMPIC- III (600mg/hr) to generate the gaseous ozone (218ppm/W-air) which bypassed around the agar plates containing on of the isolated bacteria with different time intervals (1-10 minutes). And with special aeration stone for generation of ozonized water (0.6 ppm) with different time intervals (1-15 minutes).

Results: Gaseous ozone have a significant reduction in the bacterial growth on the agar plates for (A.a) was 7 minutes and (P.g) was 4 minutes. While ozonated water have also a significant reduction in the bacterial growth on the agar plates for (A.a) was 5 minutes and (P.g) was 4 minutes.

Conclusion: Both gaseous ozone and ozonized water are a powerful antimicrobial effects on anaerobic microorganism isolated from chronic periodontitis patients.

Keywords: gaseous ozone, ozonized water, *Aggregatibacter actinomycetemcomitans* (A.a), *Porphyromonas gingivalis* (P.g) (**J Bagh Coll Dentistry 2017; 29(2):78-82**)

INTRODUCTION

Periodontitis is a destructive and inflammatory disease of the connective tissues that supporting the teeth and is caused either by one specific type of microorganism or by a group of specific microorganisms, leading to progressive destruction of periodontal ligament and alveolar bone with the formation of periodontal pocket, gingival recession, or both ⁽¹⁾. Bacteria are the prime etiological agents in periodontal disease, and it is estimated that more than 500 different bacterial species are capable of colonizing the adult mouth ⁽²⁾. *Aggregatibacter actinomycetemcomitans* is considered a primary pathogen in localized and generalized chronic periodontitis ⁽³⁾. While, *Porphyromonas gingivalis* is implicated in chronic and aggressive periodontitis ⁽⁴⁾. It is considered one of the main etiologic agents of destructive periodontal disease ^(5,6). Ozone is a potent oxidant and an important disinfectant, acting on microorganisms by means of oxidation of their biological material ⁽⁷⁾. It has been reported that ozone can be employed as a bactericidal agent under various forms, such as ozonized water ⁽⁸⁾, ozonized oil, ⁽⁹⁾ ozone associated with other substances ⁽¹⁰⁾, and more frequently the gaseous O₃/O₂ mixture ⁽¹¹⁾.

Gaseous ozone has a high oxidation capacity and is greater than chloride for about 1.5 times when is use as an antimicrobial agent against several bacteria, viruses, fungi, and protozoa. It has also the ability to stimulate blood circulation and the immune response. Such characteristic features can be applied in medicine and dentistry and have been indicated for the treatment of 260 different pathologies ⁽¹²⁾. Ozonized water have a high level of biocompatibility on human oral epithelial cells, gingival fibroblast cells, and periodontal cells ⁽¹³⁾. Ozonized water strongly inhibited the accumulation of dental plaque and is effective in killing gram positive, gram-negative bacteria and oral *Candida albicans* causing periodontal disease

MATERIALS AND METHODS

1-Isolation and identification of the pathogens:

The subgingival plaque samples were collected from 10 systemically healthy patients with chronic periodontitis attending the clinic at the Department of periodontics in the teaching hospital of the College of Dentistry / University of Baghdad. The age range was (35-55) years old; the subgingival plaque samples were collected from the periodontal pocket of more

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than >6 mm depth with attachment loss of one to two mm. The subgingival plaque was put on a swab that was inserted immediately in transfer media to preserve the sample which is then spread on selective agar media (tryptic soy agar) for both A.a and P.g and incubated anaerobically using anaerobic jar and anaerobic gas packs in the incubator for 72 hours at 37°C^(15,16). The procedure must be done within a period of less than 30 minutes from collecting the sample from the patient. The identification of both A.a and P.g was done by Morphological characteristic⁽¹⁷⁾, Gram's stain⁽¹⁸⁾ and by using Analytical profile index (API) test for the biochemical testes⁽¹⁹⁾

2. General description of the experiments:

The gaseous ozone was generated from ozone generator OLYMPIC- III (600mg/hr). In this experiment a small plastic jar was used. The plastic cover has one ozone gas inlet port to inject the ozone gas and distribute it evenly throughout the jar, and one gas outlet for the release of the ozone gas. The ozone generator was fed with 1 LPM of dry compressed air as a feed gas. Ozonized air was bypassed around the agar plates to supply a total air flow of ozone of 218 ppm/w-air. The ozone gas/dry air mixture flowed into the jar for different times (1-10 minutes) according to the experimental design the ozone level inside the plastic jar was kept consistent during the time periods by adjusting the outlet port (Fig. 1).



Figure 1: General description of the device

While ozonized water was generated by using special aeration stone, with concentration (0.6 ppm) measured by special CHEMets-Kit. As shown in (Fig 2).



Figure 2: shows the: 1) Ozone Generator 2) plastic container 3) CHE Mets Kit

3. Procedure:

Five well-isolated colonies of the same morphological type which was incubated anaerobically at 37°C for 24hrs, were selected from tryptic soy agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 5 ml of tryptic soy broth medium. This results in a suspension containing approximately 1×10^8 CFU/ml (equivalent to a 0.5 tube McFarland standard)⁽²⁰⁾. Using a sterilized Loop, a loopful of bacterial growth was streaked on agar petri plate (A.A. / P.g. agar). Each plate was exposed to the ozonated gas for a specific time, and then incubated anaerobically at 37°C for 24hrs. The bacterial growth corresponding to each exposure time on each plate was performed visually, and recorded⁽²¹⁾. While the ozonized water experiment was done with the same suspension by adding 1.0ml of bacterial broth in a test tube and then 1.0ml of ozonized water (0.6ppm) was added to the first tube and mixed well then by streaking on agar petri plate (A.A. / P.g. agar) for 1,2,3,4,5,10, and 15 minutes respectively. The plates were sealed and incubated in an anaerobic jar at 37°C overnight. The bacterial growth corresponding to each contact time on each plate was performed visually, and recorded⁽²¹⁾. Plates showing no bacterial growth means highly efficient exposure time of both ozonized water and the gaseous ozone.

RESULTS

The results for A.a for colony morphology were white radiating star shaped with no black pigmentation with Gram negative, coccobacilli and give appositve reaction to API NH test (According to API, A.a is listed as (Haemophilus actinomycetemcomitans). While the results for P.g for colony morphology were appeared as

round spherical in shape with raised or convex surface, black-pigmented colonies with Gram negative, coccobacilli and give appositive reaction to API 20A(According to API, P.g is listed as (*p.saccharolytica*) with index number (10000004).

The results obtained for the qualitative evaluation of ozonated gas (218 ppm/W-Air), is presented in Table 1. The inactivation effect of ozonated gas was observed on both A. a, and P.g colonies. After ozonated gas exposure, the numbers of bacterial colonies on the agar surface decreased in a time-dependent manner and the colony's growth was no longer detected in 7, and 4 minutes of treatment against A.a and P.g. Respectively. The analysis of the results of Table 1 verified that the P.g bacteria were much more sensitive toward ozonated gas compared to A.a.

Table 1: Efficiency of ozonated gas at different exposure times on A.a and P.g. colonies growth.

ExposurTimes(minutes)	Antimicrobial agent	
	Ozonated gas (218 ppm/W-air)	
	A.a	P.g
1	+ve	+ve
2	+ve	+ve
3	+ve	+ve
4	+ve	-ve
5	+ve	-ve
6	+ve	-ve
7	-ve	-ve
8	-ve	-ve
9	-ve	-ve
10	-ve	-ve

+ve =Bacterial growth, -ve = no bacterial grow.

While the results obtained for the ozonized water is presented in Table 2. The inactivation effect of ozonized water was observed on both A. a, and P.g colonies. After ozone exposure, the numbers of bacterial colonies on the agar surface decreased in a time-dependent manner and the colony's growth was no longer detected in 5 and 4 minutes of treatment against A.a and P.g. respectively.

Table 2: Efficiency of ozonated water at different exposure times on A.a and P.g. colonies growth.

Contact Times (minutes)	Antimicrobial agents	
	Ozonated Water(0.6 ppm)	
	A.a	P.g
1	+ve	+ve
2	+ve	+ve
3	+ve	+ve
4	+ve	-ve
5	-ve	-ve
10	-ve	-ve
15	-ve	-ve

+ve = Bacterial growth (Uncountable colonies).

-ve= No bacterial growth (no colonies).

DISCUSSIONS

Ozone has been proposed in clinical practice of dentistry and medicine due to its several actions such as antimicrobial, anti-inflammatory, immunostimulating, etc. (22). Ozone has been used for treatment of early carious lesions, periodontal pockets, wound healing such as ulceration, bleaching and in treatment of peri-implantitis(23). The antimicrobialaction of ozone is by damaging the cytoplasmic membrane of the bacteria and cell lysis (24).The results of gaseous ozone experiment was showed that ozonated gas was highly effective in eliminating of both A.a and P.g, (7 and 4 minutes respectively).These results could be explained by the conclusions reached by Hauser et al.,in 2011(25)who investigated the use of gaseous ozone on bacteria adhering to implant surfaces and showed a selective reduction in bacteria, concluding that gaseous ozone may have a role in treatment of peri-implantitis.

Huth et al., in 2011(26) similarly showed significant results with gaseous and aqueous ozone and concluded that they merit further investigation.

Pereira et al., in 2005(27)reported that application of a gaseous O3/O2 mixture (0.4%/99.6%) for 1 h, at constant pressure and flow (11 mm Hg and 2 L/min, respectively) and controlled temperature, in plates containing 10⁴ CFU/mL of *E. coli*, *S. aureus*, and *P. aeruginosa* lead to total inhibition of growth of these bacteria. And Fontes et al.,in 2012(28), concluded that the application of a low dose of gaseous ozone (dose of 20 µg of O3/mL in a gaseous O3/O2 mixture) for 5 minutes completely prevented the in vitro growth of gram-positive and negative pathogenic bacteria commonly present in patients with

severe nosocomial infections, with known resistance to antibiotics.

While, the result of ozonized water (0.6 ppm) was highly effective in eliminating of both A.a and P.g (5 and 4 minutes respectively).This results were in agreement with kshitish and vandana in 2010 ⁽²⁹⁾.It was reported that ozone at low concentration of 0.1 ppm, is sufficient to inactivate bacterial cells including their spores ⁽⁸⁾.This reacts could explained by the parcons of various chemical compounds in two different and coexisting modes, one involving direct reactions of molecular ozone and the other a free radical-mediated reaction ,both these mechanisms may be involved in the destruction of bacteria by ozone⁽³⁰⁾.Ozonated water had nearly the same antimicrobial activity as 2.5% sodium hypochlorite and also the metabolic activity of fibroblasts was high when the cells were treated with ozonated water. The aqueous form of ozone, as a potential antiseptic agent, showed less cytotoxicity than gaseous ozone or established antimicrobials like chlorhexidine digluconate, sodium hypochlorite or hydrogen peroxide under most conditions. Therefore, aqueous ozone fulfils optimal cell biological characteristics in terms of biocompatibility for oral application ⁽³¹⁾.

In conclusions, gaseous ozone and ozonized water form therapy was very efficient against *A. actinomycetemcomitans* and *P. gingivalis*, and can be employed as a useful antimicrobial for periodontal therapy. This form of application can be used singly or in combination to treat dental disease.

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المستخلص

المقدمة: يعتبر التجويف الفمي كنظام بيئي مفتوح مع التوازن بين البكتيريا الداخلة والنظام الدفاعي للجسم. فهذا يعتبر تطور التهاب اللثة مع انواع محدده من البكتيريا اللاهوائية وخاصة (البورفوريموناسا للثوية والاجريجاتيبياكتر اكتينومايسيتيمكوميتانس) الموجودة في الصفيحة الجرثومية في داخل الجيوب اللثوية. الاوزون له تأثير بايولوجي على البكتريا من خلال اكسده ماده البايولوجيه والتوكسين للبكتريا. هدف الدراسة : لتحديد ومقارنه تاثير المضاد البكتيري لغاز الاوزون دقيقه) و ماء الاوزن على البكتريا اللاهوائية المعزوله عند تعرضها لاورقات مختلفه

المواد والطريقه: في دراسته مختبريه ,اجريت التجربه من خلال جهاز الاوزون (اولمبك 3) لتوليد غاز الاوزون بوقت مابين (1-10 دقيقه) ومع استعمال حجر خاص لتوليد ماء الاوزون بوقت مابين (1-5 دقيقه) والذي سوف يمر على الاطباق المزروعه بالبكتريا المعزوله. النتيجة: وجد بأن غاز الاوزون و ماء الاوزون قد قلل بشكل ملحوظ على نمو البكتريا المتواجده بالاطبق لكلا النوعين. الاستنتاج: غاز الاوزون وماء الاوزون يعتبر مضاد بكتيري فعال جدا ضد البكتريا اللاهوائية المعزوله من المرضى المصابين بالتهاب اللثة المفتاح: غاز الاوزون, ماء الاوزون, البورفوريموناسا للثويه , الاجريجاتيبياكتر اكتينومايسيتيمكوميتانس.