Original Article

Evaluation of antibacterial activity of ozonated oil and ozonated water against *Neisseria gonorrhoeae* and *Neisseria meningitidis* by broth microdilution method



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1. Introduction

<u>ABSTRACT</u>

The necessity of using ozonated oil and water for treating infections such as gonorrhea and meningitis which their treatment by common antibiotics is sometimes difficult and it is becoming more apparent every day. In this study, the antibacterial effect of ozonated oil and water against Neisseria gonorrhoeae (N. gonorrhoeae) and N. meningitidis were investigated using Broth microdilution methods at zero, 24 and 48 hours after incubation at 37°C. The results were determined by culturing bacteria on their specific culture medium and using an ELISA reader to determine minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively). The results showed that ozonated oil with PI=500 had an expressive effect on N. meningitidis. The concentration of 0.09PI was determined as MIC and the concentration of 0.19PI was determined as MBC. The ozonated oil with PI=1000 had an expressive effect on N. gonorrhoeae. The concentration of 3.12 PI was determined as MIC and the concentration of 6.25 PI was determined as MBC. Ozonated water containing 2.5 mg/L of ozone had no significant antimicrobial effect on the studied bacterial species. The results of this study showed that ozonation of oil may improve its chemical properties. In addition, by increasing the incubation time to 24 hours, ozonated oil showed a favorable antibacterial effect against N. gonorrhoeae and N. meningitidis.

The genus Neisseria is a group of Gramnegative bacteria within the family *Neisseriaceae*. Although most members of this genus are not, or only very rarely pathogenic, *Neisseria meningitidis* (*N. meningitidis*) and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) cause infections that are of worldwide importance. *N. gonorrhoeae* or gonococcus is the causative agent of the sexually transmitted disease gonorrhea. The incidence of gonorrhea varies greatly in both developed and developing countries. Gonococci invade the mucous membranes of the genitals, eyes, rectum, and throat, causing secretory lesions that may lead to tissue invasion [<u>1</u>, <u>2</u>]. Moreover, it can cause ophthalmia neonatorum, an infant's eye infection acquired during vaginal delivery [<u>3</u>]. Mechanical prevention provides relative protection, and pharmacological prevention offers limited efficacy due to the increased antibiotic resistance of gonococci [<u>4</u>]. Despite medical advances in recent years, the previously recommended drugs for the first-

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line empirical treatment of gonorrhea have failed to cure the disease due to antimicrobial resistance^[5]. Ν. meningitidis or meningococcus are often found in the upper respiratory tract and cause meningitis. Gonococcal meningitis is characterized as an epidemic wave and, in a few cases, as a sporadic infection, both of which cause major morbidity and mortality in children and young people globally [6, 7]. With the development of sulfonamide-resistant meningococci and reduced susceptibility to third-generation cephalosporins, drug prophylaxis is of little value to people with close contact $[\underline{8}, \underline{9}]$.

Due to the ineffectiveness of drug therapy in a number of patients who resist antibacterial treatments, scientists have sought alternative methods. Ozone therapy, a well-studied method for therapeutic purposes, may be a possible alternative treatment against resistant bacteria [10-13].

Ozone, a colorless and unstable gas with a pungent odor, is composed of three atoms and has an unstable structure due to its mesomeric states. Ozone was previously known mostly as a shield for the Earth against ultraviolet radiation in the stratosphere, and presence in the troposphere was considered harmful. The therapeutic roles of this molecule have not been considered for a long time, but in recent years, the therapeutic effects of ozone combined with oxygen have been considered [10-13]. For example, ozone can trigger fibroblasts' migration to wound sites and, therefore, aids the wound-healing process^[14]. Moreover, ozone therapy is used for the treatment of decubitus ulcers, burns, ulcerations, inflammation of skin and bone tissue, or radiation therapy-related changes in patients^[15]. Additionally. cancer ozone affects inflammatory tissue metabolism, increases the body's immunological response, and destroys bacteria, fungi, and viruses[15].

Ozone's antibacterial effects are a result of its oxidative capabilities. By oxidizing glycoproteins and glycolipids, ozone inhibits bacteria's enzymatic activity and then oxidizes the phospholipids and lipoproteins in the bacterial cell envelope, causing the cytosolic membrane integrity to be disrupted, allowing it to enter the bacterial cell[10, 16]. In this manner, previous studies have evaluated the antibacterial potential of ozonated water and vegetable oils. Based on a previous study, was ozonated water found to have antibacterial effects in vitro against bacterial and fungal strains [15]. Also, another study indicated that bacterial strains were susceptible to ozonated vegetable oils. especially mature biofilms and adhered cells [17]. Therefore, these ozonated substances may be used as an alternative treatment against drug-resistant bacteria. This study assessed the antibacterial potential of ozonated water and vegetable oils against N. gonorrhoeae and N. meningitidis and their corresponding minimum inhibitory concentration (MIC) minimum and bactericidal concentration (MBC) for the first time.

2. Materials and Methods

Standard strains of *N. meningitidis* (PTCC: 1507) and *N. gonorrhea* (PTCC: 1773) were purchased from the Iranian Research Organization for Science and Technology. *N. gonorrhoeae* was cultured on GC medium agar and *N. meningitidis* on blood agar. Colonies developed after 24 hours of incubation at 37 ° C.

2.1. Preparation of GC medium agar

GC medium agar was prepared with IsoVitaleX supplement plus vancomycin, trimethoprim, colistin sulfate, and amphotericin B antibiotics.

2.2. Bacterial identification

To identify the received strains, motility (negative for both species) and catalase tests (positive for both species) were performed.

2.3. Ozonated Oil preparation method

Ozonated Oil with PI = 500 and PI = 1000was purchased from Exan Company. The peroxide index (PI) was defined as milliequivalents of active oxygen in 1 kg of the sample. Due to the insolubility of ozonated oil in a culture medium containing water, an emulsifier with negligible antimicrobial effects was required to dissolve ozonated oil, therefore, dimethyl sulfoxide (DMSO) was used as a solvent. In order to obtain the best solubility for ozonated oil and achieve a clear solution, different dilutions of DMSO solution and ozonated oil have been made.

2.4. Ozonated Water preparation

To prepare ozonated water Dr. Aydin's device was used. First, ¾ a glass cylinder was filled with distilled water and ozone gas was continuously blown into the water using a diffuser for 20 minutes until saturated. The water produced contained 2-3 mg/ml ozone. The produced water could only be used for up to 5 minutes.

2.5. Preparation of bacterial suspension

To prepare the bacterial suspension, samples with a turbidity of 0.5 McFarland were prepared. In order to prepare 0.5 McFarland turbidity standard, 0.5 ml of 0.048 mol / l barium chloride dihydrate (BaCl₂•2H₂O) was added to 99.5 ml of 0.18 mol / l sulfuric acid (H₂SO₄) (1% V / V). Then the opacity density was determined using a spectrophotometer with a wavelength of 625 nm. The obtained light absorption was 0.08 up to 0.13 which it was equivalent to 1.5×10^8 CFU/mL of bacteria.

2.6. Broth microdilution test

2.6.1. Determination of MIC

MIC is the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic and can inhibit significant growth of microorganisms after an incubation period. In order to determine the MIC, first, two 96-plate microplates were prepared. One microplate was specified for Ozonated water and another for ozonated oil. The first four rows of each microplate were assigned to N. meningitidis and the last four rows of each microplate were assigned to *N. gonorrheae*. In the ozonated oil microplate, well No. 11 in each row was used as a negative control containing Müller Hinton broth culture medium, Ozonated Oil and DMSO while in the ozonated water microplate the same well was filled with Muller Hinton broth and ozonated water. Well, No. 12 in each row contained Müller Hinton broth and bacteria as a positive control.

First, 50 μ L of Müller-Hinton Broth culture medium was poured into two 96-microplate wells. A mixture of DMSO and ozonated oil

was put into the first well of each row in the ozonated oil microplate, and ozonated water was poured into the first well of each row in the ozonated water microplate and passaged. Then 50μ L of 0.5 McFarland bacterial suspension was added to all wells except well No. 11. The microplate light absorption was then read at zero time by the ELISA reader at 630 nm wavelength. The microplates were then incubated at 37°C. Turbidity was examined visually and by an ELISA reader after 24 and 48 hours to determine MIC and MBC.

2.6.2. Determination of MBC

MBC refers to the lowest concentration of antibacterial, which can reduce the bacterial population by 9.99% after 24 hours. In order to determine the MBC, 10μ L mixed suspension was taken from all wells and cultured on Müller Hinton agar and blood agar for *N. meningitidis* and Müller Hinton agar and GC agar for *N. gonorrhea*. After 24 hours of incubation at 37°C, the lowest ozone concentration that was able to kill 99.9% of bacteria was determined as MBC. All assays were done in triplicate.

2.7. Statistical analysis

Analysis of variance and Tukey's post-hoc tests were performed for bacterial growth rate analysis using SPSS statistics software version 22. P <0.05 was considered statistically significant.

3. Results

3.1. The effect of ozonated oil on *N. meningitidis* and *N. gonorrhoeae*

After broth dilution and culture in Müller-Hinton agar and GC medium agar, we read the culture results once after 24 and 48 hours. Ozonated oil with PI=500 was effective on *N. meningitidis* (p<0.05)(Figure 1). Well No. 6 (PI = 0.09) was determined as MIC and well No. 5 (PI = 0.19) was determined as MBC for *N. meningitidis.* On the other hand, Ozonated oil with PI=500 was not effective on *N. gonorrhoeae*(Figure 2).

Using ELISA, we observed a lower number of *N. gonorrhoeae* in the wells with higher ozone concentrations after 24 and 48 hours although bacterial growth was not inhibited. As a result, we assumed that ozonated oil with greater PI might have a considerable effect on N. gonorrhoeae. Therefore, we performed another test on this bacterium using ozonated oil with PI=1000(Figure 3). Well No. 2 (PI = 3.12) was determined as MIC and well No. 1 (PI = 6.25) was designated as MBC for N. gonorrhoeae.

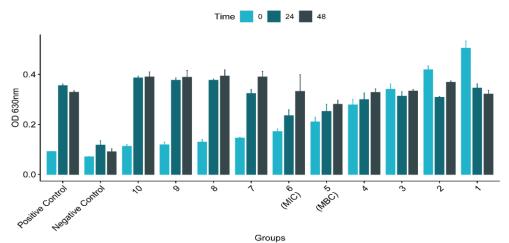


Fig. 1. The Effect of ozonated oil on N. meningitidis PI= 500

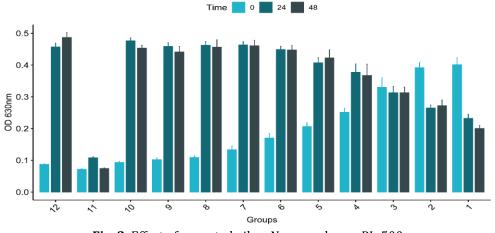


Fig. 2. Effect of ozonated oil on N. gonorrhoeae PI=500

0

24 48

Time

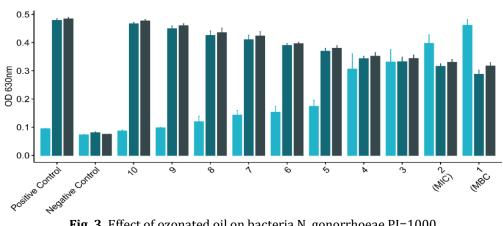
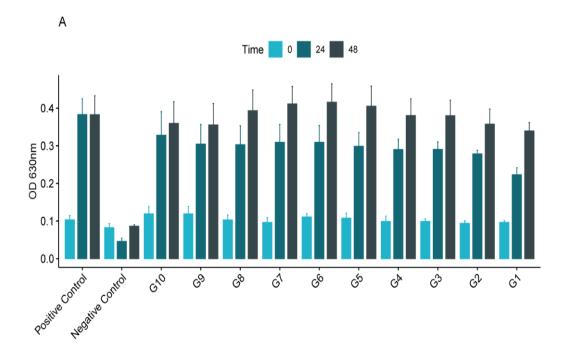


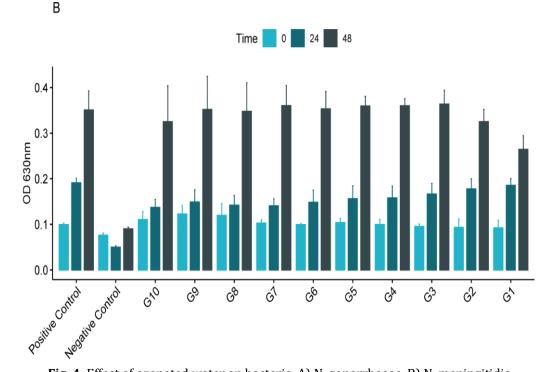
Fig. 3. Effect of ozonated oil on bacteria N. gonorrhoeae PI=1000

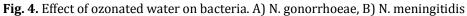
3.2. The effect of ozonated water on *N. meningitidis* and *N. gonorrhoeae*

Ozonated water containing 2-3 mg/L ozone failed to have a significant effect on either of the strains after 48 hours. We saw a significant effect in wells G1 and G2 of N.

gonorrhoeae after 24h (p = 1.12E-05 and 0.041, respectively) but it was compensated after 48 hours. In the *N. meningitidis* group, ozonated water showed a significant effect against the bacteria in G1 compared to controls only after 48 hours (p = 4.139514e-02)(Figure 4).







4. Discussion

We performed the present study to evaluate and compare the antibacterial potential of ozonated water and oil against standard strains of *N. gonorrhoeae* (PTCC: 1773) and N. meningitidis (PTCC: 1507). Based on the results of ELISA and cultures, we found that although ozonated vegetable oil had significant antibacterial effects on both studied strains, ozonated water did not follow this trend. The most significant inhibitory effect of ozonated oil was seen after 24 h, which significantly inhibited the growth of *N*. meningitidis. For N. meningitidis, PI=0.09 and PI=0.19 were determined as MIC and MBC, respectively (P<0.05). Additionally, For N. gonorrhoeae, we calculated PI=3.12 and PI=6.25 as MIC and MBC, respectively (P<0.05). The therapeutic roles of ozone have been considered in recent years. For instance, previous research has shown that ozone is a potent oxidant that increases the cellular antioxidant enzymes inhibiting oxidative stress, which may effectively treat various diseases such as acquired immunodeficiency syndrome (AIDS) [16]. Also, studies have highlighted ozone therapy's efficacy in the elimination of bacteria strains. It has been showed that 2% chlorhexidine digluconate (CHG) and 53gm ozone gas destroyed Tannerella forsythia and Parvimonas micra. High concentrations of oxygen gas were shown to be more effective as a disinfectant [18-20]. Moreover, it has been showed that 2 min exposure of 10 ppm ozone significantly decreased Escherichia coli and Listeria spp on spinach and the pathogens did not grow after nine days of storage. They also found that the age of the colonies affected ozone resistance [21, 22].

Ozone and its combination with oils could eliminate bacteria, making them viable substitutes for chemical substances. Ozonated oil and ointments, such as ozonated sunflower oil, may reduce swelling and hasten healing in parasite infections and acne by slowing pathogen spread and reproduction [15]. It has been evaluated the effect of ozonated sunflower oil on various bacterial species. Ozonated oil showed valuable antimicrobial activity against all tested microorganisms. However. Mycobacterium showed less sensitivity to ozonated oil compared to other genera [<u>17</u>]. It has been showed that ozonated oil was more effective on Gram-positive bacteria than those Gram-negative species and had lower antibacterial activity than factors related to CHX [<u>10</u>].

In agreement with our results, Silva et al. demonstrated that an increase in peroxide radicals in oil enhanced the oil's antibacterial activity. The use of high ozone doses may raise some questions regarding toxic effects. However, studies have shown that ozone is biocompatible with human gingival, fibroblast, and periodontal cells. The effect of ozone on bacterial cell membranes may also be because of its highly unstable trait, resulting in its rapid decomposition in free radicals, which spread quickly through the bacterial cell, disturbing the usual cellular activity. It seems that this action does not harm human cells due to the antioxidant mammalian capacity of cells. The antimicrobial effect of ozone-containing oils may vary depending on the oil used and the number of carbon-carbon double bonds. Ozonation of vegetable oils reduces saturated fatty acids and increases the amount of peroxide radicals [10, 23, 24].

Based on the results of ELISA and cultures. the bacterial strains continued to grow in the culture medium containing ozonated water after 24 and 48 hours. The number of bacteria the first wells where the ozone in concentration was higher decreased but the growth did not stop. Therefore, it can be concluded that the antibacterial effect of ozonated oil on *N. meningitidis* is significantly higher than ozonated water. The lack of ozonated water's antibacterial efficacy on the studied bacterial strains can be due to the short half-life of ozone in water. It has been found that ozonated water had antibacterial properties on Staphylococcus aureus biofilm after 30 seconds of exposure, however, some strains of *Pseudomonas aeruginosa* were resistant to ozonated water. On the other hand, ozone gas had a more negligible effect, and therefore, it appears that it cannot be used extensively for disinfection. Prolonged exposure (40 min) failed to reduce resistant cell count [25-28]. Widespread access to oils such as sunflower, olive, and sesame, makes ozonated oil a competitive antimicrobial compound. Our findings should pave the way for clinical trials comparing the efficacy of ozone oil to other antibacterial treatment approaches. We propose further research to be conducted to determine the effect of ozonated water on *N. gonorrhoeae* and *N. meningitidis* by increasing the quantity of ozone in the water.

5. Conclusion

Ozonated oil may help treat gonorrhea and meningitis. Further studies are needed to better understand ozonated oils' function on bacteria and identify and quantify the compounds responsible for antibacterial activity. Ozonation of vegetable oils is a viable and ecologically friendly option against bacteria especially when the overuse of antibiotics to treat infectious diseases has resulted in increased antibiotic resistance. Novel compounds having antibacterial characteristics, ideally natural and nonsynthetic, must be developed. Given the growing trend of antibiotic resistance, we suggest that the effects of ozonated oils on resistant bacteria be investigated deeply.

Conflict of Interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors had equal role in study design, work, statistical analysis and manuscript writing.

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