



Original paper

Ozonized water, background, general use in medicine and preclinic support

Gregorio Martínez-Sánchez

Pharm. Dr., Ph.D., Freelance, Ancona, Italy

Keywords

ozonized water,
ozone,
ozonized solution,
ozone therapy.

Abstract

The use of ozonized water (O3W) is now gaining in importance in medicine. Ozone is dissolved in its molecular form (O₃). In this review, the bibliographical data concerning the general use of O3W in medicine and preclinical support is analyzed. The search included a review of scientific articles and experimental results papers in the MEDLINE and Zotero ISCO3 Ozone Database, between the years 1980-2018. Descriptors used was: ozonized water, ozonized solutions. The primary (original articles) sources of information were located. Main indications of O3W involve the control of local infections: ulcus cruris, decubitus ulcers, mycosis, mycotic infections, herpes simplex and herpes zoster, burns, also superinfected burns, intraoperative rinsing, eye injuries and infections, surgical scars (healing: primary or secondary), and edemas of traumatic or bacterial origin. In addition, O3W is also applied on account of its pain-relieving and anti-inflammatory effects, as well as its tissue-activating properties in acute and chronic injuries with and without infection. O3W is also being used intraoperatively for rinsing (disinfection), as in hand disinfection in surgery, in dental medicine (including the disinfection of tools and water), rinsed in conditions such as thrush and periodontal disease, in primary root canal disinfection and particularly in oral surgery. O3W also can play an important role as a potential disinfectant in controlling the problem of contamination of dental unit waterlines. O3W may also be swallowed in cases of gastritis or gastric carcinoma, or irrigated in chronic intestinal or bladder inflammation. In balneotherapy, O3W bubbled in warm baths, provides stimulation of local circulation and disinfectant action to varicosities, peripheral circulatory disorders and dermatological conditions (eczema, ulcers). Preclinical evidence supports the use of O3W in medicine and shown the importance of it use in daily medical practice...

Abbreviations:

AIDS, acquired immunodeficiency syndrome
ARE, antioxidant response element
BHY, human oral epithelial cells
CHX, chlorhexidine digluconate
COX-2, cyclooxygenase-2
CBP, CREB binding protein
DUWL, water disinfection of dental unit waterlines
ECM, extracellular matrix
EDTA, ethylenediaminetetraacetic acid
GSH, reduced glutathione
HDAC3, histone deacetylase 3
HGF-1, human gingival fibroblast
HAT, histone acetyl transferases
HO1, haem-oxygenase-1
HNE, hydroxynonenal
ICAM, intracellular adhesion molecule
IKK, I κ B kinase
Keap1, Kelch-like ECH-associated protein 1
iNOS, inducible nitric oxide synthase
LSP, lipopolysaccharide
MRSA, methicillin resistant *Staphylococcus aureus*
NETosis, a novel form of programmed neutrophil death that resulted in the formation of neutrophil extracellular traps (NETs)
Nrf2, nuclear erythroid 2 related factor 2
NF- κ B, nuclear factor kappa light chain enhancer of B cell
O3SS, ozonated saline solution
O3W, ozonated water
PMN, polymorphonuclear leukocytes
SOD, superoxide dismutase
TNF- α , tumor necrosis factor- α .

Suggestion on how to quote this paper:

Martínez Sánchez, Gregorio. (2019). Ozonized water, background, general use in medicine and preclinic support, *Ozone Therapy Global Journal*, Vol. 9, n° 1, pp 33-60

General background

The industrial applications of ozone exploit its antiseptic properties making it very useful in the disinfection of water, both for its purification and for the treatment of sewage (Fig. 1). It is more effective than chlorine and also eliminates the viruses that are able to survive in presence of high concentrations of chlorine. In October 1893, in Holland (Ousbaden), the first system of water treatment with ozone was invented and realized and is currently extended to more than 3.000 municipalities, including some big cities, all over the world.¹

In 1902 the London practical dictionary of medicine contained some notes by J.H Clark who enthusiastically described the use of ozonized water (O₃W), called *Oxygenium*, in the treatment of anemia, cancer, diabetes, flu, morphine poisoning and aphtha. The Swiss dentist E.A Fish (1899-1966) was the first medical doctor to exploit the benefits of ozone in local treatment. He treated his patients with ozone and O₃W from 1932 obtaining good results in cases of gangrenous pulpitis with a simple injection of the gas. One of the patients he treated, was Dr. E. Payr (1871-1946) who immediately understood the usefulness of ozone and was very excited about its application in general surgery. In 1935, Dr. Payr published a work of 290 pages entitled *Ozone Treatment in Surgery* and presented it at the 59th Meeting of the German Surgical Society.

In terms of water purification, the use of ozone is also important in the treatment of the water used in medical environments. During the last decade nosocomial infections have become common because the resistance of pathogens to antibiotics has increased to a point where we no longer have an effective drug for some strains. Recent studies suggest that environmental contamination plays a significant role in healthcare-associated infections and in the unrecognized transmission of nosocomial pathogens during outbreaks, as well as ongoing sporadic transmission. Several pathogens can persist in the environment for extended periods and serve as vehicles of transmission and dissemination in the hospital setting. Cross-transmission of these pathogens can occur via hands of healthcare workers, who become contaminated directly from patient contact or indirectly by touching contaminated environmental surfaces. Less commonly, a patient could become colonized by direct contact with a contaminated environmental surface.² This is a complex problem, partly due to the extensive use of antibiotics in animal food and the improper use in patients. O₃W, due to its high antimicrobial potential could eliminate the resistant bacteria³ and as consequence several thousand deaths could be avoided each year.⁴

In spite of a large use of chlorine, 4.5 billion people (6 out of 10 peoples in the world) do not have access to adequate water sanitation (Fig. 2).⁵ Unfortunately, chlorine has unsatisfactory organoleptic characteristics and it is being widely substituted by ozone all over the world. Ozone is possibly an even more potent drinking water disinfectant able to inactivate several human pathogens, e.g. as many as 63 different bacteria (*Salmonella*, *Shigella*, *Vibrio*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Legionella*, *Staphylococcus aureus*,⁶ etc.), some 15 viruses (polio, echo, Coxsackie viruses, etc.), some 25 fungi and mould spores (*Aspergillus*, *Penicillium*, *Trichoderma*, etc.), several yeast varieties, and up to 13 fungal pathogens (*Alternaria*, *Monilinia*, *Rhizopus*, etc.). More recently, due to contamination of groundwater with faecal material, the problem of disinfection has become more complex, since encysted protozoa, such as *Giardia lamblia*, *Cryptosporidium parvum* oocysts and helminth eggs (*Ascaris suum* and *Ascaris lumbricoides*), require a much longer time of contact with ozone than bacteria and viruses.⁴ Every year *Cryptosporidium spp.* causes outbreaks of sickness, which can be fatal for elderly and very ill patients (AIDS).⁷

We can define *ozonized water* as the water obtained after the *ozonation* (a process of infusing water with ozone) of water. During the ozonation of water there are no chemical reactions between ozone and pure water. The only chemical reactions take place between ozone and organic, inorganic, or biological material present in the water. The solubility of ozone in water depends on different variables: pH, temperature, salts contents, and others. The half-life of ozone in water also depends on these variables.

The treatment of domestic well drinking water⁸ and waste waters⁹ is also an important application of ozone. It is estimated that today, over 1 100 water treatment plants around the world use ozone for disinfection. Ozone it has been used for water purification prior to chlorine and only its high cost has hampered its wider application.¹⁰ However, considering the mode of effect of the various oxidizing agents and the longer flow time through the biological activated carbon filters (as a result of the ozonation), ozone is – according to studies made by Grob and the Zurich Water Supply with ozonized Lake Zurich water – the most economical oxidation process with the best effect, i.e., without formation of toxic or carcinogenic substances. The pre – and/or intermediate oxidation with ozone surely will be the right way in the foreseeable future.¹¹ Chemical and pharmaceutical industry also use ozone in order to treat residual water, as a bleaching agent,¹² and other applications.

Food industry and agricultural industry also employed successfully O3W. For example: Ozonation of water used for rinsing prevented fruits from consecutive contamination by pesticide residues present in water after several rinsing cycles. It was proven that applying O3W as a part of post-harvest treatment of apples may be beneficial for the fruit quality.¹³ A food toxin “Patulin” can be present in pear juice and especially in apple juice, this toxin can be destroyed by the action of ozone.¹⁴ In addition, the effect of ozonized water in *Xanthomonas oryzae pv. oryzae*, a seed borne bacterium causing leaf blight disease in Paddy (*Oryza sativa*) was study. Washing the paddy seeds with O3W would help control the bacterial blight of rice, the most serious disease of rice in Asia.¹⁵ Moreover, washing tomatoes (*Lycopersicon esculentum* Mill) with O3W 1 mg/L by 15 min, achieve an adequate disinfection.¹⁶ However, in case of tomatoes slices it is recommended to wash with 0.4 mg/L O3W for 3 min only.¹⁷

Salmonella inactivation by O3W washing was also demonstrated in tomatoes, lettuces and green onions. Mild heat and low pH improved inactivation efficacy on tomatoes and lettuces, but not on green onions. Processors should consider adjustments that are most appropriate for their produce.¹⁸

Ozone facilitates the extraction of some heavy metals from soil using ethylenediaminetetraacetic acid (EDTA). EDTA forms strong, water-soluble coordination compounds with some heavy metals (Pb, Zn) thereby making it possible to dissolve them out from contaminated soil. If contaminated soil is pre-treated with ozone, the extraction efficacy of Pb, [Am](#) and Pu increases by 11.0–28.9%,¹⁹ 43.5%²⁰ and 50.7%²⁰ respectively.

In aquaculture, O3W can be used to facilitate organic breakdown. Ozone is also added to recirculating systems to reduce nitrite levels²¹ through conversion into nitrate. If nitrite levels in the water are high, nitrites will also accumulate in the blood and tissues of fish, where it interferes with oxygen transport and causes oxidation of the heme-group of haemoglobin from ferrous (Fe²⁺) to ferric (Fe³⁺), making haemoglobin unable to bind O₂.²² Despite these apparent positive effects, ozone use in recirculation systems has been linked to reducing the level of bioavailable iodine in salt water systems, resulting in iodine deficiency symptoms such as goitre and decreased growth in Senegalese sole (*Solea senegalensis*) larvae.²³

Ozonate seawater is used for surface disinfection of haddock and Atlantic halibut eggs against *nodavirus*. *Nodavirus* is a lethal and vertically transmitted virus which causes severe mortality in fish. Haddock eggs should not be treated with high ozone level as eggs so treated did not hatch and died after 3–4 days.²⁴

O3W is also used successfully in the disinfection of drinking water, high purity water for Pharmaceuticals industries, and swimming pools.^{25,26} The use of ozonized seawater to reduce and eliminate bacterial pathogens in mariculture facilities and to extend shelf life of marine food products is demonstrated. After treatment, *Vibrio* counts and shrimp disease were eliminated, ozonized seawater decreased the time required for normal molting of shrimp and the total growth cycle was reduced by three days versus control water.²⁷

General applications in medicine

The main medical applications of O3W are: the washing of wounds, surgical areas, ulcers and to eliminate pus and clean necrotic areas, as a mouthwash for periodontitis, gingivitis, and for the washing of cavities during surgical operations. When swallowed, it can be used in the treatment of gastric ulcers, *Helicobacter pylori*,²⁸ *Giardia lamblia* or gastritis.

Another application of O3W is in dentistry. Those applications are supported by pre-clinical studies. One study examined the effect of O3W on oral microorganisms and dental plaque. Almost no microorganisms were detected after being treated with O3W (4 mg/L) for 10 s. When the experimental dental plaque was exposed to O3W, the number of viable *S. mutans* remarkably decreased. O3W strongly inhibited the accumulation of experimental dental plaque *in vitro*. After the dental plaque samples from human subjects were exposed to O3W *in vitro*, almost no viable bacterial cells were detected. These results suggest that O3W should be useful in reducing the infections caused by oral microorganisms in dental plaque.²⁹

One study examined the effect of O3W against *Enterococcus faecalis* and *Streptococcus mutans* infections *in vitro* in bovine dentin. After irrigation with O3W, the viability of *E. faecalis* and *S. mutans* invading dentinal tubules significantly decreased. Notably, when the specimen was irrigated with sonication, O3W had nearly the same antimicrobial activity as 2.5% sodium hypochlorite (NaOCl). The study also compared the cytotoxicity against L-929 mouse fibroblasts between O3W and NaOCl. The metabolic activity of fibroblasts was high when the cells were treated with O3W, whereas that of fibroblasts significantly decreased when the cells were treated with 2.5% NaOCl.³⁰

O3W was found effective in reducing the bacterial load in both the 24 h plaque and stimulated whole saliva samples, but it did not eliminate it completely. In addition, there was no statistically significant effect of the O3W rinse on the salivary flow rate, salivary calcium and protein concentration. As consequence O3W rinse can be an effective adjunct to tooth brushing and flossing to maintain plaque and salivary bacterial load.³¹In biofilms containing *Actinomyces naeslundii*, *Streptococcus mutans* and *Lactobacillus paracasei* O3W had a level of antimicrobial activity equivalent to chlorhexidine.³²

One study compared the antimicrobial efficacy of O3W and minocycline against mixed microbial cultures after their initial adherence to tooth surfaces. Teeth were incubated in saliva suspension containing reference strains of *Streptococcus mitis*, *Fusobacterium nucleatum*, *Actinomyces naeslundii* and *Candida albicans*. The teeth were immersed either in O3W or in minocycline solution. Sterile saline served as control. Adherent microorganisms were resuspended, serially diluted, cultured, and statistically analyzed. In comparison to the control group, O3W showed significant reduction of all four strains, whereas minocycline was effective only on two strains tested.³³

Different *in vitro* assessments demonstrate the antibacterial activity of O3W against microorganisms associated with illnesses such as abscesses, bacteremia, endocarditis and osteomyelitis, which were resistant to multiple drugs.³⁴ Significant reduction of the microorganisms *Staphylococcus aureus*,³⁵⁻³⁷ *Pseudomonas aeruginosa*,^{36,37} *Enterococcus faecalis*,³⁶ *Candida albicans*,³⁸ was also found. However, a study shown that the irrigation of infected human root canals with O3W, 2.5% NaOCl, 2% chlorhexidine and the application of gaseous ozone for 20 min was not sufficient to inactivate *E. faecalis*.³⁹ Moreover, a study comparing the effect of O3W, O3W with ultrasonication, NaOCl, and chlorhexidine in human primary root canals contaminated by *E. faecalis*, provided evidence of its antibacterial effect *in vitro* conditions, but NaOCl showed the best effects.⁴⁰

Water disinfection of dental unit waterlines (DUWL) using ozone was also study with excellent results. When ozone disinfection methods were compared to hydrogen peroxide/silver ion, best results were obtained using ozone in particular against *Pseudomonas aeruginosa*.⁴¹ In addition, a study of *Acanthamoeba* strains, isolated from DUWL shown high levels of disinfection *in vitro* by the application of ozone as disinfectant.⁴²

One study analysed the bactericidal and fungicidal activity of O3W in multidrug-resistant bacterial strains. The scope was the application of O3W during intraoperative ozone treatment for tissue protection against infection with pathogenic bacteria. O3W was useful to kill almost all cells of the bacterial and yeast strains tested after 30 s (60 clinical bacterial isolates were analysed). Effective action against *Aspergillus brasiliensis* spores required a longer time than those required in the case of bacterial cells or vegetative cells of yeast.⁴³

The effect of O3W in remineralizing artificially created initial enamel caries was also investigated using laser fluorescence and polarized light microscopy in an *in situ* study. Results shown that O3W can be used to remineralize incipient carious lesions, and it enhances the remineralizing potential of nano-hydroxyapatite thereby preventing the tooth from entering into the repetitive restorative cycle.⁴⁴

Because of the antiseptic properties of O3W, its efficacy in the treatment of wounds has been studied. An *in vivo* study in diabetic rats, showed that wounds irrigated with O3W revealed diffuse haemorrhage and increased blood vessel neoformation. There was no statistically significant difference in bone trabeculae neoformation. After seven and 14 days, the number of osteoclasts was higher in O3W groups than in those treated with pure water.⁴⁵ The use of O3W are also extended to keratomycosis⁴⁶ and contact dermatitis.⁴⁷

The effect of ozonated saline solution (O3SS) in was also tested *in vitro*⁴⁸ and *in vivo*⁴⁹ against deformed strains of *Mycobacterium tuberculosis*. The O3SS was found to produce bactericidal and bacteriostatic effects, including multiresistant strains. In addition, it was investigated the anti-biofilm potential of O3SS against biofilms of *Staphylococcus aureus*, a microorganism commonly implicated in wound infections. The results shown a 99 % reduction in bacterial numbers within 15 min of exposure. These results suggesting that O3SS may be suitable as an adjuvant therapy to treat patients as an instillation fluid for wound irrigation and sterilization.⁵⁰

The action mechanism of O3SS is complex and involved mediators such as hydroxynonenal (HNE) and H₂O₂ and the activation of the Nrf2 pathways. A study involved different non-toxic doses of O3SS ranging from 2 µM to 300 µM, in an *in vitro* wound scratch model by the use of human keratinocytes supports that hypothesis. The results showed that O3SS is able to improve *in vitro* wound healing by stimulating cell proliferation.⁵¹

Disinfectant effect of ozonized water. Preclinical support

The disinfectant effect of O₃W is a direct consequence of the presence of dissolved ozone in water. Different variables such as: pH, ion content, water temperature, exposure time, and ozone concentration may impact in the germicide effect. For example, a concentration of ozone of 0.1 mg/L yields a low impact in oral cavity bacterial control,³¹ however using 8 mg/L yielded good results.³⁶ Although the inhibitory and lethal effects of ozone on pathogenic organisms has been observed since the latter part of the 19th century, the mechanisms for these actions have not yet been satisfactorily elucidated. Ozone is a strong germicide needing only a few micrograms per liter for measurable action. At low concentrations, ozone rapidly inactivates, virus, coliform bacteria, *Staphylococcus aureus*, *Aeromonas hydrophilia*, and many other microorganisms.⁵²

Virus inactivation by ozone

Viruses are parasites at the genetic level, separated into families based on their structure, type of nucleic genome and mode of replication. Many virions contain a phospholipid envelope with glycoprotein spikes encasing the nucleocapsid which contains nucleic acids (DNA or RNA), and structural proteins (including enzymes).

Lipid-containing viruses are sensitive to treatment with ether, assorted organic solvents, and ozone, indicating that disruption or loss of lipids results in impaired or destroyed infectivity. Viruses containing lipid envelopes include the *Herpes viridae* a large family grouping the *Simplex*, *Varicella-Zoster*, *Cytomegalovirus* and *Epstein-Barr* viruses; the *Paramyxoviridae* (mumps, measles); the *Orthomyxoviridae* (influenza); the *Rhabdoviridae* (rabies); and the *Retroviridae* (HIV). The HIV virus has an outer envelope made of a double layer of lipids penetrated by proteins of several types encasing two molecules of RNA.⁵³

Many of the above viruses have complex, sometimes baffling life cycles and replicative strategies with progressions from host cell attachment of the virus particle, to penetration, uncoating of the viral envelope, synthesis of molecular components, and release of new generations of virions to the surrounding medium, most often through cell lysis. Many chronic viruses have eclipse phases alternating with phases of viremia, when waves of viral particles flood the bloodstream.

Viruses differ in their susceptibility to destruction by ozone. The resistance of polio virus type 2 was 40 times that of coxsackie AS,^{54,55} and in an experiment using a continuous flow mixed reactor under controlled laboratory conditions, relative resistance in descending order was found to be: polio virus type 2, echovirus type 1, polio virus type 1, coxsackie virus type B5, echovirus type 5, coxsackie virus type A9. In pure water, at maximal solubility of ozone and room temperature, *Echovirus* type 29 is inactivated in one minute, polio virus type 1 in two, type 3 in three and type 2 in seven minutes.⁵⁵ The mechanism of enteroviral inactivation by ozone was investigated with poliovirus 1 (Mahoney) as the model virus. Ozone was observed to alter two of the four polypeptide chains present in the viral protein coat of poliovirus 1. However, the alteration of the protein coat did not significantly impair virus adsorption or alter the integrity of the virus particle. Damage to the viral RNA after exposure to ozone was demonstrated by velocity sedimentation analysis. It was concluded that the damage to the viral nucleic acid is the major cause of poliovirus 1 inactivation by ozone.⁵⁴ The inactivation rate of enteroviruses⁵⁶ is more rapid than for *E. coli*, takes place in relatively small concentrations of ozone, and is influenced by pH, temperature, and the presence of ambient organic compounds. On the basis of the measured second-order ozone inactivation rate constants, typical ozone exposures applied in water and wastewater treatment, are considering as a highly effective disinfectant for virus control.⁵⁷

In view of the above considerations, what part can ozone play as an antiviral agent? In one study,⁵⁴ polio virus 1 was exposed to 0.21 mg/L of ozone at pH 7.2. After 30 seconds 99 % of the viruses were inactivated (lost their ability to replicate within host cells), but appeared to maintain their structural integrity. Analysis of viral components showed damage to polypeptide chains and envelope proteins, which could result in attachment capability compromise, and breakage of the single-stranded RNA into two parts, producing replicating dysfunction at its root level. Other researchers⁵⁸ in similar experiments concluded that in ozonation, it is the viral capsid which sustains damage. It is to be noted however, that the polioviridae (Picornavirus family) contain four structural proteins encapsulating a single RNA strand and are devoid of lipids (Fig. 3).

Norwalk virus and other human caliciviruses (noroviruses) are major agents of gastroenteritis, and water is a major route of their transmission. In an effort to control Norwalk virus in drinking water, Norwalk virus reduction by bench-scale ozone disinfection was determined using quantitative reverse transcription (RT)-PCR for virus assays. Two other enteric viruses, poliovirus 1 and coliphage MS2, were included for comparison, and their reductions were assayed by infectivity assays as well as by RT-PCR. Virus reductions by ozone were determined using a dose of 0.37 mg of ozone/L at pH 7 and 5 °C for up to 5 min. Based on two RT-PCR assays, the reductions of Norwalk virus were $>3 \log_{10}$ within a contact time of 10 s, and these were similar to the reductions of the other two viruses determined by the same assay methods.⁵⁹

The inactivation of simian rotavirus SA-11 and human rotavirus type 2 (Wa) by ozone was compared at 4 °C by using single-particle virus stocks. Although the human strain was clearly more sensitive, both virus types were rapidly inactivated by ozone concentrations of 0.25 mg/L or greater at all pH levels tested. Comparison of the virucidal activity of ozone with that of chlorine in identical experiments indicated little significant difference in rotavirus-inactivating efficiencies when the disinfectants were used at concentrations of 0.25 mg/L or greater.⁶⁰

There is evidence of inactivation of some others virus by ozone as: Hepatitis A virus,⁶¹ papillomavirus (HPV),⁶² adenovirus, norovirus, sapovirus, parechovirus, hepatitis E virus, astrovirus, pecovirus, picobirnavirus, parvovirus, gokushovirus,⁶³ coxsackievirus B5 (CVF, CVEnv1, and CVEnv2), human adenovirus (HAdV), echovirus, bacteriophages (MS2, Q β , T4, and Φ 174),⁵⁷ bacteriophage lambda⁶⁴ human H5N1/H1N1 influenza viruses,⁶⁵ herpes simplex virus type-1 (HHV-1, strain McIntyre), vesicular stomatitis Indiana virus (VSIV), vaccinia virus (VACV, strain Elstree), adenovirus type-2 (HAdV-2), and the PR8 strain of influenza A virus (FLUAVA/PR/8/34/H1N1; FLUAV),⁶⁶ and others.

Bactericidal effects of ozone

The most cited explanation for ozone's bactericidal effects centres on disruption of envelope integrity through peroxidation of phospholipids and lipoproteins. There is evidence for interaction with proteins as well,⁶⁷ enzymes⁶⁸ and DNA^{68,69} (Fig. 4).

The cell envelope of Gram-negative microorganisms such as *E. coli* is a complex multilayer system composed of an inner cytoplasmic membrane made of phospholipids and proteins invaginating into the cytoplasm, a peptidoglycan layer, and an outer membrane of polymers such as polysaccharides. Gram positive cells have a less complex, three-layer envelope with a thick peptidoglycan middle layer.

In one study⁶⁹ exploring the effect of ozone on *E. coli*, evidence was found for ozone's penetration of the cell membrane, reacting with cytoplasmic substances and converting the closed circular plasmid DNA to open circular DNA, which would presumably lessen the efficiency of bacterial proliferation. It is notable that higher organisms have enzymatic mechanisms to restabilize disrupted DNA and RNA, which could provide a partial explanation for why, in clinical treatment with ozone at doses prescribed, ozone appears to be toxic to infecting organisms and not to the patient.⁷⁰

Vibrio parahaemolyticus is a bacterium in the same family as those that cause cholera. It lives in brackish saltwater and causes gastrointestinal illness in humans. *Vibrio parahaemolyticus* contamination, causes serious foodborne illness, and it has become a global health problem. A study, shown that under low aqueous ozone concentrations (less than 0.125 mg/L), the bacterial cell membranes remained intact, and the ozone was detoxified by intracellular antioxidant enzymes (e.g., superoxide dismutase and catalase). Under high aqueous ozone concentrations (more than 1 mg/L), cell membranes were damaged by the degree of peripheral electro negativity at the cell surface and the concentration of lactate dehydrogenase (LDH) released into the extracellular space, and the ultra-structures of the cells were confirmed by transmission electron microscopy. O₃W penetrated the cells through leaking membranes, inactivated the enzymes, inhibited almost all the genes, and degraded the genetic materials of gDNA and total RNA, which eventually lead to cell death.⁶⁸

Staphylococcus aureus (*S. aureus*) belongs to the normal flora of the skin, mucosa and nasopharynx of several animal species, including man, but it is also associated to illnesses such as abscesses, bacteremia, endocarditis and osteomyelitis, and can show resistance to multiple drugs. O₃W reduces this bacterium.^{35,36,50} Treatment of *Staphylococcus aureus* infections, particularly that of methicillin resistant *Staphylococcus aureus* (MRSA), is a challenge in clinical practice. A study shown that 100 % *S. aureus* and 100 % MRSA were eliminated by O₃W (1.5 mg/L) in 1 min.⁶

Other bacteria that shown sensibility to ozone treatment are: *Mycobacterium smegmatis*,⁷¹ *Mycobacterium tuberculosis*,⁴⁸ *Enterococcus faecalis*,^{36,40,72} *Legionella pneumophila*, *Streptococcus faecalis*, *Streptococcus mutans*,²⁹ *Pseudomonas aeruginosa*,^{36,37,73} *Helicobacter pylori*,⁷⁴ *Salmonella typhimurium*,⁷⁵ *Lactobacillus paracasei*,³² *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*,⁷⁶ *Actinomyces naeslundii*, *Streptococcus mitis* and *Fusobacterium nucleatum*.⁶⁴

Fungicidal effects of ozone

Ozone possesses fungicidal effects, through poorly understood mechanisms. In one study, *Candida utilis* cell growth inhibition with ozone was greatly dependent on phases of their growth, budding cells exhibiting the most sensitivity to its presence.⁷⁷ In addition, with an increase in the ozone dose, neutral lipids (sterols) and nitrogen-containing phospholipids (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) were modified to a greater extent.⁷⁸ The bacterial action of ozone consists mainly in damaging the surface cell structures.⁷⁹

Interestingly, in another study,⁸⁰ different doses of ozone were shown either to stimulate (1-2 x 10⁸ molecules O₃/cell) or inhibit (1 x 10⁹ molecules O₃/cell) respiration and reproduction of *C. utilis* yeast. The observed changes were preserved for several hours after treatment with ozone. Possible role of structural rearrangements of cell membranes in the ozone-induced effects has been involved. Moreover, low doses of ozone stimulated the growth and development of *Monilia fructigen* and *Phytophthora infestans*, while higher doses were inhibitory.⁸¹

The cell wall of fungi is multilayered and composed of approximately 80 % carbohydrates and 20 % of proteins and glycoproteins. The presence of many disulfide bonds making this a possible site for oxidative inactivation by ozone (Fig. 5). Ozone has the capacity to diffuse through the fungal wall, enters into its cytoplasm and disrupting vital cellular functions.⁸² The inhibitory effect of ozone on spore germination, spore production and biomass production in two *Aspergillus* species was also examined.⁸³

Dermatophytes are classified in three genera, *Epidermophyton*, *Microsporum* and *Trichophyton*. They have the capacity to invade keratinized tissue to produce a cutaneous infection known as dermatophytoses. Ozone is effective in killing keratinophilic dermatophyte fungi *Trichophyton rubrum* and *Trichophyton mentagrophytes*; two organisms commonly isolated as the etiological agent in onychomycosis and *tinea pedis* patients.⁸⁴ In addition, other study demonstrated that *Microsporum gypseum* and *Microsporum canis* were the most susceptible, while *Trichophyton interdigitale* and *Trichophyton mentagrophytes* were relatively resistant.⁸²

A study shown that *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Candida albicans* were shown to survive at least 123 days in chlorinated swimming-pool water of 28-30 °C, at least 18 days in O₃W at 34-35 °C, and at least 25 days in pipe water at room temperature of 23-25 degrees.⁸⁵

C. albicans is an emerging multidrug-resistant fungal pathogen representing an important source of invasive disease in humans and generating high healthcare costs worldwide. This fungus is frequently found in different anatomical sites of healthy persons and could induce systemic and superficial infections under optimal environmental conditions. Invasive candidiasis, is an important nosocomial infection with high morbidity and mortality rates in hospitalized children. It represents a major source of prolonged infections in intensive care unit, particularly in immunosuppressed or elderly patients.⁸⁶ Ozone was highly effective in killing *C. albicans*,^{38,64,87} but It seems that ozone therapy induces resistance to amphotericin B.⁸⁷ However, disinfection protocol combining chlorhexidine and ozone may act in synergic, promoting complete elimination of *C. albicans*.⁸⁸ Moreover, the combination of O₃W and ultrasonication had a strong effect on the viability of *C. albicans* adhering to the acrylic resin plates.³⁸

Wound healing and anti-inflammatory effect of ozonized solution. Preclinical support

Although ozone therapy can be applied when wound repair and antimicrobial effect are necessary, little is known about cellular mechanisms regarding this process (Fig. 6). An *In vitro* evaluation of wound healing and antimicrobial potential of ozone therapy in fibroblasts (L929) and keratinocytes (HaCaT) cell lines demonstrated that ozone (ozonated phosphate-buffered saline 8, 4, 2, 1, 0.5 and 0.25 µg/mL ozone), showed no cytotoxicity for the cell lines. In addition, a considerable increase in fibroblasts migration was noticed on cells treated with 8 µg/mL ozonated solution.⁸⁹ Moreover, a study using human keratinocytes evaluated the effect of different non-toxic doses of ozonated saline ranging from (0.096-0.144) µg/mL, in an *in vitro* wound scratch model. The results showed that ozonated saline is able to improve *in vitro* wound healing by stimulating cell proliferation [optimal dose 10 µM (0.48 µg/mL)]. Furthermore, nuclear erythroid 2 related factor 2 (Nrf2) pathways were also activated as determined by its translocation to the nucleus and the increased haem-oxygenase-1 (HO1) gene expression. It was suggested that the ozonated saline effect on wound closure is the result of the combination of more molecules among which HNE and H₂O₂ play a key role.⁵¹ The release of growth factors by ozone from platelets⁹⁰ is another mechanism that probably facilitates wound healing.

O₃W also allowed an increase in blood vessel neoformation and osteoclast migration without affecting bone trabeculae neoformation in an animal model of irrigation over bone healing in hyperglycemia-induced rats.⁴⁵

The anti-inflammatory effect of O₃W is also an important factor in wound healing. A study, evaluated the anti-inflammatory effects of O₃W that was intraperitoneally injected into an experimental inflammatory mouse model. The 10 µg/mL O₃W was injected intraperitoneally into mice with LPS-induced acute inflammation. The results showed that the intraperitoneal injection of O₃W decreased the levels of TNF-α and increased the activity of superoxide dismutase (SOD). The results suggest that O₃W has anti-inflammatory properties and is a potential therapeutic option for acute inflammation.⁹¹

A study focused on developing an antimicrobial agent without any toxicity to dental pulp tissue found that lipopolysaccharide (LPS)-induced expression of cyclooxygenase-2 (COX-2), IL-6, tumor necrosis factor- α (TNF- α) and p38 in rat odontoblastic cell line, KN-3. The activation was markedly suppressed when LPS was pretreated with O3W. The study suggest that odontoblastic cells exhibit inflammatory responses against LPS and that O3W has the ability to improve LPS-induced inflammatory responses and suppression of odontoblastic properties of KN-3 cells through direct inhibition of LPS.⁹² The action mechanism of O3W is not limited to its antimicrobial power and its high biocompatibility with mammalian cells. New therapeutic strategies for the treatment of infectious disease should consider not only its antibacterial effects, but also its influence on the host immune response. The action of O3W on the nuclear factor kappa light chain enhancer of B cell (NF- κ B) system, a paradigm for inflammation-associated signaling/transcription was investigated. Results shown that NF- κ B activity in oral cells stimulated with TNF, and in periodontal ligament tissue from root surfaces of periodontally damaged teeth, was inhibited following incubation with an ozonized medium. Under this treatment, I κ B α proteolysis, cytokine expression, and κ B-dependent transcription were prevented. Specific ozonized amino acids were shown to represent major inhibitory components of the ozonized medium. This study establishes a condition under which aqueous ozone exerts inhibitory effects on the NF- κ B system, suggesting that it has an anti-inflammatory capacity.⁹³

The mechanism of action of O₃W involves an interaction with the Nrf2 and NF- κ B pathways (Fig. 7). Oxidative stimulus of O₃ mediated Nrf2 activation can lead to the production of antioxidant enzymes and cytoprotective enzymes such as SOD, catalase, glutathione reductase (GPx), HO-1, NADPH quinone oxidoreductase (NQO1), and increased synthesis of GSH, NADPH and multidrug transporters. Nrf2 binds to the antioxidant response element (ARE) on DNA and maintains redox balance in body. Normally Nrf2 is bound to cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1) and labelled for polyubiquitination mediated proteasomal degradation. However, during oxidative insult, the sulfhydryl groups on Keap1 are oxidised, causing an alteration in the configuration of Keap1, which then releases Nrf2, which then binds with ARE elements of the genome along with small Maf proteins. NF- κ B is another redox regulated transcription factor, involved in inflammation, immune function, cellular growth and apoptosis. p65 is a Rel protein with transactivation efficiency whereas its partner p50 does not possess transcriptional activity. Oxidative stress can cause the activation of I κ B kinase (IKK). Activation of IKK causes phosphorylation of an inhibitor of NF- κ B, I κ B and hence targets the later for polyubiquitination mediated proteasomal degradation, resulting in the release of NF- κ B, which then migrates into the nucleus and binds with the κ region of genome. With the help of

other coactivators and histone acetyl transferases (HAT), NF- κ B causes the transcription of proinflammatory mediators such as interleukin-6 (IL-6), TNF- α , COX-2, interleukin-1 (IL-1), intracellular adhesion molecule (ICAM) and inducible nitric oxide synthase (iNOS).

Further, these two pathways are proposed to inhibit each other at their transcription level via protein–protein interactions or through secondary messenger effects. Nrf2 pathway inhibits the activation of NF- κ B pathway by increasing antioxidant defences and HO-1 expression, which efficiently neutralizes ROS and detoxifies toxic chemicals and hence, reduces ROS mediated NF- κ B activation. Nrf2 pathway also inhibits NF- κ B mediated transcription by preventing the degradation of I κ B- α . Similarly, NF- κ B mediated transcription reduces Nrf2 activation by reducing the ARE gene transcription and decreasing free CREB binding protein (CBP) by competing with Nrf2 for CH1-KIX domain of CBP. NF- κ B also enhances the recruitment of histone deacetylase 3 (HDAC3) to the ARE region by binding to Mafk and hence interferes with the transcriptional facilitation of Nrf2.

Moreover, O₃W (1.2 μ g/mL, p.o.) in gastric ulcers models induced by stress, promote a significant reduction in the incidence of ulcers types I, II and III ($p < 0.05$, Student's t -test).⁹⁴ Using a tumor-bearing mouse model and normal controls the local administration of O₃W (208 mM) was not associated with any detrimental effects in normal tissues. On the other hand, local administration of O₃W (20.8, 41.6, 104, or 208 mM) directly into the tumor tissue induced necrosis and inhibited proliferation of tumor cells. These results indicate that O₃W does not adversely affect normal tissue and damages only the tumor tissue by selectively inducing necrosis. Based on these results, it was suggested that the administration of O₃W is a safe and potentially simple adjunct or alternative to existing antineoplastic treatments.⁹⁵

The safety of O₃W was also tested using human oral epithelial (BHY) cells and gingival fibroblast (HGF-1) cells. The study investigated whether gaseous ozone ($4 \cdot 10^6$) μ g/m³ and aqueous ozone (1.25-20 μ g/mL) exert any cytotoxic effects on BHY and HGF-1 compared with established antiseptics [chlorhexidine digluconate (CHX) 2 %, 0.2 %; sodium hypochlorite (NaOCl) 5.25 %, 2.25 %; hydrogen peroxide (H₂O₂ 3 %], over a time of 1 min, and compared with the antibiotic, metronidazole, over 24 h. Ozone gas was found to have toxic effects on both cell types. Essentially no cytotoxic signs were observed for O₃W. CHX (2 %, 0.2 %) was highly toxic to BHY cells, and slightly (2 %) and non-toxic (0.2 %) to HGF-1 cells. NaOCl and H₂O₂ resulted in markedly reduced cell viability (BHY, HGF-1), whereas metronidazole displayed mild toxicity only to BHY cells. Taken together, O₃W represents the highest level of biocompatibility of the tested antiseptics.⁹⁶

Conflict of interest:

The author report no conflicts of interest.

Acknowledgement:

Author gratefully acknowledge the support from Dr. Frank Shallemberger in the grammatical correction of the manuscript (English version). Author also express his gratitude to Dr. Fadi Sabbah and Dr. Carlos Nogales for their input to write about this topic. This manuscript will be part of the up-coming book “Ozonated Water. A practical guide for healthcare professionals” by Fadi Sabbah, Carlos Nogales and Gregorio Martínez-Sánchez.

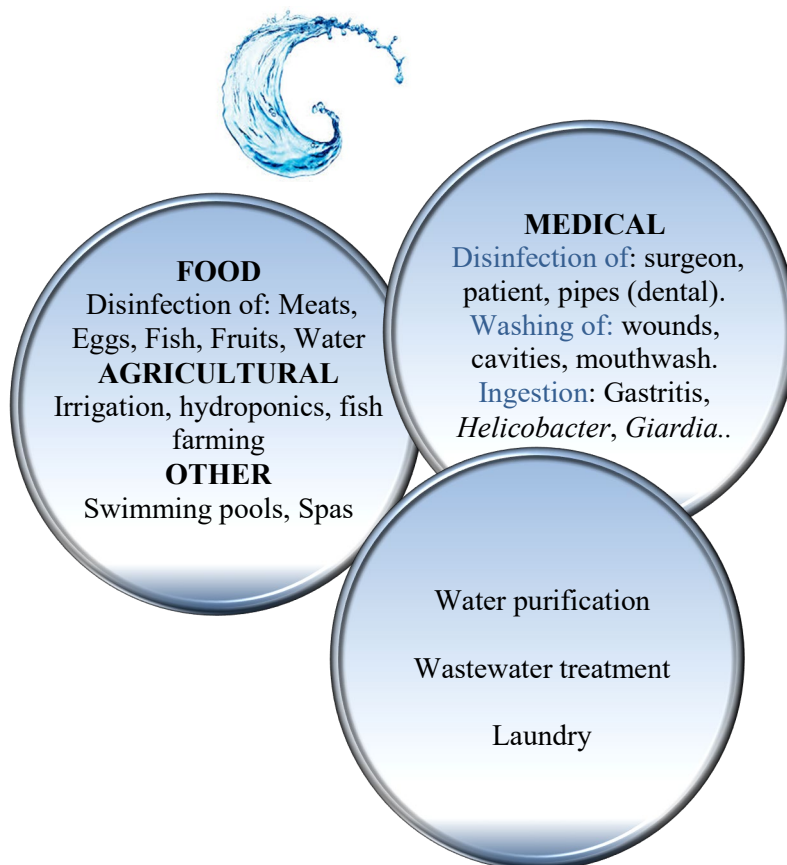


Figure. 1. Summary of industrial and medical applications of ozonized water.

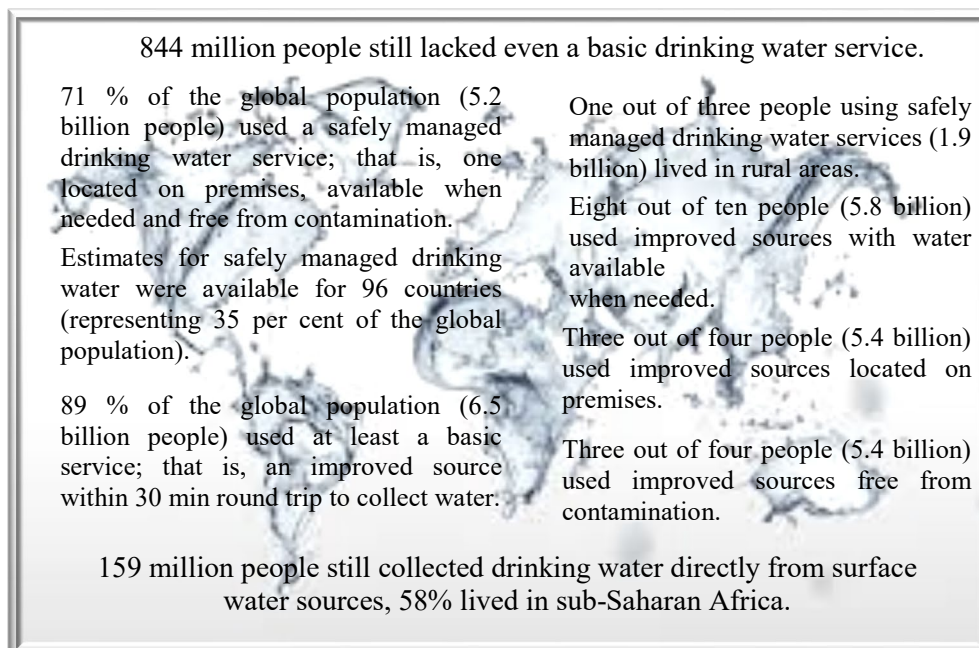


Figure 2. Global indicators for drinking water in 2015 according to WHO/UNICEF Joint Monitoring Programme for Water Supply 2017.⁵

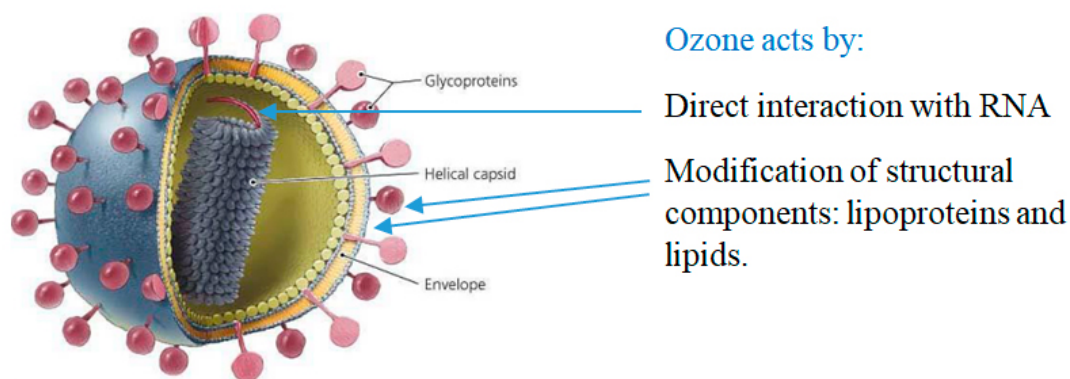


Figure 3. Possible point of interaction of ozone with virus. The example shown a representation of influenza virus particle (RNA virus).

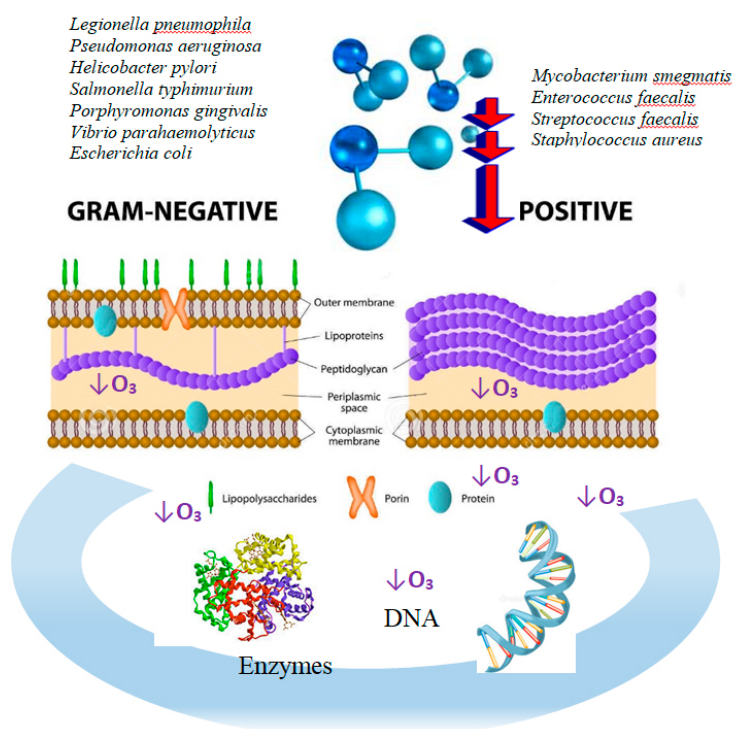


Figure 4. Mechanism of interaction of ozone with bacteria. Disruption of envelope integrity through peroxidation of phospholipids, lipoproteins, and peptidoglycan. The result is enzyme inactivation, gene inhibition, and degradation of genetic materials, all of which interrupt the normal cell activity of the bacteria.

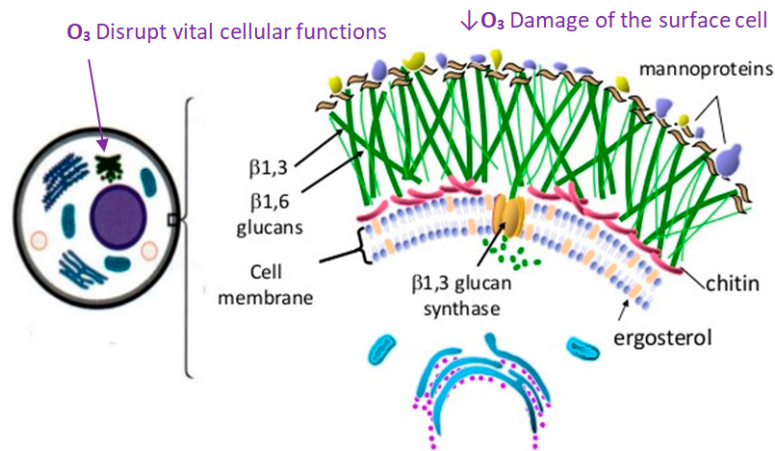


Figure 5. Representation of the main mechanism involved in the interaction of ozone with fungi. Modified from Introduction to Medical Mycology (2001).⁹⁷

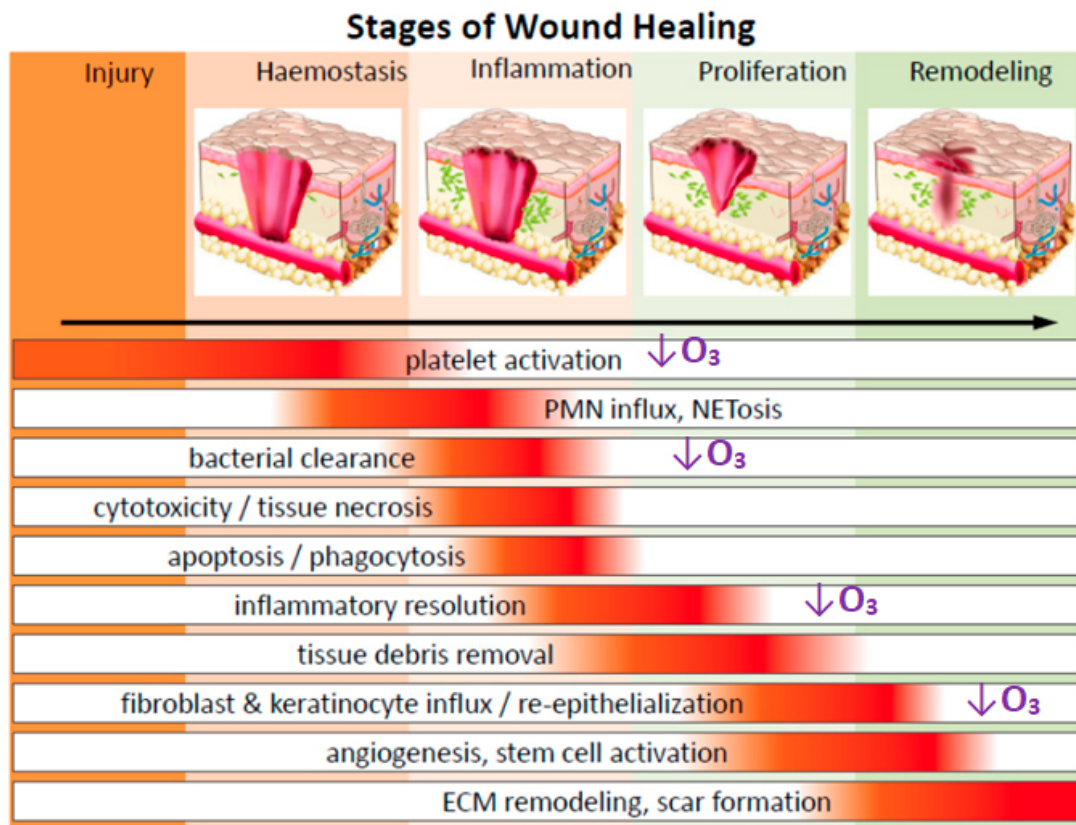


Figure 6. The function of ozone throughout the stages of wound healing. After injury, wounded tissue must establish haemostasis via coagulation and clot formation. Injury is quickly followed by immune infiltration and inflammation as a means to clear the wound of damaged tissue and microbes, thus preventing infection and allowing room for granulation. Fibroblast, epithelial cells, keratinocytes and endothelial cells, will proliferate and migrate into wounds to deposit Extracellular matrix (ECM) and re-populate the injury site, facilitating wound closure. Finally, matrix deposition and clearance regulate the development of scar formation. Legend: PMN, polymorphonuclear leukocytes; NETosis, a novel form of programmed neutrophil death that resulted in the formation of neutrophil extracellular traps (NETs); ↓O₃, point of intervention of ozone. Modified from Pei-Hui *et al.* (2018).⁹⁸

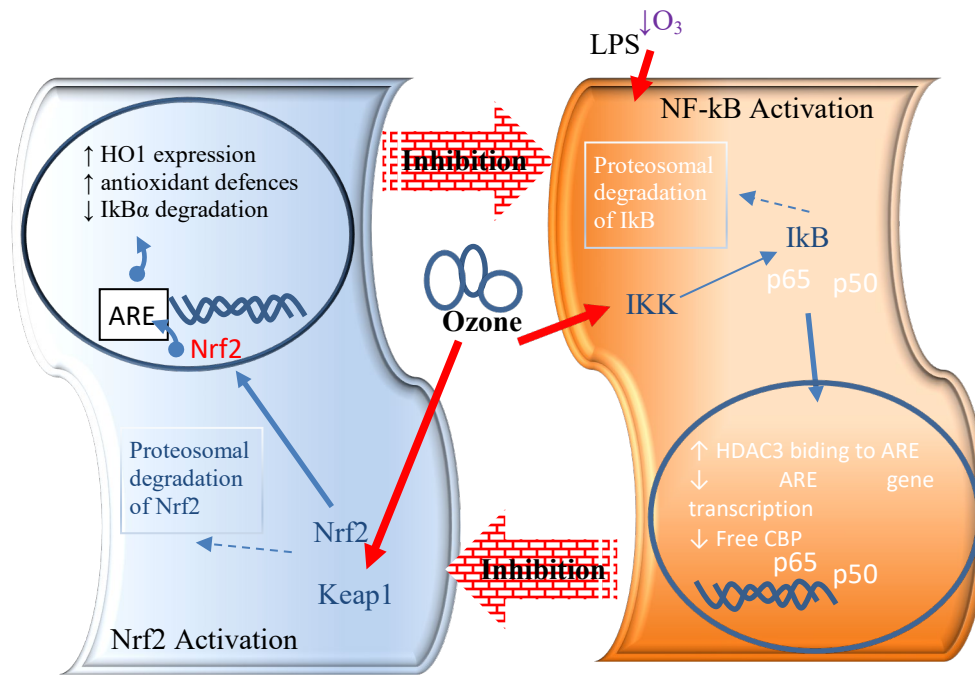


Figure 7. Representation of the anti-inflammatory mechanism of ozonized water mediated by interacting with the crosstalk between the Nrf2 and NF-κB pathways. Legend: HO-1, haem-oxygenase-1; ARE, antioxidant response element; Keap1, Kelch-like ECH-associated protein 1; IKK, IκB kinase; CBP, CREB binding protein; HDAC3, histone deacetylase 3. Nrf2, nuclear erythroid 2 related factor 2; NF-κB, nuclear factor kappa light chain enhancer of B cell; LPS, lipopolysaccharide. (Modified from Veera Ganesh Y. *et al*).⁹⁹

Reference

1. *Emerging therapies: ozone. What the patient should know and how the doctor must act.* [computer program]: Aracne Editrice, Roma; 2012.
2. Suleyman G, Alangaden G, Bardossy AC. The Role of Environmental Contamination in the Transmission of Nosocomial Pathogens and Healthcare-Associated Infections. *Curr Infect Dis Rep.* Apr 27 2018;20(6):12.
3. Terao R, Terao C, Mori K, Matsui M. Technical Note: 100% Ozone-treatment System of Bath Water. *Ozone: Science & Engineering.* 2010;25(4):345-349.
4. Bocci V, ed *Ozone: A new medical drug.* ISBN-10: 9048168058. ISBN-13: 978-9048168057. Netherlands: Springer; 2010.
5. WHO, INICEF. *Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines.* Geneva2017.
6. Song M, Zeng Q, Xiang Y, et al. The antibacterial effect of topical ozone on the treatment of MRSA skin infection. *Mol Med Rep.* Feb 2018;17(2):2449-2455.
7. Adamu H, Petros B, Zhang G, et al. Distribution and clinical manifestations of Cryptosporidium species and subtypes in HIV/AIDS patients in Ethiopia. *PLoS Negl Trop Dis.* Apr 2014;8(4):e2831.
8. Demir F, Atguden A. Experimental Investigation on the Microbial Inactivation of Domestic Well Drinking Water using Ozone under Different Treatment Conditions. *Ozone Science & Engineering.* 2016;38(1):25-35.
9. Schneider W, Rump HH. Experiments for Waste Water Treatment with OZONE in Combination with Adsorption Procedures. *Ozone Science & Engineering.* 2008;4(1):3-14.
10. Diamant BZ. Recent Development in the Role of Ozone in Water Purification and its Implications in Developing Countries. *Ozone Science & Engineering.* 1980;2(3):241-250.
11. Schalekamp M. Pre-ndintermediate Oxidation Of Drinking Water With Ozone, Chlorine and Chlorine Dioxide. *Ozone Science & Engineering.* 1986;8(2):151-186.
12. Archibald F, Roy-Arcand L. The use of ozone to decolorize residual direct paper dyes in kraft paper machine whitewater. *Ozone Science & Engineering.* 1997;19(6):549-565.
13. Sadło S, Szpyrka E, Piechowicz B, Antos P, Józefczyk R, Balawejder M. Reduction of Captan, Boscalid and Pyraclostrobin Residues on Apples Using Water Only, Gaseous Ozone, and Ozone Aqueous Solution. *Ozone: Science & Engineering.* 2016;39(2):97-103.
14. Cataldo F. Ozone Decomposition of Patulin—A Micotoxin and Food Contaminant. *Ozone Science & Engineering.* 2008;30(3):197-201.
15. Mohan N, Patel K, Padmanabhan K, Ananthi S. Ozone for Plant Pathological Applications. *Ozone: Science & Engineering.* 2014;27(6):499-502.
16. Bataller-Venta M, Cruz-Broche SS, Fernández-Torres I, et al. Ozone Application for Postharvest Disinfection of Tomatoes. *Ozone Science & Engineering.* 2010;32(4):361-371.
17. Aguayo E, Escalona V, Silveira AC, Artes F. Quality of tomato slices disinfected with ozonated water. *Food Sci Technol Int.* Apr 2014;20(3):227-235.
18. Xu W, Wu C. Different efficiency of ozonated water washing to inactivate Salmonella enterica typhimurium on green onions, grape tomatoes, and green leaf lettuces. *J Food Sci.* Mar 2014;79(3):M378-383.
19. Lestan D, Hanc A, Finzgar N. Influence of ozonation on extractability of Pb and Zn from contaminated soils. *Chemosphere.* Nov 2005;61(7):1012-1019.
20. Plaue JW, Czerwinski KR. The influence of ozone on ligand-assisted extraction of 239Pu and 241Am from rocky flats soil. *Radiochim. Acta.* 2003;91(6-2003):309-313.
21. Noble AC, Summerfelt ST. Diseases encountered in rainbow trout cultured in recirculating systems. *Annual Review of Fish Diseases.* 1996;6:65–92.
22. Ferreira O, de Costa OT, Ferreira S, Mendonca F. Susceptibility of the Amazonian fish, *Colossoma macropomum* (Serrasalminae), to short-term exposure to nitrite. *Aquaculture.* 2004;232:627-636.
23. Ribeiro ARA, Ribeiro L, Saele Ø, Hamre K, Dinis MT, Moren M. Iodine-enriched rotifers and Artemiaprevent goitre in Senegalese sole (*Solea senegalensis*) larvae reared in a recirculation system. *Aquaculture Nutrition.* 2009;17(3):248-257.

24. Buchan K, Martin-Robinchaud D, Benfey TJ, MacKinnon A, Boston L. The efficacy of ozonated seawater for surface disinfection of haddock (*Melanogrammus aeglefinus*) eggs against piscine nodavirus. *Aquacultural Engineering*. 2006;35:102–107.
25. Masschelein WJ. Belgian Experiences in the Ozonization of Water. *Ozone Science & Engineering*. 1984;7(4):327-350.
26. Steinbruchel A, Rice RG, Spangenberg R. First Year Operation Report of the Corona Discharge Ozone Swimming Pool Water Treatment Systems at the Peck Aquatic Facility, Milwaukee, Wisconsin. *Ozone Science & Engineering*. 1989;13(4):463-478.
27. Blogoslowski WJ, Stewart ME. Some Ozone Applications in Seafood. *Ozone Science & Engineering*. 2011;33(5):368-373.
28. Fedorov AA, Gromov AS, Sapronenok SV, Kurochkin V, Zhernakova ZM. [Ozone therapy in gastroduodenal pathology associated with *Helicobacter pylori*]. *Vopr Kurortol Fizioter Lech Fiz Kult*. Nov-Dec 2006(6):34-37.
29. Nagayoshi M, Fukuizumi T, Kitamura C, Yano J, Terashita M, Nishihara T. Efficacy of ozone on survival and permeability of oral microorganisms. *Oral Microbiol Immunol*. Aug 2004;19(4):240-246.
30. Nagayoshi M, Kitamura C, Fukuizumi T, Nishihara T, Terashita M. Antimicrobial effect of ozonated water on bacteria invading dentinal tubules. *J Endod*. Nov 2004;30(11):778-781.
31. Sadatullah S, Mohamed N, Razak F. Qualitative analyses of the antimicrobial effect of ozonated water on supragingival plaque and salivary microbes. *Ann Med Health Sci Res*. Jul 2014;4(4):526-531.
32. Kollmuss M, Kist S, Obermeier K, Pelka AK, Hickel R, Huth KC. Antimicrobial effect of gaseous and aqueous ozone on caries pathogen microorganisms grown in biofilms. *Am J Dent*. Jun 2014;27(3):134-138.
33. Zimmermann D, Waltimo T, Filippi A. Ozonized Water in Dental Traumatology - A Preliminary Study on the Treatment of Avulsed Teeth, in Vitro. *Ozone: Science & Engineering*. 2012;34(6):484-488.
34. Onouchi T. A study on the bactericidal action of aqueous solution of O₃ in dentistry. *J Nihon Univ Sch Dent*. Dec 1965;7(4):200-208.
35. Velano HE, do Nascimento LC, de Barros LM, Panzeri H. [In vitro assessment of antibacterial activity of ozonized water against *Staphylococcus aureus*]. *Pesqui Odontol Bras*. Jan-Mar 2001;15(1):18-22.
36. Nogales CG, Ferreira MB, Lage-Marques JL. Comparison of the Antimicrobial Activity of Three Different Concentrations of Aqueous Ozone on *Pseudomonas Aeruginosa*, *Staphylococcus Aureus*, and *Enterococcus Faecalis* - in Vitro Study. *Revista Española de Ozonoterapia*. 2014;4(1):9-15.
37. Bialoszewski D, Pietruczuk-Padzik A, Kalicinska A, et al. Activity of ozonated water and ozone against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. *Med Sci Monit*. Nov 2011;17(11):BR339-344.
38. Arita M, Nagayoshi M, Fukuizumi T, et al. Microbicidal efficacy of ozonated water against *Candida albicans* adhering to acrylic denture plates. *Oral Microbiol Immunol*. Aug 2005;20(4):206-210.
39. Estrela C, Estrela CR, Decurcio DA, Hollanda AC, Silva JA. Antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in infected human root canals. *Int Endod J*. Feb 2007;40(2):85-93.
40. Goztas Z, Onat H, Tosun G, Sener Y, Hadimli HH. Antimicrobial effect of ozonated water, sodium hypochlorite and chlorhexidine gluconate in primary molar root canals. *Eur J Dent*. Oct 2014;8(4):469-474.
41. Filippi A. Water Disinfection of Dental Units using Ozone – Microbiological Results after 11 Years and Technical Problems. *Ozone Science & Engineering*. 2002;24(6):479-483.
42. Hikal W, Zaki B, Sabry H. Evaluation of Ozone Application in Dental Unit Water Lines Contaminated with Pathogenic *Acanthamoeba*. *Iran J Parasitol*. Jul-Sep 2015;10(3):410-419.
43. Bialoszewski D, Bocian E, Bukowska B, Czajkowska M, Sokol-Leszczynska B, Tyski S. Antimicrobial activity of ozonated water. *Med Sci Monit*. Sep 2010;16(9):MT71-75.

44. Samuel SR, Dorai S, Khatri SG, Patil ST. Effect of ozone to remineralize initial enamel caries: in situ study. *Clin Oral Investig*. Jun 2016;20(5):1109-1113.
45. Frascino AV, Mantesso A, Correa L, Deboni MC. Aqueous-ozone irrigation of bone monocortical wounds in hyperglycemic rats. *Acta Cir Bras*. May 2013;28(5):327-333.
46. Xiao CW, Jing XM, X. S, et al. The experimental study of keratomycosis treated by Ozone solution. *Chinese Journal of Practical Ophthalmology*. 2004;12.
47. Qin G, Huang J, Pan Y, et al. [Topical ozone application: An innovative therapy for infantile atopic dermatitis]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*. Feb 28 2018;43(2):163-167.
48. Belianin, II, Safonova SG. [In vitro bactericidal action of dissolved ozone on different strains of Mycobacterium tuberculosis]. *Probl Tuberk*. 1999(2):48-49.
49. Belianin, II, Nikolaeva GM, Martynova LP. [Action of dissolved ozone on mycobacterium tuberculosis and alveolar macrophages in experimental tuberculosis]. *Probl Tuberk*. 1997(1):56-59.
50. Al-Saadi H, Potapova I, Rochford ET, Moriarty TF, Messmer P. Ozonated saline shows activity against planktonic and biofilm growing Staphylococcus aureus in vitro: a potential irrigant for infected wounds. *Int Wound J*. Oct 2016;13(5):936-942.
51. Valacchi G, Sticozzi C, Zanardi I, et al. Ozone mediators effect on "in vitro" scratch wound closure. *Free Radic Res*. Aug 3 2016:1-25.
52. Lohr A, Gratzek J. Bactericidal and paracitidal effects of an activated air oxidant in a closed aquatic system. *J Aquaric Aquat Sci* 1984;4(41):1-8.
53. Albertini A, Bressanelli S, Lepault J, Gaudin Y. Structure and working of viral fusion machinery. *Curr Top Membr*. 2011;68:49-80.
54. Roy D, Wong PK, Engelbrecht RS, Chian ES. Mechanism of enteroviral inactivation by ozone. *Appl Environ Microbiol*. Mar 1981;41(3):718-723.
55. Roy D, Engelbrecht R, Chian E. Comparative inactivation of six enteroviruses by ozone. *Am Water Works Assoc J*. 1982;74(12):660-664.
56. Ivanova OE, Bogdanov MV, Kazantseva VA, Gabrilevskaia LN, Kodkind G. [Inactivation of enteroviruses in sewage with ozone]. *Vopr Virusol*. Nov-Dec 1983;28(6):693-698.
57. Wolf C, von Gunten U, Kohn T. Kinetics of Inactivation of Waterborne Enteric Viruses by Ozone. *Environ Sci Technol*. Feb 20 2018;52(4):2170-2177.
58. Riesser V, Perrich J, Silver B, McCammon J. Possible mechanism of poliovirus inactivation by ozone, in Forum on Ozone Disinfection. *Proceedings of the International Ozone Institute*. 1977;Syracuse, NY:186-192.
59. Shin GA, Sobsey MD. Reduction of Norwalk virus, poliovirus 1, and bacteriophage MS2 by ozone disinfection of water. *Appl Environ Microbiol*. Jul 2003;69(7):3975-3978.
60. Vaughn JM, Chen YS, Lindburg K, Morales D. Inactivation of human and simian rotaviruses by ozone. *Appl Environ Microbiol*. Sep 1987;53(9):2218-2221.
61. Hall R, Sobsey M. Inactivation of hepatitis A virus and MS2 by ozone and ozone-hydrogen peroxide in buffered water. *Wat. Sci. Technol*. 1993;27:371-378.
62. Peirone C, Mestre VF, Medeiros-Fonseca B, et al. Ozone therapy prevents the onset of dysplasia in HPV16-transgenic mice-A pre-clinical efficacy and safety analysis. *Biomed Pharmacother*. Aug 2018;104:275-279.
63. Wang H, Sikora P, Rutgersson C, et al. Differential removal of human pathogenic viruses from sewage by conventional and ozone treatments. *Int J Hyg Environ Health*. Apr 2018;221(3):479-488.
64. Komanapalli IR, Lau BH. Inactivation of bacteriophage lambda, Escherichia coli, and Candida albicans by ozone. *Appl Microbiol Biotechnol*. Jun 1998;49(6):766-769.
65. Lenes D, Deboosere N, Menard-Szczebara F, et al. Assessment of the removal and inactivation of influenza viruses H5N1 and H1N1 by drinking water treatment. *Water Res*. Apr 2010;44(8):2473-2486.
66. Murray BK, Ohmine S, Tomer DP, et al. Virion disruption by ozone-mediated reactive oxygen species. *J Virol Methods*. Oct 2008;153(1):74-77.
67. Mudd JB, Leavitt R, Ongun A, McManus TT. Reaction of ozone with amino acids and proteins. *Atmos Environ*. Nov 1969;3(6):669-682.
68. Feng L, Zhang K, Gao M, et al. Inactivation of Vibrio parahaemolyticus by aqueous ozone. *J Microbiol Biotechnol*. Jun 6 2018.

69. Ishizaki K, Sawadaishi D, Miura K, Shinriki N. Effect of ozone on plasmid DNA of Escheria coli in situ. *Water Res.* 1987;21(7):823-828.
70. Cech TR. RNA as an enzyme. *Sci Am.* 1986;255(5):64-75.
71. Seki M, Ishikawa T, Terada H, Nashimoto M. Microbicidal Effects of Stored Aqueous Ozone Solution Generated by Nano-bubble Technology. *In Vivo.* Jul-Aug 2017;31(4):579-583.
72. Bitter K, Vlassakidis A, Niepel M, et al. Effects of Diode Laser, Gaseous Ozone, and Medical Dressings on Enterococcus faecalis Biofilms in the Root Canal Ex Vivo. *Biomed Res Int.* 2017;2017:6321850.
73. Martinelli M, Giovannangeli F, Rotunno S, Trombetta CM, Montomoli E. Water and air ozone treatment as an alternative sanitizing technology. *J Prev Med Hyg.* Mar 2017;58(1):E48-E52.
74. Orta de Velasquez MT, Yanez Noguez I, Casasola Rodriguez B, Roman Roman PI. Effects of ozone and chlorine disinfection on VBNC Helicobacter pylori by molecular techniques and FESEM images. *Environ Technol.* Mar 2017;38(6):744-753.
75. Song WJ, Sung HJ, Kang DH. Inactivation of Escherichia coli O157:H7 and Salmonella Typhimurium in apple juices with different soluble solids content by combining ozone treatment with mild heat. *J Appl Microbiol.* Jan 2015;118(1):112-122.
76. Hayakumo S, Arakawa S, Takahashi M, Kondo K, Mano Y, Izumi Y. Effects of ozone nano-bubble water on periodontopathic bacteria and oral cells - in vitro studies. *Sci Technol Adv Mater.* Oct 2014;15(5):055003.
77. Matus V, Nikava A, Prakopava Z, Konyew S. Effect of ozone on the survivability of Candida utilis cells. *Vyestsi AkadNauuk Bssr Syer Biyal Navuk.* 1981;0(3):49-51.
78. Matus VK, Martynova MA, Skorynko EV, Melnikova AM, Konev SV. Different modes of ozone-induced lipid oxidation in Candida utilis yeast cells and isolated membrane preparations. *Membr Cell Biol.* 1999;13(1):59-67.
79. Konev SV, Matus VK, Mel'nikova AM, Rudenok AN. [Action of ozone on the membrane-dependent functions of Candida utilis yeast cells]. *Mikrobiologija.* Mar-Apr 1982;51(2):220-223.
80. Mel'nikova AM, Kaler GV, Babich GV, Romanov SL, Matus VK. [The conflicting action of low and high doses of ozone on the reproductive capacity and respiratory activity of Candida utilis yeast cells]. *Zh Obshch Biol.* Nov-Dec 1989;50(6):815-818.
81. Matus V, Lyskova T, Sergienko I, Kustova A, Grigortsevich T, Konev V. Fungi; growth and sporulation after a single treatment of spores with ozone. *Mikol Fitopatot.* 1982;16(5):420-423.
82. Ouf SA, Moussa TA, Abd-Elmegeed AM, Eltahlawy SR. Anti-fungal potential of ozone against some dermatophytes. *Braz J Microbiol.* Jul-Sep 2016;47(3):697-702.
83. Antony-Babu S, Singleton I. Effect of ozone on spore germination, spore production and biomass production in two Aspergillus species. *Antonie Van Leeuwenhoek.* Nov 2009;96(4):413-422.
84. Gupta AK, Brintnell W. Ozone gas effectively kills laboratory strains of Trichophyton rubrum and Trichophyton mentagrophytes using an in vitro test system. *J Dermatolog Treat.* Jun 2014;25(3):251-255.
85. Fischer E. [How long do dermatophytes survive in the water of indoor pools?]. *Dermatologica.* Oct 1982;165(4):352-354.
86. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K. Candida albicans - Biology, molecular characterization, pathogenicity, and advances in diagnosis and control - An update. *Microb Pathog.* Apr 2018;117:128-138.
87. Zargarán M, Fatahinia M, Zarei Mahmoudabadi A. The efficacy of gaseous ozone against different forms of Candida albicans. *Curr Med Mycol.* Jun 2017;3(2):26-32.
88. Noites R, Pina-Vaz C, Rocha R, Carvalho MF, Goncalves A, Pina-Vaz I. Synergistic antimicrobial action of chlorhexidine and ozone in endodontic treatment. *Biomed Res Int.* 2014;2014:592423.
89. Borges GA, Elias ST, da Silva SM, et al. In vitro evaluation of wound healing and antimicrobial potential of ozone therapy. *J Craniomaxillofac Surg.* Mar 2017;45(3):364-370.

90. Valacchi G, Bocci V. Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets. *Mediators Inflamm.* 1999;8(4-5):205-209.
91. Azuma K, Mori T, Kawamoto K, et al. Anti-inflammatory effects of ozonated water in an experimental mouse model. *Biomed Rep.* Sep 2014;2(5):671-674.
92. Noguchi F, Kitamura C, Nagayoshi M, Chen KK, Terashita M, Nishihara T. Ozonated water improves lipopolysaccharide-induced responses of an odontoblast-like cell line. *J Endod.* May 2009;35(5):668-672.
93. Huth KC, Saugel B, Jakob FM, et al. Effect of aqueous ozone on the NF-kappaB system. *J Dent Res.* May 2007;86(5):451-456.
94. Cardoso CC, Carvalho JC, Ovando EC, Macedo SB, Dall'Aglio R, Ferreira LR. Action of ozonized water in preclinical inflammatory models. *Pharmacol Res.* Jul 2000;42(1):51-54.
95. Kuroda K, Azuma K, Mori T, et al. The Safety and Anti-Tumor Effects of Ozonated Water in Vivo. *Int J Mol Sci.* Oct 22 2015;16(10):25108-25120.
96. Huth KC, Jakob FM, Saugel B, et al. Effect of ozone on oral cells compared with established antimicrobials. *Eur J Oral Sci.* Oct 2006;114(5):435-440.
97. Merch. *Introduction to Medical Mycology*: Merch & Co., Inc; 2001.
98. Lin PH, Sermersheim M, Li H, Lee PHU, Steinberg SM, Ma J. Zinc in Wound Healing Modulation. *Nutrients.* Dec 24 2017;10(1).
99. Ganesh Yerra V, Negi G, Sharma SS, Kumar A. Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-kappaB pathways in diabetic neuropathy. *Redox Biol.* Aug 1 2013;1:394-397.