Ozone therapy: An overview of pharmacodynamics, current research, and clinical utility

Article *in* Medical Gas Research · October 2017

DOI: 10.4103/2045-9912.215752

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REVIEW

Ozone therapy: an overview of pharmacodynamics, current research, and clinical utility

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Abstract

The use of ozone (O_3) gas as a therapy in alternative medicine has attracted skepticism due to its unstable molecular structure. However, copious volumes of research have provided evidence that O_3's dynamic resonance structures facilitate physiological interactions useful in treating a myriad of pathologies. Specifically, O_3 therapy induces moderate oxidative stress when interacting with lipids. This interaction increases endogenous production of antioxidants, local perfusion, and oxygen delivery, as well as enhances immune responses. We have conducted a comprehensive review of O_3 therapy, investigating its contraindications, routes and concentrations of administration, mechanisms of action, disinfectant properties in various microorganisms, and its medicinal use in different pathologies. We explore the therapeutic value of O_3 in pathologies of the cardiovascular system, gastrointestinal tract, genitourinary system, central nervous system, head and neck, musculoskeletal, subcutaneous tissue, and peripheral vascular disease. Despite compelling evidence, further studies are essential to mark it as a viable and quintessential treatment option in medicine.

Key words: ozone; ozone therapy; ozone gas; autohemotherapy; oxidative stress; reactive oxidative species; lipid ozonation products; oxidative preconditioning

doi: 10.4103/2045-9912.215752
How to cite this article: Smith NL, Wilson AL, Gandhi J, Vatsia S, Khan SA. Ozone therapy: an overview of pharmacodynamics, current research, and clinical utility. Med Gas Res. 2017;7(3):212-219.

Introduction

Ozone (O_3) gas was discovered in the 1840s, and soon after that, the scientific community began to expand past the notion that it was just another gas of the Earth's atmosphere. Though the migration of O_3 into the medical field has taken a circuitous road since the 19th century, its medicinal value is currently controversial despite compelling research.[1] O_3 is highly water-soluble inorganic molecule composed of three oxygen molecules. O_3's inherently unstable molecular structure, due to the nature of its mesomeric states, tends to make it difficult to obtain high concentrations. O_3 will often experience transient reactions with itself or water. Thus, it was initially problematic to achieve desired levels and even more difficult is to assess the therapeutic effects of such a transient state.[1,2] These mesomeric states create a conundrum within the scientific community. A divide has formed between those who believe the volatile nature of these mesomeric states can foster positive responses and those who are wary of its seemingly dangerous effects.

Despite suspicions, a multitude of O_3 therapies have shown substantial benefits that span a large variety of acute and chronic ailments. O_3 is currently prevalent in dentistry to treat diseases of the jaw.[1] O_3 has also proven itself beneficial as a disinfectant for drinking water and sterilization of medical instruments.[1,3] The function of O_3 shares similarities to that of a prodrug, as it is modified upon reacting with

molecules to create more active substrates, thus stimulating an endogenous cascade of responses. On the other hand, it is hard to classify O_3 as simply a prodrug, due to its capability to directly interact with phospholipids, lipoproteins, cell envelopes of bacteria, and viral capsids. The physiology of these biological responses is herein discussed.

Despite the various benefits, O_3 toxicity and clinical utility depends on the concentration and administration to the appropriate site.[1,2,4,5] One of the major contraindications of O_3 therapy is lung inhalation. O_3 therapy significantly increases airway resistance without changing the compliance or elastic characteristics of the lung.[1] Additionally, direct contact of O_3 with the eyes and lungs is contraindicated because of the low antioxidant capabilities in these specific locations.[6]

Literature Retrieval

A MEDLINE® database search of literature extended from 1980 to 2017 to obtain current information regarding O_3 therapy, its routes of administration, and mechanism of action. Subsequently, trials pertaining to the clinical implications of O_3 therapy were paired by pathology and anatomical system. The most important points refer to the type of pathology, route of O_3 administration, type of research trial, result(s) of the trial, side effect(s), and proposed physiological mechanism(s). Literature retrieval was performed in July 2017 and included the term "ozone therapy" combined with the following search criteria: "routes of administration", "mechanism of action", "cardiovascular", "subcutaneous tissue", "peripheral vascular disease", "neurological", "head and neck", "orthopedic", "musculoskeletal", "gastrointestinal", and "genitourinary". We did not formulate any exclusion criteria.

Routes of Administration

O_3 therapy combines a mixture of oxygen (O_2)-O_3, with a diverse therapeutic range (10–80 μg/mL of gas per mL of blood).[5-7] O_3 therapy administration is variable based on treatment goals and location of therapy. The first and most popular is O_3 autohemotransfusion (O_3-AHT). O_3-AHT has grown in popularity because it allows for a predetermined amount of blood to be taken and thus, using stoichiometric calculations, a precise concertation of O_2-O_3 can be infused. This small amount of blood is subjected to O_2-O_3 *ex vivo* is then administered to the patient.[5,6] Extracorporeal blood oxygenation and ozonation are very similar techniques. However, its goal is to obtain higher blood volume than the 200–300 mL seen in O_3-AHT.[5]

Other modalities of therapies include direct injection *via* the intramuscular, intradiscal, and paravertebral site of administration. Rectal insufflation of O_2-O_3 is another common site of administration. However, insufflation of the nasal, tubal, oral, vaginal, vesical, pleural, and peritoneal cavities have proven to be prudent routes of administration. Cutaneous exposure has also had likely outcomes and can be achieved by sealing the portion of the body in a chamber or bag and insufflating with O_2-O_3 mixture. Saline with O_2-O_3 dissolved is used to avoid the risk of embolism when administered intravenously.[5]

Mechanism of Action

Antioxidant capacity

Upon beginning O_3 therapy, a multifaceted endogenous cascade is initiated and releases biologically active substrates in response to the transient, and moderate, oxidative stress that O_3 induces. O_3 can cause this mild oxidative stress because of its ability to dissolve in the aqueous component of plasma.[8] By reacting with polyunsaturated fatty acids (PUFA) and water, O_3 creates hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS). Simultaneously, O_3 forms a mixture of lipid ozonation products (LOP).[9] The LOPs created after O_3 exposure include lipoperoxyl radicals, hydroperoxides, malonyldialdeyde, isoprostanes, the ozonide and alkenals, and 4-hydroxynonenal (4-HNE). Moderate oxidative stress caused by O_3 increases activation of the transcriptional factor mediating nuclear factor-erythroid 2-related factor 2 (Nrf2). Nrf2's domain is responsible for activating the transcription of antioxidant response elements (ARE). Upon induction of ARE transcription, an assortment of antioxidant enzymes gains increased concentration levels in response to the transient oxidative stress of O_3. The antioxidants created include, but are not limited to, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), catalase (CAT), heme oxygenase-1 (HO-1), NADPH-quinone-oxidoreductase (NQO-1), heat shock proteins (HSP), and phase II enzymes of drug metabolism. Many of these enzymes act as free radical scavengers clinically relevant to a wide variety of diseases.[9]

O_3, as well as other medical gases, *e.g.*, carbon monoxide (CO) and nitric oxide (NO), has twofold effects depending on the amount given and the cell's redox status. There is a complex relationship between these three medical gases as O_3 overexpresses HO-1, also referred to as HSPs of 32 kPa (Hsp32),[10] the enzyme responsible for CO formation, and downregulates NO synthase, which generates NO. Furthermore, O_3 upregulates the expression levels of Hsp70 which, in turn, is strictly related to HO-1. O_3 may have a developing role in Hsp-based diagnosis and therapy of free radical-based diseases. HO-1 degrades heme, which can be toxic depending on the amount produced, into free iron, CO, and biliverdin (*i.e.*, precursor of bilirubin), a neutralizer of oxidative and nitrosative stress due to its ability

to interact with NO and reactive nitrogen species.[11,12] Recently, it is becoming clear the heat shock response (HSR) provides a cytoprotective state during inflammation, cancer, aging, and neurodegenerative disorders.[13] Given its extensive cytoprotective properties, the HSR is now a target for induction *via* pharmacological agents.[1] Hsp70 is involved in co- and post-translational folding, the quality control of misfolded proteins,[14] folding and assembly of *de novo* proteins into macromolecular complexes, as well as anti-aggregation, protein refolding, and degradation.[15] HO isoforms are acknowledged as dynamic sensors of cellular oxidative stress and regulators of redox homeostasis throughout the phylogenetic spectrum. The effect of O_3 on these cell activities remains to be evaluated. Hormesis is a potent, endogenous defense mechanism for lethal ischemic and oxidative insults to multiple organ systems.[13] O_3 may have a hormetic role in regulating the anti-inflammatory and proinflammatory effects of CO, including prostaglandin formation akin to NO, which has been shown to exert some of its biological actions through the modulation of prostaglandin endoperoxide synthase activity.[16] Inhibiting HO activity prevents CO biosynthesis and its downstream effects[17]; the effect of O_3 on this cascade is yet to be determined.

Animal models have postulated the beneficial effects of prophylactic O_3 therapy in controlling the age-related effects of oxidative stress.[18,19] Evidence was provided to show that low O_3 dose administration provided beneficial effects on age-related alterations in the heart and hippocampus of rats. Additional research has been performed and provided room for speculation that O_3 therapy may provide the mediation of a mechanism involved in rebalancing the dysregulated redox state that accumulates as individuals age.[20] There was an apparent reduction of lipid and protein oxidation markers, lessening of lipofuscin deposition, restoration of glutathione (GSH) levels, and normalization of GPx activity in aged heart tissue. O_3 was demonstrated to decrease age-associated energy failure in the heart and hippocampus of rats. Researchers suspect that the improved cardiac cytosolic calcium and restoration of weakened Na^+-K^+ ATPase activity in the heart and hippocampus, respectively, were associated with the improvements seen.[20]

In hopes of attaining a sense of the possible toxic components of O_3 therapy, a study was done to assess the extent of lesions on human hematic mononucleated cells (HHMC), human thymic epithelium, murine macrophages, mouse splenocytes, and B16 melanoma murine cells. A significant finding of the study was that Hsp70 exhibited an O_3-induced increase in biosynthesis in HHMC. Hsp70s are synthesized in response to thermal shock and other stressing agents to cope with the damage that stimulates their biosynthesis.[21] Additionally, they stimulate several immune system responses in lymphocytes and macrophages. The study provided evidence that O_3 is a stressing agent capable of upregulating the biosynthesis of Hsp70, without toxicity to membranes. However, the membranes of macrophages are highly resistant to the possible toxicity of O_3 at high concentrations; HHMC is less resistant at the high end of the spectrum. The statement above should not discount the effectiveness of O_3 as a therapy because Hsp70s are induced in HHMCs without lesions up to 20 μg/mL— a typical dose given in O_3-AHT.[21]

Cisplatin (CDDP), a treatment used in a variety of cancers has been observed to have nephrotoxicity in 25% of the patients as a side effect. The occurrence of this nephrotoxicity is thought to be secondary to the free radical generation and the inability of ROS scavengers to ameliorate these molecules, leading to acute renal failure. O_2-O_3 therapy was used to increase the antioxidant capacity of rats exposed to CDDP and compared to control groups. Serum creatine levels were significantly reduced compared to control groups, illustrating the decreased nephrotoxicity indirectly in the rats with CDDP and O_2-O_3 therapy. In addition to attenuating the nephrotoxicity, O_2-O_3 therapy also restores the levels of antioxidant defense constituents (GSH, SOD, CAT, and GSH-Px), which are usually decreased by CDDP. Also, thiobarbituric acid reactive substances (TBARS) were reduced, which is a marker of lipid peroxidation in the kidney.[22,23]

Additional human studies examined the beneficial effects of O_3 therapy employed *via* O_3-AHT, in conjunction with coenzyme Q10, administered orally. The study evaluated SOD levels, a powerful antioxidant and catalase enzyme, an additional antioxidant enzyme in a control group, a group of O_3 therapy by itself, and O_3 therapy combined with Q10. Evidence has implied that SOD was significantly increased and catalase enzyme insignificantly increased in the O_3 + Q10 group when compared to the control group. Malondialdehyde, a product of lipid peroxidation, is an indicator of oxidative membrane damage. Malondialdehyde levels were significantly decreased concentrations in the O_3 + Q10 group when compared to the control group. Taken together, this study provides evidence of the beneficial effects of O_3 therapy in combination with Q10 in combatting and the prevention of damage elicited by oxidation.[9]

Multiple studies have provided evidence that O_3 therapy increased activation of the Nrf2 pathway *via* the induction of moderate oxidative stress.[15,24] By doing so, a transient increase in H_2O_2 and LOPs enhances the number of antioxidants and therefore can be used for a longer time frame to re-establish the balance of the redox system. Additionally, the creation of these antioxidant enzymes has effects, not only at the level of O_3 radical metabolism, but on the

whole body.[22,23]

Researchers have argued that knowing the total antioxidant status and plasma protein thiol group levels of a blood sample are indicators of the precise amount of O_3 required to optimize treatments. By developing more accurate antioxidant status indicators, an individual treatment would achieve the correct dosage on a day and case basis.[7,23,25] Systems have been proposed to have a more precise measurement of the redox state of a patient to achieve this goal. One system proposes simultaneously measuring different biological markers in the blood such as GSH, GPx, GST, SOD, CAT, conjugated dienes, total hydroperoxides, and TBARS. Using an algorithm, information can be gathered about the total antioxidant activity, total pro-oxidant activity, redox index, and grade of oxidative stress. Systems like this can provide insights to the correct dosage and response to O_3 therapy based on oxidative stress levels seen in the patient.[7,23,25]

Vascular and hematological modulation

O_3 is a stimulator of the transmembrane flow of O_2. The increase in O_2 levels inside the cell secondary to O_3 therapy makes the mitochondrial respiratory chain more efficient.[26] In red blood cells, O_3-AHT may increase the activity of phosphofructokinase, increasing the rate of glycolysis. By enhancing the glycolytic rate, there is an increase in ATP and 2,3-diphosphoglycerate (2,3-DPG) in the cell. Subsequently, due to the Bohr effect, there is a rightward shift in the oxyhemoglobin dissociation curve allowing for the oxygen bound hemoglobin to be unloaded more readily to ischemic tissues. Combined with the increase in NO synthase activity, there is a marked increase in perfusion to the area under stimulation by O_3-AHT.[27] With repeated treatment, sufficient enough LOP may be generated to reach the bone marrow acting as repeated stressors to simulate erythrogenesis and the upregulation of antioxidant enzyme upregulation. O_3 also causes a reduction in nicotinamide adenine dinucleotide (NADH) and assists in the oxidation of cytochrome c.[1,28]

O_3 has also been shown to improve blood circulation and oxygen delivery to ischemic tissues.[29] Multiple studies have provided evidence that the correction of chronic oxidative stress *via* the increase of antioxidant enzymes in O_3 can increase erythroblast differentiation. This leads to a progressive increase in erythrocytes and preconditions them to having resilience towards oxidative stress. This is known as "oxidative preconditioning".[1,30] Also, O_3 increases levels of prostacyclin, a known vasodilator.[1]

Additionally, it was speculated that O_3's oxidative capabilities would interfere with the endothelial production of NO and thus hinder vasodilation. However, studies have provided evidence that because NO is not substantially transported in the vasculature of the blood, a deleterious interaction is unlikely.[29] Since HO-derived bilirubin[31] has been demonstrated to interact with NO,[11,12] O_3-induced HO upregulation could modify NO production and alter vasodilation.

Unpredictably, studies have shown an increase of NO, which led to speculation of O_3's ability to activate genes associated with NO synthase expression to further promote higher levels of NO formation. Moreover, O_3's stimulation of antioxidant enzymes are also speculated to increase NO levels. While endothelial generation of superoxide disrupts the activity of NO, O_3 upregulates the enzymes to ameliorate the downstream effects of ROS responsible for deleterious vasoconstriction.[29,32]

The prophylactic role of O_3 has been explored with hepatic ischemia/reperfusion (I/R) injury, a phenomenon associated with liver transplantation. Hepatic I/R is a clinically unsolved problem mainly due to the unknown mechanisms that are the foundations of this ailment. In summary, O_3 oxidative preconditionings (ozoneOPs) were found to protect against liver I/R injury through mechanisms that promote a regulation of endogenous NO concentrations and the maintenance of an adequate cellular redox balance. OzoneOPs are postulated to upregulate endogenous antioxidant systems and generate an increase in NO molecule generation, both of which are protective orders against liver and pancreas damage. The results in this animal model provided evidence that ozoneOPs protected against liver I/R *via* an increase in concentrations of endogenous NO and prime cells to have a more balanced redox system.[32] Additionally, enhanced activation of adenosine A_1 receptors in rat models have been observed with ozoneOPs in liver I/R.[33]

Further studies have expanded upon this postulation by applying O_3 therapy to renal I/R in rats. Renal I/R is a primary cause of acute renal failure after transplantation surgery. The findings of a study by Orakdogen et al.[34] indicated that the ozoneOPs allowed for a protective element when facing I/R. Following an increase in endothelial NO synthase and inducible NO synthase expression, it was concluded that ozoneOPs were intimately related to the increasing NO production as well as reducing renal damage by suppressing endothelin 1.[34]

Cerebral vasospasm after subarachnoid hemorrhage is a significant detriment to the recovery of patients. An animal model examined the effects intravenous O_3 therapy on vasospasms in the rat femoral artery. Histopathological and morphometric measurements provided evidence that O_3 therapy decreased morphometric changes, disruption of endothelial cells, and hemorrhages that are a result of

vasospasm. The study speculated the anti-oxidative and anti-inflammatory effects of O_3 might be a prudent treatment for posthemorrhagic vasospasm.[35]

Pathogen inactivation

When bacteria are exposed to O_3 *in vitro*, the phospholipids, and lipoproteins that are within the bacterial cell envelope are oxidized. As this occurs, the stability of the bacterial cell envelope is attenuated. Moreover, evidence has demonstrated O_3 to interact with fungal cell walls like bacteria. This disrupts the integrity of the cytosolic membrane and infiltrates the microorganisms to oxidize glycoproteins, glycolipids, and block enzymatic function. The combination of these reactions causes inhibition of fungi growth and mortality of bacteria and fungi.[1,3,5] *In vitro*, O_3 has been shown to interfere with virus-to-cell contact in lipid-enveloped viruses *via* oxidation of lipoproteins, proteins, and glycoproteins, thus interfering with the viral reproductive cycles.[1,3,36]

Specifically, animal models have shown that O_3 therapy as an adjunct to vancomycin enhances the animal's capability to eliminate methicillin-resistant *Staphylococcus aureus* mediastinitis.[37]

Immune system activation

In vivo, O_3 therapy has been shown to have multifaceted effects when interacting with PUFA. As stated previously, O_3 reacts with PUFA and other antioxidants, H_2O_2 and varies peroxidation compounds are formed. H_2O_2 readily diffuses into immune cells has been shown to act as a regulatory step in signal transduction and facilitating a myriad of immune responses.[36,38] Specifically, increases in interferon, tumor necrosis factor, and interleukin (IL)-2 are seen. The increases with IL-2 are known to initiate immune response mechanisms.[1] Additionally, H_2O_2 activates nuclear factor-kappa B (NF-κB) and transforming growth factor beta (TGF-β), which increase immunoactive cytokine release and upregulate tissue remodeling. H_2O_2 mediates the action of NF-κB by enhancing the activity of tyrosine kinases that will phosphorylate IκB, a subunit of the transcription factor NF-κB.[34,37] Low doses of O_3 have been shown to inhibit prostaglandin synthesis, release bradykinin, and increase secretions of macrophages and leukocytes.[34] Having the correct amount of either of these oxidative markers can be used to create a sufficient rise in H_2O_2 and NO levels to stimulate the most notable increase in IL-8. IL-8 also activates NF-κB, allowing production of ROS scavengers.[7]

Animal models using O_3 have shown to reduce and prevent inflammatory responses steming from the presence of *E. coli* in the renal system.[26,38] Additional studies have provided evidence of the anti-inflammatory effects of O_3. A study by Chang et al.[25] purified rheumatoid arthritis synovial fibroblast cells from human patients and injected them into immunocompromised mouse joints. Using an Ozonsan-α generator to deliver precise gas flows to vessels in the localized area, the authors discovered that 3% and 5% O_3 application significantly decreased the proinflammatory cytokines IL-1β, IL-6, and TNF-α without any toxicity or severe side effects.[25]

Studies have shown that human cancer cells from lung, breast, and uterine tumors are inhibited in a dose-dependent manner by O_3 therapy *in vitro*. O_3 concentrations of 0.3 and 0.5 ppm inhibited cancer cell growth by 40% and 60%, respectively. Furthermore, the noncancerous cell controls were not affected by these levels of O_3. At 0.8 ppm, cancer cell growth was inhibited by more than 90%. However, the control cell growth was less than 50%. Additionally, as control cells aged, they exhibited further growth inhibition and morphological changes. The study speculated that as the healthy cells matured, there was a decrease in growth due to the increased cellular damage incurred by each division.[39]

Clinical Utility

With its ever-growing ubiquity, O_3 therapy is finding a place in many branches of medicine and medical specialties. In fact, its clinical use can be arranged systematically into cardiovascular (**Additional Table 1**), subcutaneous tissue (**Additional Table 2**), peripheral vascular disease (**Additional Table 3**), neurological (**Additional Table 4**), head and neck (**Additional Table 5**), orthopedic (**Additional Table 6**), gastrointestinal (**Additional Table 7**), and genitourinary (**Additional Table 8**). These indications are a product of human clinical trials conducted for specific pathologies related to the aforementioned systems. Despite a lack of direct support of O_3 therapy, the current Food and Drug Administration regulations do not restrict the use of it in situations where it has proven its safety and effectiveness. Nonetheless, there has been support for its safety and effectiveness in multi-international studies.

Conclusions

O_3 therapy can alter the natural history of several disease and disorders, with potentially many more yet untested. A plethora of laboratory studies have provided evidence of O_3's antioxidant capabilities, as well as vascular, hematological, and immune system modulations. This evidence has been further substantiated in clinical trials with O_3 therapy being useful in the cardiovascular, subcutaneous tissue, peripheral vascular disease, neurological, head and neck, orthopedic, gastrointestinal, and genitourinary pathologies. O_3 therapy has proven especially beneficial in the diabetic foot, ischemic wounds, and peripheral vascular disease, areas in which O_3

use is most prevalent. Upcoming laboratory and translational research should begin to develop protocols for O_3-AHT in attempts to establish a dose-response relationship as it has demonstrated high utility in a myriad of pathologies at varying concentrations. Despite the presently compelling evidence, future studies should include more double-blind, randomized clinical trials with greater sample sizes, determination of longevity in benefits produced, as well as methods of measurements and analysis.

Acknowledgments
The authors are thankful to Drs. Kelly Warren, Inefta Reid, Todd Miller, and Peter Brink (Department of Physiology and Biophysics, Stony Brook University School of Medicine, Stony Brook, NY, USA) for departmental support, as well as Mrs. Wendy Isser and Ms. Grace Garey (Northport VA Medical Center Library, Northport, NY, USA) for literature retrieval.

Author contributions
NLS designed, organized, and wrote the article; ALW designed the outline, wrote the article, and solved queries related to scientific publications from the journals; JG performed literature searches, critiqued the literature findings, and wrote the article; SV critiqued and applied logical reasoning to the literature findings; SAK applied clinical concepts, revised the article to add logical reasoning, and cross-checked the referencing. All authors have read and approved the manuscript provided.

Conflicts of interest
The authors have no conflicts of interest to declare.

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Additional files
Additional Table 1: Cardiovascular indications for O_3 therapy.
Additional Table 2: Subcutaneous tissue indications for O_3 therapy.
Additional Table 3: Peripheral vascular disease indications for O_3 therapy.
Additional Table 4: Neurological indications for O_3 therapy.
Additional Table 5: Head and neck indications for O_3 therapy.
Additional Table 6: Orthopedic indications for O_3 therapy.
Additional Table 7: Gastrointestinal indications for O_3 therapy.
Additional Table 8: Genitourinary indications for O_3 therapy.

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Additional Table 1: Cardiovascular indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Martínez-Sánchez et al.[14]	Coronary artery disease	57 patients with massive cerebral infarction	Ungrouped; cocktail therapy: nimodipine (10 mg) intravenously, once per day, for 10 consecutive days	Prothrombin time	Significantly improved ($P < 0.001$)	None	Upregulation of adenosine A_2 receptor
				Oxidative stress levels	Increased antioxidant activity	None	Increased SOD and catalase enzyme activity
Hernandez et al.[40]	Previous myocardial infarction (3 months to 1 year)	200 mL of blood subjected to O_3-AHT, for a final concentration of 50 mg/L; treatment was given 5 days a week for up to 15 sessions	Pretest-posttest design (n = 22)	Serum lipid pattern	Cholesterol and low-density lipoprotein were significantly reduced with no changes in high-density lipoprotein and triglycerides	Not reported	Initiating radical formation which increasing lipid peroxidation
				Activity of antioxidant defense system	Biologically significant increases on erythrocyte GPx and glucose-6-phosphate dehydrogenase	Not reported	O_3-AHT stimulates ROS scavenger enzymes

Note: O_3: Ozone; O_2: oxygen; O_3-AHT: O_3 autohemotransfusion; GPx: glutathione peroxidase; SOD: superoxide dismutase; ROS: reactive oxidative species.

Additional Table 2: Subcutaneous tissue indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Wainstein et al.[41]	Diabetic foot ulcer	A noninvasive sealed chamber was used in two phases. Phase 1 delivered 96% O_2 and 4% O_3 (80 μg/mL) for up to 4 times a week for 4 weeks. Phase II delivered 98% O_2 and 2% O_3 (40 μg/mL) until the 12th week	Double-blind, randomized, placebo-controlled clinical trial (n = 61)	Wound closure	Of the patients completing per protocol, wound closure was significantly greater than controls (P = 0.03), expressly in patients with small ulcers initially (≤ 5 cm^2)	Control group (n = 2) O_3 group (n = 5); none of the adverse events were linked causally with the O_3 treatment used	Induced negative pressure by the device may enhance fluid removal and increase perfusion; O_3 bactericidal capabilities and a reduction of blood viscosity improves perfusion
Martinez-Sanchez et al.[42]	Diabetic foot ulcer	20 sessions of O_3 *via* rectal insufflation (50 mg/L) and local treatment (60 mg/L) *via* sealed bag with O_3	Randomized controlled clinical trial (n = 101)	Wound size	Significant decrease in area and perimeter	None	Activation of SOD, control of hyperglycemia, and decreased endothelial damage
				Glucose levels	Reduced hyperglycemia (P < 0.05)	None	Increased antioxidant properties allowing for increase in insulin sensitivity, facilitating increased glucose uptake
				Oxidative stress	Increased antioxidant enzyme defense	None	Increased SOD and catalase enzymes and activation of NF-κB *via* normalizing levels of H_2O_2
Elvis et al.[1]; Bertolotti et al.[43]; Moore et al.[44]	Buruli ulcer (*Mycobacterium ulcerans*)	Insufflation of a sealed bag with an O_3-O_2 mixture with an O_3 concentration of 30 μg/mL	Case study (n = 1)	Wound closure	No visible necrosis (with granulations) after the first week; ulcer was eventually eradicated (without granulations)	None	Oxidizes phospholipids and lipoproteins on the bacteria's cell envelope, thus attenuating its integrity, changing the permeability of the membrane. Lysis and cell death ensues
				Histological and PCR analysis	Absence of *M. ulcerans*		
Shah et al.[45]	Non-healing or ischemic wounds	Insufflation of a sealed bag O_3-O_2 (70 μg) mixture in conjunction with O_3-AHT (50 mL of blood with an O_3 concentration of 70 μg)	Case study (n = 1)	Regression of necrotic tissue	On the 5th day of treatment, necrosis regressed enough for surgeons to perform surgery, implementing a biological cover over the location of the previous non-healing wound	None	Attenuates bacterial cell walls *via* oxidation; stimulates formation of LOP, which acts on endothelium to release prostacyclin, IL-8 and NO, to increase vasodilation; ROS causes the release of TGF-β, IL-8, and PDGF *via* platelet aggregation to stimulate wound healing; O_3-AHT increases O_2 delivery and increase antioxidant enzymes to help reperfusion and avoid excessive inflammation

Note: O_3: Ozone; O_2: oxygen; SOD: superoxide dismutase; LOP: lipid ozonation products; IL-8: interleukin-8; NO: nitric oxide; ROS: reactive oxidative species; TGF-β: transforming growth factor beta; PDGF: platelet-derived growth factor; NF-κB: nuclear factor-kappa B; H_2O_2: hydrogen peroxide; O_3-AHT: O_3 autohemotransfusion.

Additional Table 3: Peripheral vascular disease indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Tafil-Klawe et al.[46]; Romero Valdes et al.[47]	Obliterative atheromatosis (without diabetes)	Normal saline with dissolved O_3 intravenously (500 mL with an O_3 60 µg/mL) and aerosol O_3 baths of lower extremities (O_3 concentration 19 µg/L)	Pretest-posttest design (n = 64)	Lysosomal hydrolase activity	Lysosomal hydrolase activity returned to within normal limits	None reported	Improvement of blood supply to hypoxic areas to increase oxygen inflow *via* increases in 2,3-DPG. Immune cells have increased access to damaged tissue. Increased access allows for lysosomal enzymes to digest damaged cells. Increased antioxidant levels change the activity of lysosomal enzymes
				General condition	Patients general condition improved		
Verrazzo et al.[48]	Peripheral occlusive arterial disease	O_3-AHT (32 µg/mL) every other day compared to HBOT	Randomly controlled trial (n = 30)	Blood viscosity	Decrease in blood viscosity was present in O_3-AHT treatments compared to HBOT	None reported	Increase in plasma malonydialdehyde levels supports that O_3-derived free radicals increase. These are hypothesized to be selective for more rigid hematic cells, causing cell lysis. Selectively improving blood viscosity and filterability without decreasing Hct. Changes in fibrinogen and thrombin are seen to be transient effects of O_3-AHT
				Hct	Unchanged		
				Erythrocyte filterability	Increased in O_3-AHT treatments compared to HBOT		
Giunta et al.[49]	Peripheral occlusive arterial disease	O_3-AHT (100 mL exposed to O_3 for 10 minutes)	Pretest-posttest design (n = 27)	Blood viscosity	Blood viscosity decreased	None reported	Increases oxidative stress and lipid peroxidation, contributing to selective cellular lysis of rigid erythrocytes. Additionally, lipid peroxidation of erythrocyte membranes alters pH, increasing oxygen unloading
				Oxygen delivery	Increase in oxygen delivery		
				Erythrocyte filterability	Erythrocyte filterability increased		
				Hct	No significant change		
				Fibrinogen levels	Plasma fibrinogen levels decreased		
Di Paolo et al.[50,51]	Peripheral artery disease	Extracorporeal blood oxygenation and ozonation (O_3 concentrations 40–100 µg/mL)	Randomly controlled study (n = 28)	Skin lesions, pain, improvement in quality of life	Significant regression of skin lesions, decreased pain, and increases sense of well-being	None	Stimulates cytokine secretion of leukocytes to digest cellular derbis build up and allows vasodilation *via* NO

Note: O_3: Ozone; O_2: oxygen; SOD: superoxide dismutase; LOP: lipid ozonation products; IL-8: interleukin-8; NO: nitric oxide; ROS: reactive oxidative species; TGF-β: transforming growth factor beta; PDGF: platelet-derived growth factor; NF-κB: nuclear factor-kappa B; H_2O_2: hydrogen peroxide; O_3-AHT: O_3 autohemotransfusion; HBOT: hyperbaric oxygen therapy; Hct: hematocrit.

Additional Table 4: Neurological indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Zanardi et al.[2]; Molinari et al.[52,53]; Lintas et al.[54]	Multiple sclerosis	240 g blood mixed with 180 mL O_2/O_3 (O_3 at 40 μg/mL) and re-injected; O_3-AHT	Pretest-posttest design (multiple case studies) ($n = 9$)	Cerebral oxygenation *via* near-infrared spectroscopy system and cyt c levels	Increased cyt c levels and oxygenation levels and increase in brain metabolism	None reported	O_3-AHT decreases oxidative stress *in vivo*, lowering mitochondrial damage and inflammation to reverse the impairments on cyt c seen in afflicted patients
Leon Fernandez et al.[30]; Clavo et al.[55]	Refractory headache	O_3-AHT (220–300 mL at a concentration between 30–60 μg/mL)	Case-control design ($n = 5$)	Number of headaches Pain intensity on the visual analog scale	Significantly decreased unchanged Significantly reduced	Ecchymosis at the site of injection	Induces regulation of cerebral blood flow and oxygen delivery to ischemic tissues, in part due to the increase 2,3-DPG in erythrocytes and release of NO by the endothelium, fostering a regulation of metabolism. Upregulation of cytokines from lymphocytes and increased antioxidant enzymes balance oxidation levels. O_3's enhancement of adenosine A_1 receptors provides evidence for its ability to act as a self-regulator of cortical electrical activity and neurotransmitters *via* reduction of glutamate release
Valacchi et al.[23]; Ajamieh et al.[32]; Clavo et al.[56,57]	Radiation-induced brain ischemia	O_3-AHT (300 mL at a concentration of 60 μg/mL of O_3/O_2)	Case-control design ($n = 7$) and case report ($n = 1$)	Cerebral blood flow	Improved after treatment	None reported	Induces ROS and LOP to stimulate NO, IL-8 release while inhibiting ET-1 and E-selectin, which could potentially improve cerebral blood flow. May also improve erythrocyte flexibility and blood rheology

Note: O_3: Ozone; O_2: oxygen; O_3-AHT: O_3 autohemotransfusion; cyt c: cytochrome-c: ROS: reactive oxidative species; LOP: lipid ozonation products; 2,3-DPG: 2,3-diphosphoglycerate; NO: nitric oxide; IL-8: interleukin-8; ET-1: endothelin 1.

Additional Table 5: Head and neck indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Bocci et al.[58]; Ragab et al.[59]	Sensorineural hearing loss	O_3-AHT (100 mL of blood with a 1:1 gaseous mixture O_2-O_3)	Randomized controlled trial (n = 45)	Multiple methods assessing hearing outcomes (mean hearing gain, PTA, SRT, and subjective recovery rates)	All improved significantly with O_3 compared to placebo	None	Multifaceted stimulation of cellular metabolism and increase of erythrocyte activity, which increases 2,3-DPG, may attenuate cellular stress. Shift in the oxyhemoglobin dissociation curve and an increase NO allows for increase oxygen supply to tissues of hypoxia in the inner ear
Clavo et al.[60]	Head and neck tumors	O_3-AHT (60 µg/mL) and rectal insufflation (60 µg/mL)	Controlled case study (n = 19)	Patient outcome	No significant difference in overall survival between O_3 and traditional treatment	Transient meteorism and constipation	Increased production of 2,3-DPG in RBCs *via* increase of malondialdehyde and lipid peroxidation, allowing for a shift in the oxyhemoglobin dissociation curve to increase unloading of O_2 to tissues. Changes in RBC cell membranes *via* addition/removal of charges allows for increased membrane flexibility and decreased blood viscosity. Thus, with an added tissue perfusion, increased oxygenation, and increased antioxidant levels, O_3 is suspected to be a pivotal adjunct therapy
Clavo et al.[60,61]		O_3-AHT (60 µg/mL)	Controlled case study (n = 14)	Levels of oxygenation (hypoxic values, tumor pO_2, and [Hb])	All improved with O_3 therapy	None	
Menéndez et al.[62]	Vestibulocochlear syndrome	Paravertebral O_3 injection at C2-3 vertebrae (8 mg/L, flow of 60 mL/min)	Pretest-posttest design (n = 50)	Tinnitus	Improved by 65%	None reported	Increases in SOD, GSH, GPx, and CAT levels, while observing low lipid peroxidation provides evidence that O_3 helps balance cellular redox. The cellular redox balance may improve symptoms of these syndromes
				O_2 delivery	Increase in O_2 delivery		
				Nystagmus	Improved by 100%		
				Vertigo	Improved by 90%		
				Hearing loss	Improved by 80%		
Borrelli et al.[63]	Dry form of AMD	O_3-AHT (200 mL of blood with a total O_3 does equivalent to 4.0 mg)	Two clinical studies (n = 217)	Progression of disease Visual acuity	Stops progression Significantly improved	None	Improves blood rheology, glycolytic metabolism in RBCs that can increase O_2 delivery *via* increased ATP and 2,3-DPG, increase NO and vasodilation, release growth factors, and have an increase of antioxidant enzymes that can minimize the death of photoreceptors seen in dry AMD

Note: O_3: Ozone; O_2: oxygen; O_3-AHT: O_3 autohemotransfusion; AMD: age-related macular degeneration; 2,3-DPG: 2,3-diphosphoglycerate; RBC: red blood cell; SOD: superoxide dismutase; GPx: glutathione peroxidase; GSH: glutathione; PTA: pure-tone average; SRT: speech reception threshold; CAT: catalase; NO: nitric oxide; pO_2: partial pressure of oxygen; Hb: hemoglobin.

Additional Table 6: Orthopedic indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Steppan et al.[64]; Paoloni et al.[65]; Oder et al.[66]; Magalhaes et al.[67]	Herniated lumbar discs	Intradiscal and extradiscal injection (1–3 mL O_2/O_3)	Meta-analysis (n = 12)	Meta-analysis for pain levels (visual analog scale)	Significant mean improvement of 3.9	Significantly low complication rate (0.064%)	Redox capabilities allow proteoglycans in the nucleus pulposus to be oxidized, leading to a small decrease in volume of the nucleus pulposus. Decreased volume decreases pressure and attenuates pain. O_3's anti-inflammatory effects due to the redox properties are also speculated to have analgesic effects. O_3's disinfectant properties are beneficial when using intra- and extradiscal injections because it lessens the risk of infection
				Meta-analysis for functionality (ODI)	Significant mean improvement of 25.7		
				Meta-analysis for functionality (modified MacNab)	Likelihood of showing improvement was 79.7%		
Al-Jaziri et al.[68]	Spine and joint osteoarthritis	Intra-articular and paravertebral muscle injections (20 µg/mL)	Prospective study (n = 220)	Pain level after 4, 8, and 12 sessions	Significantly decrease (P = 0.005, P = 0.005, P = 0.0043, respectively)	None	Ability to activate enzymes catalyzing peroxide reactions allowing for protection against ROS and peroxides. O_3's anti-inflammatory, analgesic effects, and anti-oxidative effects, taken together with the significantly decreased pain levels long-term, allows for speculation on possible histological changes after using O_3 therapy
				Follow-up pain levels (mean follow-up time is ~10 months)	Significantly decrease (P = 0.0048)		
Bonetti et al.[69]	First degree spondylolisthesis and spondylolysis	CT-guided bilateral periganglionic infiltration of O_2-O_3 and O_2-O_3 injection into lysis point of neural arch ¾ mL O_2-O_3 gas mixture at 25 µg/mL)	Prospective study (n = 18)	Pain levels after treatments using modified MacNab	15 patients (83.3%) had complete remission of pain. 3 patients (16.7%) had poor levels of improvement	None	By injection, the gas mixture directly proximal to the lysis points allows for analgesic and anti-inflammatory actions on the meningeal branches of a spinal nerve. Also, prostaglandin and cytokine levels are balanced because of O_3's ability to increase SOD production and to reduce ROS. Local improvement in circulation after treatment allows for increased eutrophic delivery
				Pain levels at 1-month follow-up using modified MacNab	15 patients (83.3%) had complete remission of pain. 3 patients (16.7%) had poor levels of improvement		
				Pain levels at 3-month follow up using modified MacNab	13 patients (72.2%) had complete remission of pain. 2 patients (11.1%) had satisfactory levels of improvement of pain. 3 (16.7%) patients had poor levels of improvement		
				Pain levels at 3-month follow up using modified MacNab	13 patients (72.2%) had complete remission of pain. 2 patients (11.1%) had satisfactory levels of improvement of pain. 3 patients (16.7%) had poor levels of improvement		

Note: O_3: Ozone; O_2: oxygen; ODI: Oswestry Disability Index; CT: computed tomography; ROS: reactive oxidative species; SOD: superoxide dismutase.

Additional Table 7: Gastrointestinal indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Zanardi et al.[2]; Bocci et al.[5,27,70]; Zaky et al.[71]	Chronic hepatitis C	O_3-AHT (150 mL with a concentration of 25% O_2/O_3 raised by 5% every week for 5 weeks) and rectal O_3 insufflation (300 mL at 40% O_3/O_2	Case-control design (n = 52)	Presenting symptom progression (7 clinical symptoms assessed)	Significantly improved symptoms	None reported	Uses peroxidation to damage the viral capsid and disrupts the reproductive cycle of viruses by dismantling virus-to-cell contact. Formation of peroxides from O_3 stimulates the release of leukocytes and cytokines. Decreased viral load fosters liver enzymes replenishment and improved liver function
				ALT and AST	Normalized significantly more than conventional therapy		
				PCR analysis for HCV RNA	Disappearance of HCV RNA in 25% of O_3-AHT patients after 30 sessions and 44.4% after 60 sessions		
Zaky et al.[72]	Liver cirrhosis	Rectal O_3 insufflation (12 sessions, 300 mL at 40% O_3) as an adjunct to propranolol	Case-control design (n = 15)	Propranolol clearance	Increased elimination of propranolol	None reported	Propranolol metabolism is carried out by an oxidative enzyme in the CYP family, which is contingent on oxygenation. Increased portal vein oxygenation reported in the study would, therefore, optimize propranolol metabolism. This perfusion is forested by the release of mediators of NO
				Liver function tests		Significant reduction in prothrombin time	
				Portal vein oxygenation		Significantly increased after rectal insufflation of O_3	
Peretyagin et al.[74]	Gastrointestinal tract ulcers	O_3 therapy courses *via* intragastral, intravenous, biopuncture, cutaneous routes (200 mL at 3 mg/L of O_3)	Case-control design (n = 71)	Clinical symptoms (assessment of 6)	Significantly improved	In treatment group (n = 34), 1 participant had skin itch, 4 had sickness, 2 vomited and 5 had constipation. However, all of these were significantly lower than the control group	Decreases ischemia in developing ulcers and activates the immune response to increase recovery of persistent ulcers

Note: O_3: Ozone; O_2: oxygen; O_3-AHT: O_3 autohemotransfusion; CTCAE: common terminology for adverse events; AST: aspartate aminotransferase; ALT: alanine aminotransferase; HCV: hepatitis C virus; CYP: cytochrome P450; NO: nitric oxide.

Additional Table 8: Genitourinary indications for O_3 therapy

Study	Pathology	Concentration and route of O_3 administration	Type of study	Measured parameter(s)	Results	Side effect(s)	Mechanism of action
Neimark et al.[75]; Gu et al.[76]	Chronic cystitis	Ozonated saline (1,000 μg/L)	Controlled clinical trial (n = 65)	Laser Doppler flowmetry used to determine perfusion	Significantly increased, close to control levels	None reported	Microcirculation and structural reorganization of the bladder mucosa
				Cystoscopy with biopsy of the bladder mucosa	More positive shifts in hyperemia and edema then standard treatment alone		
				PRA	Significantly decreased		
				Ang II	Significantly decreased		
				ALD	Significantly decreased		
Gu et al.[76]; Clavo et al.[77]	Renal complications secondary to hepatitis	O_3-AHT (100 mL, 35 μg/mL)	Randomly controlled trial (n = 85)	Renal blood flow	Significantly increased with O_3 therapy compared to control	No obvious side effects were seen	Increased oxygen carrying and releasing capacity of Hb, can activate metabolism in RBCs, and improve microcirculation to the liver and kidney. O_3's activation of the immune and free radical removal systems can reduce the work load of the liver while improving immune response to viruses. By improving the oxygen and blood supply to the kidney, there is a decrease in PRA, Ang II, ALD caused by hepatitis, thus reducing renal damage
					Damage to renal function	Seen in lower proportion with O_3 therapy	
					Survival rate	Significantly higher proportion survived with O_3 treatment compared to control	
					Presence of hematuria	Post-1-week macroscopic hematuria disappeared. Post-8 weeks, microscopy showed about 10 RBCs/microscopic field. After 6 months, there was no evidence of macroscopic hematuria	
					Hb levels	After week 2, Hb concentration increased by 0.5 g/dL per week	
Clavo et al.[77]; Bonforte et al.[78]	Radiation-induced cystitis with hematuria	Intravesical instillation of ozonated water (35 μg/mL)	Case study (n = 1)	Cystoscopy	After week 3, significant improvement was seen	Soft bladder pruritus after initial sessions	Local and transient increase in oxidative stress causes an increase in synthesis of antioxidants, thus increase protection against free-radical tissue damage. O_3 can also increase local repair mechanisms, affecting hematological parameters and increasing tissue oxygenation
					Presence of bacteria causing UTI	Regression of bacteria and UTI symptoms	
Bonforte et al.[78]	UTI	Ozonated saline catheter injection into urinary bladder	Case series report (n = 3)	Presence of bacteria	Decreased presence of bacteria	None	Antiseptic ability *via* lipid peroxidation, DNA damage and cell death, in addition to its immune system stimulation may account for its ability to combat bacterial UTIs

Note: O_3: Ozone; O_3-AHT: O_3 autohemotransfusion; UTI: urinary tract infection; PRA: plasma renin activity; Ang II: angiotensin II; ALD: aldosterone; Hb: hemoglobin; RBC: red blood cell.