



Oxygen-Ozone (O₂-O₃) Therapy in Peripheral Arterial Disease (PAD): A Review Study

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Abstract: The purpose of this study is to review the current knowledge of oxygen-ozone (O₂-O₃) therapy and its effects on peripheral artery disease (PAD) risk factors, symptoms, as well as on PAD patients' quality of life. From the in vitro studies, it has been concluded that the oxygen-ozone therapy exerts a positive effect on the platelet aggregation, cell remodeling, cytoskeletal elements organization and mitochondria structure. In animal studies, it has been shown that the O₂-O₃ therapy is an effective method in hypertension, and it diminishes the hypoxia state of various tissues. Clinical studies have provided evidence on the oxygen-ozone therapy effectiveness in low perfusion syndromes and hyperglycemia, as well as conditions with oxidative stress and inflammation. The oxygen-ozone therapy promotes faster recovery and enhances healing processes. It appears to be an effective adjunctive therapy in preventing peripheral artery disease complications such as occurrence of cardiovascular event, amputation or other extreme surgical solutions. It has been concluded that the O₂-O₃ therapy improves the quality of life of PAD patients. The oxygen-ozone therapy appears to have no adverse events or side effects. Moreover, it is very cost-effective, as standard treatment costs can be reduced by 25%. Easy clinical protocols allow the implementation of oxygen-ozone therapy into the usual care of PAD patients. Finally, the O₂-O₃ therapy may be meaningful especially for older patients and patients who are not eligible for standard revascularization.

Keywords: amputation, diabetes mellitus, oxidative stress, wound healing

Introduction

A burden of peripheral arterial disease (PAD) prevalence has been observed over the last decade. Globally in 2010, about 202 million people were diagnosed with PAD.¹ PAD is now the third most common cause of death from cardiovascular diseases, while in the first two places, there are coronary heart diseases and stroke. Statistical data from 1990 and 2010 reveal that the prevalence of PAD greatly increased among men. On the other hand, the number of PAD-related deaths is much more frequent among women. Similarly, in 2010 women experienced a greater level of disability in comparison with 1990. PAD patients have numerous symptoms lowering their quality of life. Intermittent claudication concerns 10–20% of patients, while 50% of them have atypical leg symptoms.² Social and economic consequences of PAD need to be acknowledged. There is a great need for developing global prevention programs, as well as cost-effective treatments and management policies.

The oxygen-ozone (O₂-O₃) therapy has been proposed as one of the treatments that may bring health and quality of life-related benefits for patients with hypoxic

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syndromes and diabetes mellitus. The O₂-O₃ therapy has been evaluated as a method for wound disinfection as well as treatment of dental caries and diabetes mellitus.³⁻⁵ However, looking at the available literature, a need to review the evidence of the effectiveness of the ozone therapy on the peripheral artery disease appears necessary.

The purpose of this study is to summarize current knowledge of the oxygen-ozone therapy and its effects on peripheral artery disease risk factors, symptoms, as well as on PAD patients' quality of life. Prospective, controlled studies provide evidence that through altering risk factors, the peripheral artery disease development and a course of disease can be altered.⁶ In the following section PAD risk factors, that can be altered by a treatment, will be discussed. The medical importance of the oxygen-ozone therapy will be explained as well. The paper will go onto question if the O₂-O₃ therapy is an effective method of altering PAD risk factors will be addressed. At first, the evidence from in vitro studies will be reviewed, and then evidence coming from animal studies will be evaluated. Of the three original methods, it is the clinical trials that provide the greatest insight and will be discussed at length.

Methodology

Relevant studies have been identified through the search in the Medline database up to November 2018. Three types of studies have been in the area of interest: in vitro studies, animal studies, and randomized clinical trials. The intervention needed to include oxygen-ozone therapy in order to assess the effectiveness of the treatment, the O₂-O₃ therapy compared with sham therapy, the O₂-O₃ therapy compared with any other intervention or the O₂-O₃ therapy combined with conventional treatment compared with any other intervention. The primary outcomes needed to be concerned about PAD risk factors, ischemic damages, oxidative status, pain perception or healing processes. Secondary outcomes that were analyzed included a number of complications, amputation, quality of life, satisfaction from the treatment process.

Risk Factors for Peripheral Arterial Diseases

There is a strong correlation between PAD development and type II diabetes. Intermediate claudication occurs twice more frequently among diabetic patients than among people without diabetes mellitus.⁷ However, the largest risk factor is insulin resistance. It raises PAD development risk by

40–50%. This applies also to non-diabetic patients. The course of the peripheral arterial disease appears to be more aggressive among diabetic patients. Distal symmetrical neuropathy can occur even at the early stage of PAD. Major amputations are performed five to ten times more frequently among diabetic patients. Hypertension and dyslipidemia are the following risk factors correlated with PAD. The most accurate predictor of PAD development has been identified as a ratio of total to high-density lipoprotein.⁸ Higher blood level of triglycerides (TG), very low-density lipoproteins (VLDL) and intermediate-density lipoproteins (IDL), as well as lower level of high-density lipoproteins (HDL), has been shown to be associated with PAD. The evidence shows correlation not only with PAD incidence but also with the progression of disease, intermediate claudication occurrence and systemic complications of PAD. The occurrence of intermediate claudication is twice more frequent among patients with a fasting cholesterol level above 270 mg/dl. C-reactive protein (CRP) has been also shown to be associated with PAD.⁹ Having CRP in the highest quartile accounts for twice higher PAD development risk compared with the lowest quartile. Hyperviscosity and hypercoagulability are proven to be PAD risk factors. Among PAD patients, increased plasma level of fibrinogen and raised hematocrit level have been also reported. Hyperhomocysteinemia appears to be an independent risk factor for atherosclerosis. The prevalence of this condition among young PAD patients is as high as 30%.

Ozone Therapy

Ozone therapy has been studied for more than a century. Medical O₃, used to disinfect and treat disease, has been around for over 150 years and its effectiveness has been well documented. At the same time, opinions about this method are still divided. The uncertainty of ozone therapy might come from the toxicity of ozone (O₃) present in the atmosphere. Ozone produced by the breakage of pollutants and nitrous oxides by ultraviolet light is indeed irritant to the eyes and lungs. The exact toxicity level of ozone has not been established due to a great variance of individual sensitivity to the O₃ exposure.¹⁰ This variability can be attributed to age, body mass index, physical fitness, as well as health problems such as asthma. The National Ambient Air Quality Standard states a daily maximum of 8-hour ozone exposure at the level of 80 ppb. It has been also shown that a 10-ppb ozone raise in the previous week results in a 0.52% increase in daily mortality and

a 0.64% increase in cardiovascular and respiratory mortality.¹¹ However, most of the time, at the Earth's surface, ozone occurs at the concentration of 20 $\mu\text{g}/\text{m}^3$ and this level is completely safe for human beings. It is worth noting that what is dangerous for health is a repeated exposure to low doses of O_3 . Ozone toxicity does not apply to the controlled and proper administration of medical ozone.¹² Ozone therapy is based on high doses in order to stimulate the immunology system. Numerous studies have proven that ozone therapy effects are consistent and the therapy is safe and does not cause side effects. The ozone therapy mechanism of action is based on the formation of reactive oxygen species (ROS) and lipids oxidation products (LOP). Fortunately, ROS disappear quickly, as they are short-term acting particles. LOPs are distributed throughout the tissues; therefore, only a few molecules can act on the organs. Therefore instead of causing harm, these particles stimulate antioxidant defense, as well as modify the immune system.

The first use of ozone was mainly with the purpose of treating infections and wounds. Ozone therapy has been shown to be very effective in bacterial infection of skin.¹³ Ozonated oil eliminated almost 100% of *Staphylococcus aureus* in 5 minutes, as well as almost 100% of methicillin-resistant *Staphylococcus aureus* after 15 minutes. Ozone causes oxidation of phospholipids and lipoproteins of bacterial cell walls.¹² Thus, ozone therapy disrupts the integrity of bacterial cell walls. Similarly, ozone damages the viral capsid causing inactivation of viruses.¹⁴ Moreover, viral reproductive cycle is broken as peroxidation precludes the virus-to-cell contact.¹² Ozone has been also proven to cause irreversible damage to viral DNA.³ Ozone therapy used for fungal infections is based on the mechanism of ozone inhibition of cell growth at certain stages.¹²

The knowledge of medical use of oxygen has been broadly expanding since 1929, when a book titled "Ozone and Its Therapeutic Actions" was published. This publication covered more than hundred diseases that could be treated by the ozone therapy. Ozone's hemodynamic and anti-inflammatory properties are now well documented. Through proper administration (never via inhalation) of precise and high doses (in comparison to the environmental contamination), ozone brings benefits as it decreases blood cholesterol, improves glycemic index, as well as stimulates antioxidant defence.¹² The therapeutic dosage ranges from 10 to 80 $\mu\text{g}/\text{mL}$.¹⁵ It has been shown that the immune system is altered by the ozone. The

production of interferon is greatly stimulated by ozone therapy with the parameter of 30–55 $\mu\text{g}/\text{mL}$. The release of tumor necrosis factor and interleukin-2 is also stimulated by ozone. Moreover, the oxygen-ozone therapy is a successful treatment of hypoxic and ischemic syndromes through modulation of tissue oxygenation. This particular effect seems to be most important in relation to peripheral artery disease. A recently published review has also confirmed a mechanism of action through which ozone might be involved in recovering metabolism altered by chronic diseases such as cardiovascular disease.¹⁶

Evidence of Ozone Therapy Effectiveness in vitro Studies

The detailed results of the ozone therapy effectiveness are shown in Table 1. One of the studies concerning the in vitro effect of the ozone therapy tested platelet aggregation changes due to the ozone administration.¹⁷ The purpose of the other study was to identify structural and functional cell changes that occurred due to the oxygen-ozone therapy.¹⁸ The methodology of these two studies varied a lot, as the ozone concentration used to observe cell structure changes ranged from 1 $\mu\text{g O}_3/\text{mL}$ to 20 $\mu\text{g O}_3/\text{mL}$, while the concentration used to analyze the effect on platelet aggregation ranged from 20 $\mu\text{g O}_3/\text{mL}$ to 80 $\mu\text{g O}_3/\text{mL}$. However, the time of the $\text{O}_2\text{-O}_3$ therapy with the methodology of higher concentrations was much shorter (30 seconds) compared with the time of lower concentrations of ozone therapy (10 minutes). The presence of a control group in both of these studies allows the conclusion to be drawn that observed effects are actually due to ozone. It has been shown that the oxygen-ozone therapy directly causes an increase of PDGF (platelet-derived growth factor) and TGF- β 1 (transforming growth factor-beta 1). These two molecules play an important role in healing processes. The most suitable parameters of the $\text{O}_2\text{-O}_3$ therapy to exert the effect on PDGF and TGF β 1 molecules have been proposed as 40–80 $\mu\text{g O}_3/\text{mL}$. Stimulation of IL-8 (interleukin 8) release has been also attributed to the oxygen-ozone therapy; however, this effect required a longer time as a synthesis of IL-8 was firstly inhibited by ROS scavengers. Though, even high concentration ozone therapy did not manage to change the overall oxidative status of cells. It was also shown that the $\text{O}_2\text{-O}_3$ therapy does not affect cell proliferation as well as cell viability. The effect of ozone on the cell adhesion is dependent on the concentration. At the dose of 10 μg

Table 1 In vitro Studies Concerning Ozone Therapy

Author, Year	Aim of the Study	Ozone Therapy	Results		
Costanzo et al, 2015 ¹⁸	Investigation of the effects of low ozone concentrations on structural and functional cell features	Time: 10 min Concentration of O ₂ /O ₃ mixture: Gr1: 1 µg O ₃ /mL O ₂ Gr2: 10 µg O ₃ /mL O ₂ Gr3: 20 µg O ₃ /mL O ₂ Control group – yes: GrC: air Gr0: 100% O ₂	Cell viability and proliferation: no significant differences Cell adhesion: ↑ Gr1 vs GrC & Gr0 after 30 min and after 1 hour; after 2 h, 4 h, 6 h: no significant difference ↑ Gr2 vs GrC & Gr0 ↓ Gr3 vs GrC & Gr0 Cell flattening: - no significant difference between GrC, Gr0, Gr1, Gr3 - percentage of flat cells significantly higher in Gr2	Mitochondrial membrane potential: no significant difference Distribution of protein in the cell: no significant difference Pyruvate dehydrogenase complex: no significant difference	Morphological features: Gr1: smaller mitochondria with lower amount of cristae Gr2: well developed & abundant cristae Gr2: higher density of fibrillary component in both nucleoplasm and nucleolus Gr2: higher content of Hsp70 protein in nucleolus (vs GrC), lower content of Hsp70 protein in cytoplasm (vs GrC) Gr2: higher content of mtHsp70 protein inside mitochondria (vs GrC)
Valacchi et al, 1999 ¹⁷	Investigation whether the use of heparin or citrate added to blood, before ozonation, affects the release of platelet factors differently	Before ozonation, blood samples were enriched with either heparin or citric acid. Time: 30 sec Concentration of 3% O ₂ /O ₃ mixture: Gr1: 20 µg O ₃ /mL O ₂ Gr2: 40 µg O ₃ /mL O ₂ Gr3: 80 µg O ₃ /mL O ₂ Control group – yes: GrC: air Gr0: 100% O ₂	Total antioxidant status: no significant difference Protein thiol group: ↓ Gr1 + heparin, Gr2 + heparin, Gr3 + heparin ↓ Gr2 + heparin vs Gr2 + citric acid ↓ Gr3 + heparin vs Gr3 + citric acid The higher dose of O ₃ , the larger reduction. Thiobarbituric acid reactive substances: ↑ Gr1, Gr2, Gr3 (no significant difference between heparin and citric acid) The higher dose of O ₃ , the larger increase.	PDGF: - larger increase for heparin + GrC, Gr1, Gr2, Gr3 heparin vs citric acid at all times - for citric acid increase only in Gr3 at time point of 1h, 2h, 4h; in Gr2 at time point of 4 h - for heparin increase in all groups at all time points -the higher dose of O ₃ , the larger increase of PDGF TGF-β1: - heparin: significant increase for Gr1, Gr2, Gr3 at 1h; Gr2 & Gr3 at 2h; Gr1, Gr2 & Gr3 at 4h -heparin: the higher dose of O ₃ , the larger increase of TGFβ1 - citric acid: no significant difference	IL-8: - ↑ for Gr1 + heparin, Gr2 + heparin, Gr3 + heparin for 4h - ↑ for Gr2 + citric acid, Gr3 + citric acid for 4h - the higher dose of O ₃ , the large increase of IL-8 at time point of 4h TXB2: - ↑ for Gr2 & Gr3 at 1h & 2h (both heparin and citric acid) - at 4h ↑ for Gr1, Gr2, Gr3 (both heparin and citric acid)

Abbreviations: Gr, group of cells; IL-8, interleukin 8; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor beta 1; TXB2, thromboxane B2.

O₃/mL adhesion is significantly improved, while exposure to 20 µg O₃/mL resulted in lowered adhesion and flattening ability. It is worth noting the lowest used concentration exerted positive effects; however, they were very short-termed. Concerning the side effects of the O₂-O₃ therapy, Costanzo et al study provided important evidence of minimal ROS production due to the low concentration of ozone therapy.¹⁸ Actually, small increase in ROS level stimulates remodeling of cell structure and positive changes of mitochondria. Ten-microgram O₃/mL induced reorganization of cytoskeletal elements, increased mitochondrial cristae length and prompted positive response of higher expression of proteins to engage in numerous mitochondria functions. Thus, a concentration of 10 µg O₃/mL seems to be the most appropriate for inducing structural and functional cell changes.

From the *in vitro* studies, it can be concluded that the ozone therapy exerts a positive effect on the platelet aggregation, cell remodeling, cytoskeletal elements organization, as well as mitochondria structure. At the same time, the O₂-O₃ therapy does not cause negative changes of the cell oxidative status.

Evidence of Oxygen-Ozone Therapy Effectiveness in Animal Studies

Animal studies provide evidence of the O₂-O₃ therapy effectiveness; furthermore, they give in-depth knowledge about the mechanism of the occurred changes. All the experiments presented in this review (Table 2) were performed on rats. Three of the four studies were concerned with hypoxia condition, while one study examined hypertension. All studies had a control group, the sample size varied from 20 to 30 rats. The highest dose of 3% O₃/O₂ mixture was used in the study tested oxygen-ozone therapy effectiveness on hypertension.¹⁹ This was also the longest study, as intraperitoneal ozone was administered for 10 days. As a result of the O₂-O₃ therapy with the above parameters, the direct cardiovascular effect of ozone was observed. Systolic as well as diastolic blood pressure and mean arterial pressure were lowered by ozone. The mechanism of this observation involves decreasing level of serum endothelin-1 (ET-1) and increasing levels of serum renin and nitric oxide (NO). However, it is worth noting that oxygen-ozone therapy did not manage to lower blood pressure to normative level. In the case of examining protective effect from acute myocardial infarction 3% O₃/O₂, a mixture was administered at the lowest dose

(100–300 µg/kg) and it was given only once – an hour prior to ischemia/reperfusion procedure.²⁰ Despite this, the O₂-O₃ mixture managed to decrease myocardial infarction. The protective effect was observed due to the stimulation of endothelial nitric oxide synthase-dependent endothelial progenitor cells (EPCs) recruitment. EPCs are known to induce angiogenesis.²¹ Koca et al study as well as Ozkan et al study provides an information about the mechanism of the O₂-O₃ therapy effect on oxidative status in relation to hypoxia state of bone and skeletal muscle.^{22,23} The oxygen-ozone therapy has been shown to exert protective effect from hypoxia of bone by stimulating antioxidant enzymes activity, superoxide dismutase and glutathione peroxidase. At the same time, parameters of oxidative damage, lipid peroxidation and protein carbonyl were significantly decreased by intraperitoneal administration of four doses of 0.7 mg/kg ozone mixture. Ozkan et al found similar results, but in skeletal muscle tissue.²³ Additionally, this study investigated creatinine kinase and aspartate aminotransferase serum level (tissue damage indicators), which has been found as decreased due to ozone preconditioning.

From the animal studies, it can be concluded that the ozone therapy is an effective method in hypertension, as well as ozone preconditioning exerts protective effect during myocardial infarction and hypoxia state of bone and skeletal muscles.

Evidence of Ozone Therapy Effectiveness in Clinical Trials

Effectiveness of the ozone therapy has been also examined in the clinical trials, which are presented in Table 3. The number of participants taking part in the described studies varied from seven to 151. Methods of ozone administration involved direct intravenous infusion, autohemotherapy, ozone bath, as well as rectal insufflation accompanied by the use of ozonated oil. The number of sessions in the clinical trials varied from three sessions to 20 sessions, while the whole treatment lasted from 1 week up to 22 weeks. Two of the described studies were concerned with cerebral low perfusion syndromes. Clavo et al examined the effect of the O₂-O₃ therapy on cerebral blood flow.²⁴ The oxygen-ozone therapy was found to cause an increase in diastolic velocity in arteries, accompanied by decreased vascular resistance and rheological improvement. The observation of the correlation between the stimulation of arterial blood flow and the initial values has been made.

Table 2 Animal Studies Concerning Ozone Therapy

Author, Year	Aim of the Study	Animals	Experimental Groups	Ozone Therapy	Results
Alkilar et al, 2015 ¹⁹	To determine the role of ozone administration in hypertension	n = 23 male Sprague Dawley rats with DOCA-salt-induced hypertension	GrC: n = 7, control group GrH: n = 8, hypertension GrHO: n = 8, hypertension + ozone therapy	Time: 10 days Method: intraperitoneal Dose: 3% O ₃ /O ₂ mixture 1.1 mg/kg body weight	<p>ET-1 (pg/mL): p = 0.0001 GrC: 20.9 ± 1.24 GrH: 29.8 ± 3.95 GrHO: 18.2 ± 0.51 p = 0.0001 GrH vs GrHO</p> <p>Renin (mU/mL): p = 0.001 GrC: 38.6 ± 1.44 GrH: 26.0 ± 0.58 GrHO: 38.7 ± 0.96 p = 0.0001 GrH vs GrHO</p> <p>NO (nmol/mL): p = 0.04 GrC: 1.97 ± 0.14 GrH: 1.12 ± 0.25 GrHO: 2.29 ± 0.32 p = 0.038 GrH vs GrHO</p> <p>Systolic blood pressure (mmHg): p = 0.0001 GrC: 119.9 ± 1.43 GrH: 150.9 ± 1.96 GrHO: 136.4 ± 0.75 p = 0.0001 GrHO vs GrC</p> <p>Diastolic blood pressure (mmHg): p = 0.001 GrC: 83.3 ± 1.92 GrH: 94.7 ± 0.74 GrHO: 88.5 ± 1.38</p> <p>Mean arterial pressure (mmHg): p < 0.05 GrC: 101.0 ± 1.24 GrH: 121.3 ± 1.11 GrHO: 111.1 ± 1.09 p = 0.001 GrHO vs GrC</p> <p>ET-1 mRNA expression: - heart: p = 0.004 GrC: 0.089 ± 0.29 GrH: 4.39 ± 0.88 GrHO: 0.89 ± 0.13</p> <p>- vascular tissue: p = 0.003 GrC: 0.03 ± 0.01 GrH: 0.29 ± 0.009 GrHO: 0.03 ± 0.001</p> <p>ET-1 mRNA expression was significantly higher in GrH than in GrHO: - in the heart (p = 0.004) - in the vascular tissue (p = 0.002)</p>
Di Filippo et al, 2010 ²⁰	Investigation of the ozone therapy effect on damage linked to acute myocardial	n = 20 Sprague Dawley rats after 25 minutes occlusion and 2-hour reperfusion of the left descending coronary artery	- GrC: n = 5, control group - Gr1: n = 5 - Gr2: n = 5 - Gr3: n = 5	Time: 30 min prior to ischemia/reperfusion (IR) procedure Method: intraperitoneal Dose of O ₂ /O ₃ mixture: Gr1: 100 µg/kg Gr2: 150 µg/kg Gr3: 300 µg/kg	<p>Area at risk/left ventricle: GrC: 53 ± 2% Gr1, Gr2, Gr3: 52 ± 3.2%, p > 0.05</p> <p>Infarct size/left ventricle: GrC: 33 ± 1.5% Gr2: no change, p > 0.05 Gr2: 22 ± 2%, p < 0.05 Gr3: p < 0.01</p> <p>GrC: low level of immunoprotection from CD-34 and CD117/c-kit, low level of eNOS Gr2: increase amount of CD-34 and CD117/c-kit, high level of eNOS (p < 0.01)</p>

Koca et al, 2010 ²²	To investigate protective role of hyperbaric oxygen precondition and ozone precondition on IR injury	n = 32 male Wistar Albino rats after 2-hours occlusion and 22-hours reperfusion of unilateral femoral artery	- GrS: n = 8, sham - GrC: n = 8, IR - GrHBOT: n = 8, IR + hyperbaric oxygen precondition - GrO3: n = 8, IR + ozone precondition	Time: 72, 48, 24, 0 hours prior to IR Method: intraperitoneal Dose of 3% O ₂ /O ₃ mixture: 4 doses of 0.7 mg/kg	Lipid peroxidation: - higher level in GrC than GrS, p < 0.01 - lower level in GrHBOT & GrO3 (GrHBOT < GrO3) than GrC, p < 0.01	Oxidative damage: - higher level in GrC than GrS, p<0.01 - lower level in GrHBOT & GrO3 (GrO3 < GrHBOT) than GrC, p<0.01	Antioxidant enzymes: - higher level of SOD in GrC (vs GrS, p < 0.01) and lower level of GSH-Px (p < 0.01) - GrHBOT i GrO3 (vs GrC) had higher level of SOD and GSH-Px (p < 0.01) (GrHBOT > GrO3)
Ozkan et al, 2015 ²³	To evaluate and compare the effect of hypothermia and ozone therapy on ischaemia reperfusion injury of skeletal muscle	n=18 male Wistar Albino rats after 2-hours occlusion and 22-hours reperfusion of common iliac artery	- GrS: n=6, sham - GrC: n = 6, IR - GrH: n = 6, IR + hypothermia - GrO3: n = 6, IR + ozone therapy - GrH + O3: n = 6, IR + hypothermia + ozone therapy	Time: 72, 60, 48, 36, 24, 12 hours prior to IR Method: intraperitoneal Dose of 3% O ₂ /O ₃ mixture: 6 doses of 0.7 mg/kg	Lipid peroxidation - MDA (mmol/g protein): - GrS: 0.63 ± 0.16 - GrC: 4.23 ± 0.68 - GrO3: 2.10 ± 0.49* - GrH + O3: 1.73 ± 0.45* *p < 0.05 vs GrC Oxidative stress - SOD (U/g protein): - GrS: 918.7 ± 257.7 - GrC: 312.6 ± 66.1 - GrO3: 991.9 ± 251.6*	- GrH + O3: 727.3 ± 115.1 [#] *p < 0.05 vs GrC [#] p < 0.05 vs GrO3 Oxidative stress - GSH-Px (U/g protein): - GrS: 90.9 ± 11.3 - GrC: 29.5 ± 6.9 - GrO3: 87.6 ± 17.1 [#] - GrH + O3: 86.4 ± 20.1 [#] *p < 0.05 vs GrC [#] Nitrosative stress - NOx (g/g protein):	- GrS: 0.25 ± 0.02 - GrC: 0.59 ± 0.11 - GrO3: 0.34 ± 0.2* - GrH + O3: 0.31 ± 0.7* *p < 0.05 vs GrC Inflammation - IL-1β (pg/p protein): - GrS: 13.0 ± 2.8 - GrC: 60.6 ± 12.4 - GrO3: 24.6 ± 3.4* - GrH + O3: 23.3 ± 0.7 [#] *p < 0.05 vs GrC [#] p < 0.05 vs GrO3

Abbreviations: DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; Gr, group of subjects; GSH-Px, glutathione peroxidase; IL-1β, interleukin 1 beta; IR, ischemia/reperfusion procedure; MDA, malondialdehyde; n, number of subjects; NO, nitric oxide; NOx, nitrite-nitrate; SOD, superoxide dismutase.

Table 3 Clinical Trials of Oxygen-Ozone Therapy

Author, Year	Aim of the Study	Participants	Ozone Therapy	Results
Clavo et al, 2004 ²⁴	To assess the effect of ozone therapy on the blood flow in the middle cerebral and common carotid arteries	n = 7 subjects: 2 healthy volunteers + 5 patients with cerebral vascular disease Age: 58 years (34–78 years)	Autohemotherapy: 200 mL blood + 200 mL O ₂ /O ₃ mixture (concentration 60 µg/mL) 3 autohemotherapy sessions on alternate days over one week	<p>Middle cerebral artery: - systolic velocity: t0 = 90.9 ± 6.1 cm/s t1 = 111 ± 7.3 cm/s, p = 0.001 t2 = 104.3 ± 8 cm/s, p = 0.035</p> <p>- diastolic velocity: t0 = 41.1 ± 4.4 cm/s t1 = 54.6 ± 4.6 cm/s, p < 0.001 t2 = 48.6 ± 5 cm/s, p = 0.023</p> <p>t0 – baseline t1 – after third session t2 – one week later</p> <p>Common carotid artery: - blood flow: t0 = 233 ± 19 mL/min t1 = 407 ± 38 mL/min, p < 0.001 t2 = 301 ± 22 mL/min, p = 0.039</p> <p>The percentage increase in common carotid artery blood flow was directly correlated with age (r = 0.35, p = 0.004) and inversely correlated with the initial values (r = 0.691, p = 0.009).</p>
Wu et al, 2013 ²⁵	To determine the association between major ozonated autohemotherapy and functional recovery of patients with acute cerebral infarction	n = 86 acute cerebral infarction patients GrO ₃ : autohemotherapy GrC: conventional therapy	10 ± 3 daily autohemotherapy sessions: 100 mL blood + 100 mL O ₂ /O ₃ mixture (concentration 47 µg/mL)	<p>Cortical potential rise rate: no significant difference between groups</p> <p>Motor-evoked potential amplitude: - upper limb: GrO₃: significantly higher in comparison with GrC (p < 0.05) - lower limb: no significant difference between groups</p> <p>Correlation analysis: - positive correlation between NIHSS improvement rate and central motor conduction time improvement rate of the upper limb (r = 0.78, p < 0.005) - positive correlation between NIHSS improvement rate and the improvement rate of motor-evoked potential amplitude of the upper limb (r = 0.85, p < 0.05)</p> <p>Modified Rankin Scale: GrO₃: t0 = 4.1 ± 0.8; t1 = 2.5 ± 1.1 GrC: t0 = 4.1 ± 0.9; t1 = 3.1 ± 1.3 GrO₃ vs GrC p < 0.05</p> <p>National Institutes of Health Stroke Scale (NIHSS): GrO₃: t0 = 11.7 ± 3.5; t1 = 5.4 ± 3.5 GrC: t0 = 11.5 ± 3.8; t1 = 7.3 ± 4.1 GrO₃ vs GrC p < 0.05</p> <p>Central motor conduction time: - upper limb: GrO₃ significantly shorter time in comparison with GrC (p < 0.05) - lower limb: no significant difference between groups t0 – baseline t1 – after 10 ± 3 sessions</p>

<p>Martínez-Sánchez et al, 2012²⁶</p>	<p>Investigation of the therapeutic efficacy of ozone in patients with coronary artery disease, treated with antithrombotic therapy, Aspirin and policosanol.</p>	<p>n = 53 patients with coronary artery disease GrO₃: 26 patients, regular anti-thrombotic therapy + ozone therapy GrC: 27 patients, regular anti-thrombotic therapy GrN: reference group of healthy subjects</p>	<p>20 daily sessions: - rectal insufflation - dose: 200 mL O₂/O₃ mixture, concentration 40 µg/mL</p>	<p>Prothrombin time (s): GrO₃: t0 = 11.2 ± 0.6 s; t1 = 24.6 ± 6.6 GrC: t0 = 11.0 ± 0.4 s; t1 = 18.4 ± 4.6 t1: GrO₃ vs GrC p < 0.01 Bleeding time: no difference (GrO₃ vs GrC) Advanced oxidation protein products (µM): GrO₃: t0 = 23.90 ± 0.84; t1 = 18.26 ± 3.61 GrC: t0 = 23.12 ± 5.31; t1 = 24.25 ± 7.18 t1: GrO₃ vs GrC p < 0.05 Malondialdehyde (µM): GrO₃: t0 = 12.04 ± 0.44; t1 = 8.38 ± 3.63 GrC: t0 = 13.71 ± 3.01; t1 = 10.82 ± 2.52 t1: GrO₃ vs GrC p < 0.05 Peroxidation potential (µM): GrO₃: t0 = 16.25 ± 3.62; t1 = 6.16 ± 2.51 GrC: t0 = 20.41 ± 8.16; t1 = 19.00 ± 7.03 t1: GrO₃ vs GrC p < 0.05 t1: GrO₃ vs GrN p > 0.05 t0 – baseline t1 – after 20 sessions</p>	<p>Ferric reducing ability of plasma (µM): GrO₃: t0 = 191.3 ± 57.0; t1 = 471.9 ± 115.8 GrC: t0 = 146.2 ± 62.3; t1 = 386.0 ± 104.3 t1: GrO₃ vs GrC p < 0.05 Reduced glutathione (mM): GrO₃: t0 = 1.18 ± 0.23; t1 = 1.55 ± 0.63 GrC: t0 = 1.5 ± 0.23; t1 = 1.08 ± 0.45 t1: GrO₃ vs GrC p < 0.05 Superoxide dysmutase (U/mL): GrO₃: t0 = 37.75 ± 7.13; t1 = 16.27 ± 8.39 GrC: t0 = 40.48 ± 10.12; t1 = 35.27 ± 14.21 t1: GrO₃ vs GrC p < 0.05 Catalase (U/l): GrO₃: t0 = 839.5 ± 165.3; t1 = 543.4 ± 172.3 GrC: t0 = 880.4 ± 250.1; t1 = 288.0 ± 99.6 t1: GrO₃ vs GrC p < 0.05 t1: GrC vs GrN p > 0.05 No difference between GrC and GrO₃ for total hydroperoxides.</p>
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Table 3 (Continued).

Author, Year	Aim of the Study	Participants	Ozone Therapy	Results
Martinez-Sanchez et al, 2005 ²⁷	Evaluation of the effectiveness of ozone in the treatment of patients with type 2 diabetes suffering from diabetic foot complications and its effects on oxidative stress, hyperglycaemia and some markers of endothelial damage.	n = 100 patients with diabetic foot GrO ₃ : 51 patients, ozone therapy GrA: 49 patients, antibiotic therapy GrC: 50 healthy subjects, control group	20 daily sessions: - rectal insufflation (ozone dose 10 mg, concentration 50 mg/l); - local ozone treatment (concentration 60 mg/l, time 1 hour) - Oleozon: ozonised sunflower oil	<p>Blood glucose concentration: GrO₃ > GrA (p < 0.05) Blood glucose within reference range (3.33–8.33 mM): GrO₃: t0 = 27%, t1 = 56% patients GrA: t0 = 30%, t1 = 26% patients Lesions area reduction: GrO₃: 74.58 ± 0.35% GrA: 50.3 ± 0.17%, GrO₃ vs GrA p = 0.017 GrO₃: 2.66 ± 0.03 cm²/day GrA: 1.21 ± 0.01 cm²/day, GrO₃ vs GrA p = 0.005 t0 - baseline t1 -24 h after the last session</p> <p>Total recovery time: GrO₃: 21 ± 10 days GrA: 45 ± 11 days, GrO₃ vs GrA p = 0.002 Length of hospitalisation: GrO₃: 26 days (6–58 days) GrA: 34 days (7–83 days), GrO₃ vs GrA p = 0.010 Biomarkers of oxidative damage: GrO₃: improvement (t1 vs t0) of the following biomarkers: fructosylsine, advanced oxidation protein products, peroxidation potential, superoxide dismutase, catalase. GrO₃: improvement to the GrC levels (t1 vs t0) of the following biomarkers: nitrites/nitrates, total hydroperoxides. GrO₃: no change for the following biomarkers: reduced glutathione, malondialdehyde, glutathione peroxidase</p>

<p>Tafli-Klawe et al, 2002²⁸</p>	<p>Comparison of the effect of ozone and classical balneological methods on health condition of patients with obliterative artheromatosis and on serum activity of three lysosomal enzymes.</p>	<p>n = 94 subjects GrO₃: 32 patients with lower limb ischemia Age: 65.9 ± 6.9 years GrB: 32 patients with lower limb ischemia Age: 62 ± 8 years GrC: 30 healthy subjects, control group Age: 60 years</p>	<p>GrO₃: everyday ozone therapy for 10 days: - intravenous infusions: 500 mL of normal saline satiated with ozone (60 µg O₃/mL) - 30-minute aerosol oxygen-ozone baths: ozone concentration in a chamber was 19 µg/l GrB: whirlpool massage therapy and carbonic acid baths, everyday therapy for 10 days</p>	<p>GrO₃: - arylsulphatase: t1 = 1.71 ± 0.40; t2 = 1.88 ± 0.42 (p < 0.05 vs t1), t3 = 2.71 ± 0.68 - acid phosphatase: t1 = 14.11 ± 2.56; t2 = 12.21 ± 1.9 (p < 0.05 vs t1), t3 = 9.97 ± 1.48 - cathepsin D: t1 = 1.75 ± 0.38; t2 = 3.41 ± 0.69 (p < 0.05 vs t1), t3 = 5.39 ± 1.19 - alfa-1-antitrypsin: t1 = 1.62 ± 0.22; t2 = 1.39 ± 0.22 (p < 0.05 vs t1), t3 = 1.19 ± 0.15 (p > 0.05 vs GrC: 1.07 ± 0.12) t1 – 24 h hours before first therapy t2 – one hour following therapy t3 – on 10th day of therapy</p>	<p>GrO₃: - BMI reduction by 2.4% - ankle arm index (right extremity): improved by 13.8% - ankle arm index (left extremity) improved by 14.9% - intermittent claudication distance in the corridor: improve by 34.2% - intermittent claudication distance on a track: improvement by 50.6% - total cholesterol: reduction by 5.0% - HDL: no change - LDL: reduction by 7.2% - triglycerides: reduction by 11.1%</p>
<p>Marfella et al, 2009³¹</p>	<p>Analysis of ozone therapy effect on TNF-α level, endothelial progenitor cells (EPCs) level and on limb perfusion.</p>	<p>n = 151 patients with critical limb ischemia, candidates not appropriate for standard revascularization. Age > 40 years GrO₃: 77 patients, ozone therapy GrC: 74 patients receiving injections with 10 mL sterile saline</p>	<p>Intraarterial injection of autologous blood exposed to oxygen/ozone gas mixture (ozone concentration: 15.35 g/m³ flow rate: 240 mL/min) Two treatments were given on consecutive days, followed by a third one on day 7. Subsequent treatments given at 1-week intervals for a least 22 weeks.</p>	<p>Pain perception: GrO₃: reduced pain perception in 36 patients GrC: reduced pain perception in 12 patients Wound healing: GrO₃: deterioration in wound size t1 vs t0 (p<0.05), at t1 GrO₃ vs GrC (p<0.05) GrO₃: complete ulcer healing in 32 patients at 6 months Ankle-Branchial Index: No difference among groups at any of the time points t0 – baseline t1 – 22 weeks</p>	<p>TcPO₂: GrO₃: significant increase at t1 vs t0 (p<0.01), GrO₃ vs GrC (p<0.001) GrO₃: higher number of patients with TcPO₂> 30 mmHg (t0 = 31.1%, t1 = 84.4%) GrC: lower number of patients with TcPO₂> 30 mmHg (t0 = 35.1%, t1 = 15.1%) TNF-α level: GrO₃: t0 = 225.5±28.2 ng/l, t1 = 114.8±23.1 ng/l (p<0.001) GrC: no difference EPCs: GrO₃: increase of CD34/KDR(+); No difference in CD34(+), CD133(+) or total number of mononuclear cells</p>

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Table 3 (Continued).

Author, Year	Aim of the Study	Participants	Ozone Therapy	Results
Zhang et al, 2014 ³⁰	Assessment of effectiveness of ozone therapy on the healing and the expression of growth factors of the wounds at the early stage after treatment of diabetic foot ulcer.	n = 50 patients with type II diabetes mellitus, as well as with diabetic foot ulcer of Wagner classification stages 2, 3, 4 GrO ₃ : 25 patients, ozone therapy GrC: 25 patients, standard treatment including debridement, wound dressing, moisture maintenance of the wound	Ozone gas bath: - concentration: 52 mg/mL - time: 30 min/day, 20 days	<p>VEGF levels in tissues at 11 day following up (pg/mL): GrO₃: 34.86 ± 3.00 GrC: 26.44 ± 2.02, GrO₃ vs GrC p = 0.032</p> <p>PDGF levels in tissues at 11 day following up (pg/mL): GrO₃: 31.44 ± 3.33 GrC: 20.78 ± 2.69, GrO₃ vs GrC p = 0.023</p> <p>TGF-β in wound tissues at 11 day following-up (pg/mL): GrO₃: 14.95 ± 1.39 GrC: 10.45 ± 1.07, GrO₃ vs GrC p = 0.019</p> <p>Expression of VEGF proteins in tissues at 11 day following up (a.u.): GrO₃: 3.34 ± 0.27 GrC: 2.03 ± 0.16, GrO₃ vs GrC p < 0.001</p> <p>Expression of PDGF proteins in tissues at 11 day following up (a.u.): GrO₃: 4.09 ± 0.14 GrC: 3.06 ± 0.13, GrO₃ vs GrC p < 0.001</p> <p>Expression of TGF-β proteins in tissues at 11 day following-up (a.u.): GrO₃: 7.83 ± 0.49 GrC: 6.10 ± 0.45, GrO₃ vs GrC p = 0.018</p>

Zhou et al, 2016 ³²	Comparison of the effectiveness of standard endovenous laser therapy with endovenous laser therapy combined with ozone gas bathing in patients with lower limb venous ulcers.	n = 92 patients with lower limb venous ulcers GrO ₃ : 50 patients Age: 61.1 years ± 11.2 GrC: 42 patients, control group Age: 60.2 years ± 9.7	Preconditioning with ozone gas bath: - concentration: 60 mg/l - time: 1 hour, once a day until the necrosis and infection were improved an suitable for skin puncture (mean: 8.6 days, range: 8–13 days)	Ratio of complete occlusion of the treated vein: no significant difference at any time point (1, 3, 6, 12 months of follow-up) Ratio of recovery at the 12 months following up: GrO ₃ : 92.00% GrC: 76.19%, GrO ₃ vs GrC p = 0.03	Ratio of ulcer recur at the 24 months following up: GrO ₃ : 6.52% GrC: 25.00%, GrO ₃ vs GrC p = 0.03 Patient's satisfaction assessment: GrO ₃ : 90.00% GrC: 71.43%, GrO ₃ vs GrC p = 0.03
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Note: p values above 0.05 are shown in bold.

Abbreviations: BMI, body mass index; EPCs, endothelial progenitor cells; Gr, study group; HDL, high-density lipoprotein; KDR, kinase-insert domain-containing receptor; LDL, low-density lipoprotein; n, number of subjects; p, statistical difference; PDGF, platelet-derived growth factor; t, time point; TcPO₂, transcutaneous oxygen pressure; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

Tissues with higher hypoxia state are more responsive to the O₂-O₃ therapy. What is more, a greater effect was also seen in older patients. Wu et al study provided the evidence that patients with acute cerebral infarction may gain advantage of oxygen-ozone therapy in the form of a better motor function recovery.²⁵ The mechanism enabling previously mentioned effects of the O₂-O₃ therapy involves upregulation of vasoactive substances release, enhanced erythrocyte flexibility as well as improved blood rheology. The oxygen-ozone therapy results in the rise of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate content in the red blood cells. Ozone also promotes pentose phosphate pathway reactions that result in the release of nicotinamide adenine dinucleotide phosphate. This molecule is crucial for erythrocyte membrane integrity. Moreover, ozone lowers blood viscosity and stimulates hydrogen dioxide release that breaks down thrombus, thus micro-circulation may be improved. Blood clotting is also affected due to the change of blood platelet polymerization. Finally, ozone stimulates basal metabolism by upregulating tricarboxylic acid and a stimulating fat catabolism. As a result, fatty materials on the vessel wall may be removed.

Ozone effect on hemostasis was also the main topic of Martinez-Sanchez et al.²⁶ Among 53 patients with coronary artery disease, 20 everyday sessions of rectal insufflation were performed. Platelet aggregation has been inhibited due to the platelet activation factor, collagen and adenosine diphosphate changes. The oxygen-ozone therapy activates adenosine A₂ receptor in the platelet causing enhancing platelets adenylate cyclase and rising level of platelet cyclic-3',5'-adenosine monophosphate. Moreover, the O₂-O₃ therapy has been shown to down-regulate oxidative stress and inflammatory processes. Markers such as degree of monocyte activation, dityrosine, advanced glycation end products, neopterin, inflammatory cytokines were decreased in the response to ozone. Total antioxidant capacity, measured by ferric reducing ability of plasma (FRAP) parameter, has been increased. FRAP parameter is a marker combining changes in the antioxidant activity of multiple compounds. The observed change exerted a major effect on oxidative status indicating a shift to the status of low reactive oxygen species production and an increase in H₂O₂ detoxification. Ozone can also enhance antioxidant status by inhibiting the production of reactive oxygen species through xanthine/xanthine oxidase pathway. Therefore, an effective stimulation of oxidative defense by the O₂-O₃

therapy seen in the *in vitro* studies is confirmed by the clinical trials.

Overproduction of superoxide by the mitochondrial electron-transport chain as well as disturbed antioxidant status are the processes highly correlated with a state of hyperglycemia. Patients with type II diabetes suffering from diabetic foot complication were a study group of another clinical trial examining the therapeutic effect of ozone.²⁷ It has been demonstrated that the O₂-O₃ therapy not only decreases hyperglycemia but also brings blood glucose concentration back to the healthy reference range. This observation was attributed to the ozone effect on the antioxidant system, as well as enhanced insulin sensitivity. Hydrogen peroxide causes significantly reduced glucose uptake in adipocytes. This process may be reversed by ozone, as it reduces the level of hydrogen peroxide and thus stimulates phosphatidylinositol 3 kinase activity and promotes migration of glucose transport 4 translocation from the intracellular compartment to the cellular surface. Ozone enhances metabolic actions preventing glycogen depletion, ATP depletion and providing constant availability of free glucose. Furthermore, oxygen-ozone therapy restored to normal reference values the NO⁻²/NO⁻³ ratio, a marker of nitric oxide. The most important finding of Martinez-Sanchez et al is the fact that O₂-O₃ therapy is effective in reducing the amount of amputations needed among the patients as well as decreasing the extent of the amputation.²⁷ Moreover, patients have recovered faster and have a better perceived quality of life due to the oxygen-ozone therapy.

Patients with lower limb ischemia however not being as a result of diabetes complications but due to the obliterative atheromatosis were a study group of Tafil-Klawe et al clinical trial.²⁸ The mechanism of lower limb ischemia complications involves the stimulated activity of acid phosphatase. Through normalization of lysosome membranes, reduction in phagocytosis of monocytes and macrophages, as well as through a decrease in the activity of acid phosphate, the O₂-O₃ therapy prevents cell autolysis and limits tissue damage. The oxygen-ozone therapy has been shown to have clinical effectiveness, as the intermittent claudication distance prolonged by 50.6%.

One of the most concerning complications of lower limb ischemia is the formation of ulceration. Diabetic foot ulcers can be characterized by delayed wound healing, prolonged closure time, very low collagen deposition, as well as decreased expression of endogenous growth factors in the wound.²⁹ Zhang et al investigated the

effectiveness of ozone gas bathing in relation to wound healing and expression of growth factors such as vascular endothelial growth factor (VEGF), TGF- β and PDGF.³⁰ The ozone therapy significantly stimulated wound healing, so that wound reduction occurred much more quickly. This effect can be explained by the higher content of collagen and growth factors, that has been observed in this study. Enhanced wound healing as well as reduced pain perception were the effects also observed in the clinical study concerning critical limb ischaemia.³¹

Zhou et al also examined the effectiveness of the oxygen-ozone therapy as a supplementary method of lower limb venous ulcers treatment.³² One of the most important findings of this study is that infection of the ulcer has been reduced due to the O₂-O₃ therapy. As it was stated previously, ozone disrupts the integrity of bacterial cell walls, thus demonstrating antimicrobial properties. Zhou et al provide clinical evidence that the oxygen-ozone therapy may be a successful infection treatment among diabetic patients as well.³² The observation of higher recovery ratio, higher patient's satisfaction and lower recurrence rate has been made when the patients were preconditioned with ozone gas bath. It is worth noting that just seven to 13 sessions were enough to exert long-term effects. This study provides evidence for the effectiveness of ozone gas bathing, which is a non-invasive method and a treatment showing high compliance. Ozone gas bathing is also a good method for elderly patients and patients in poor condition that make them unsuitable candidates for surgery.

Among all of the described studies, the most important effects of the O₂-O₃ therapy in relation to peripheral artery diseases were the following: improved tissue perfusion resulting in reduced hypoxia state; better rheology; better glycemic control; enhanced lipid metabolism; enhanced anti-oxidant defense; faster healing processes; neurovascular protective effect; reduced inflammation; reduced sensation of pain. An economic evaluation performed for neuroinfectious diabetic foot has shown that oxygen-ozone therapy may reduce treatment costs by one quarter compared with the standard antibiotic therapy. Taking all of those observations together the O₂-O₃ therapy appears to be an effective adjunctive therapy in preventing PAD complications such as amputation or occurrence of major cardiovascular events.

Conclusions

The consequence of risk factor modulation by the oxygen-ozone therapy, as evidenced in this paper, appears to be

very effective in terms of cardiovascular event prevention. Heart attack, stroke and major cardiovascular diseases are events resulting from deterioration over time. These changes involve arterial stenosis, elevated blood pressure, dyslipidemia, poor glycemic control, disturbed oxidative status, long-term inflammation. The oxygen-ozone therapy has been shown as an effective method of improving all of the above conditions.

Chronic ulceration is a major problem among PAD patients. It is a frequent cause of disturbed microcirculation, swelling and perivascular edema. Long healing as well as frequent recurrence of the ulcers may eventually result in non-healing venous ulcers and amputation. It has been proven that the O₂-O₃ therapy supports traditional ulcer treatment so that better therapy outcomes are achieved.

The oxygen-ozone therapy is well tolerated by patients, they experience less pain and their satisfaction from the treatment process increases when the O₂-O₃ therapy is included. All of those effects and the fact of lower risk of amputation makes the oxygen-ozone therapy effective in improving the quality of life of PAD patients. The therapy appears to have no adverse events or side effects, as none have been reported in the clinical trials. What is more, it may reduce standard treatment costs. In comparison with standard antibiotic therapy, as much as 25% of diabetic foot treatment costs can be saved. Easy clinical protocols allow the implementation of the oxygen-ozone therapy into the usual care of PAD patients. The O₂-O₃ therapy appears to be a promising treatment method especially for older PAD patients and patients, who are not eligible for standard revascularization.

Disclosure

The authors report no conflicts of interest in this work.

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