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ISCO3/MET/00/25 Ozonized Saline Solution (O3SS)

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Title

ISCO3/MET/00/21 Ozonized Saline Solution. Standard Operation Procedure. SOP

1.1. Brief background

Ozonized Saline Solution (O₃SS) is a widespread practice in Russia and was developed by the Russian school of ozone therapy in the city of Nizhny Novgorod (Volga Federal District) in 1977.¹ O₃SS is supported by pre-clinical studies,^{2 3 4 5} more than 92 clinical trials^{6 7 8 9 10 11 12 13} (mainly published in the Russian language) and an estimated number of more than 500 university theses. It is probably the most extensive and scientifically supported application method of ozone therapy. Since then, the method has evolved substantially. Dr Adriana Schwartz has improved, adapted and developed the technique to European requirements and introduced it into medical practice in the western countries with great success. She designed and patented a new method,

¹ Schwartz A. Solución Salina Ozonizada (SSO3): Fundamentos Científicos. Revista Española de Ozonoterapia. 2016;6(1):111-120.

² Volkhovskaya NB, Tkachenko SB, Belopolsky AA. Modulation of phagocytic activity of blood polynuclear leukocytes with ozonized physiological saline. Bull Exp Biol Med. Nov 2008;146(5):559-561.

³ Peretyagin S, Vorobiev A, Smirnov S, Inventors. Oxygen-ozone mixture use in traumatology. 2007.

⁴ Razumovskii SD, Konstantinova ML, Grinevich TV, Korovina GV, Zaitsev VY. Mechanism and kinetics of the reaction of ozone with sodium chloride in aqueous solutions. Kinetics and Catalysis. 2010;51(4):492-496.

⁵ Boyarinov GA, Gordetsov AS, Peretyagin SP, Matusyak KS, Ovchinnikov YV, Boyarinova LV. The analysis of interaction of ozone and sodium chloride in Aqueous solution. Rev Esp de Ozonoterapia. 2016;6(Suppl 1):77.

⁶ Kontorshchikova KN, Solopaeva IM, Peretiagin SP. [Effect of ozone on the liver state in experimental chronic hepatitis]. Biull Eksp Biol Med. Aug 1996;122(8):238-240.

⁷ Korolev BA, Boiarinov GA, Monakhov AN, Shvets NA, Peretiagin SP. [Metabolism and ultrastructure of the myocardium in protection of the heart against ischemia using an ozonized cardioplegic solution]. Grudn Khir. Nov-Dec 1983(6):27-31.

⁸ Belyaev AN, Kozlov SA, Belyaev SA, Kostin SV. Influence of ozone therapy on postoperative hemostasis system dynamics in patients with obstructive jaundice. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):13.

⁹ Utilization of Ozonated cardioplegic Solution in myocardium ischemia. Boiarinov A.; Morxov A.R.; Schbetz R. A.; Peretiagyn S.P.; Cardiología №2, 1983 C116-117

¹⁰ S.P. Peretyagin, A.G. Soloveva, A.K. Martusevich, P.V. Peretyagin, N.V. Didenko The estimation of the state of pro- and antioxidant systems of the blood and myocardium at subchronic infusion of ozonized saline.

¹¹ Korolev B.A., Medvedev .P., Monakhov A.N., Bober V.M., Gomozev I.V., Nizhny Novgorod, Centro Interregional de Cirugía Cardíaca. Moscú-Rusia. 1ª Conferencia Científico-Práctica de toda Rusia. Ozono en Biología y Medicina. 25-26 de Junio de 1992. Aplicación de solución Salina Ozonizada en pacientes cardioquirúrgicos en corrección d paro cardíaco complicado con endocarditis infecciosa.

¹² P.N. Kovalchuk, L.S. Kovalchuk. Ozone therapy in the rehabilitation of patients with coronary heart disease

¹³ P.V. Peretyagin, A.K. Martusevich, S.P. Peretyagin. A study of dose-dependent in microcirculatory response on prolonged course of ozonized saline infusions

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the ASSO3®. This is a reusable glass device with a micro bubbling plate that which can be autoclaved. This has positioned ozone therapy in a safer, more effective and privileged place.

The method consists in the prior saturation of the saline solution with a mixture of O₂/O₃ at very low concentration and its intravenous infusion into the patient. This route of application was approved by the Ministry of Health of the Russian Federation in the early 80s of the last century, specifically for the branches of orthopaedics, dermatology, gynaecology/obstetrics and neonatology. Since then the method has been officially implemented in public health hospitals.¹⁴
15 16

1.1.1 Bocci and Ozonized Saline Solution. An historical background

In September, 1995 Dr. Velio Bocci was invited to the 2nd all Russian Conference in Nizhny Novgorod. In October 1995 he wrote a letter about his impression, submitted to Dr. J. Maddox, editor of Nature in London, UK, never published. However, the letter appears in the first issue of the journal *Ozone in Biology and Medicine*.¹⁰ In this letter he informs Western scientists and physicians that ozone therapy has become of age in Russia. The letter said **“Ozonotherapy has been mainly carried out either by infusing ozonated physiological saline, by rectal insufflation (RIO3) of ozone, minor autohaemotherapy and, less frequently, major autohaemotherapy. When Prof. Peretyagin took us round two regional hospitals, I was pleasantly surprised to see that ozone therapy is carried out daily, on a routine basis, in thousands of patients yielding remarkable clinical improvements with no toxicity”**.¹⁷

However by 2005 in his book “Ozone A New Medical Drug”¹⁸ Bocci said: “As an example, physiological saline (0.9 % NaCl) should never be ozonated because of the formation of hypochlorous acid. However, after ozonating saline with an ozone concentration of 80 µg/mL, H₂O₂, and hypochlorous acid (HOCl) cause a chemical phlebitis. However, at least in Russia, it does not seem to procure significant damage because the ozonation is performed at an extremely low level (about 2 µg/mL), so that it works as a placebo. Now I must strongly recommend avoiding the use of ozonated saline owing to inherent toxicity or/and minimal activity.”

Summarising, Bocci based on a personal experience using a non-appropriate method, assumes that O₃SS is not a valid method “... after ozonating saline with an ozone concentration of 80 µg/mL, I tested it and, in spite of a considerable blood dilution during the slow infusion, the next day I felt a painful irritation along the venous path up to the axilla. I realized that the ozonated saline was somewhat caustic and could cause a chemical phlebitis. Then I went to my lab. and I measured the formation of hydrogen peroxide, that was a good thing, but also of hypochlorous

¹⁴ Peretyagin S, Vorobiev A, Smirnov S, Inventors. Oxygen-ozone mixture use in traumatology. 2007.

¹⁵ Kocheleva I, Ivanov O, Vissarionov V, Inventors. Oxygen-ozone use in dermatology and cosmetology 2005.

¹⁶ Serov N, Fedorova T, Kachalina T, Inventors. Medical ozone use in obstetrics, gynecology and neonatology 2007.

¹⁷ Bocci V. Ozonotherapy has become of age in Russia. *Ozone in Biology and Medicine*. 1996;1(Supp.).

¹⁸ . Bocci V, ed *Ozone: A new medical drug*. ISBN-10: 9048168058. ISBN-13: 978-9048168057. Netherlands: Springer; 2010.

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acid (HOCl), that was a bad thing. Traces of Fe^{++} could also catalyze the formation of hydroxyl radicals. Years later, I was surprised to learn that ozonated saline is widely used in Russia and also in Italy by a few charlatans”. In addition, Bocci assumes that the clinical results of Russian scientists were because of the placebo effect.

Surprisingly, in February 2005 Dr. Bocci's team conducted a new study: “Effects of ozone on mononuclear cells isolated from peripheral blood. Toxicol in vitro”.¹⁹ Where it was established that only a very low concentration of ozone (1.0 $\mu\text{g/mL}$) stimulated cell proliferation significantly and raised the Proliferation Index above 1, particularly evident after 48 h of incubation. Under current experimental conditions, only the lowest concentration of ozone (1.0 $\mu\text{g/ml}$) appears to slightly stimulate IFN-gamma.

In retrospect, the team reflected: "It seems obvious that we should have explored the effect of ozone concentrations below 1.0 $\mu\text{g/mL}$, to determine whether ozone might exhibit more significant stimulatory effects than that observed in the present study”.

In contrast, the action mechanism involved in O_3SS (Keap1-Nrf2- EpRE signaling pathway) was discovered first in (2011)²⁰ for O_3SS while for the autohemotherapy it was discovered in 2013 / 2014.^{21 22 23 24}

1.1.2. Ozonated Saline Solution can generate toxic compounds?

There was a discussion about the claim that the mixture of ozone with O_3SS would generate H_2O_2 and HOCl (Hypochlorous Acid), substrates that might cause toxicity in the body. However, this question has been solved a long time ago. According to research conducted by Professor Claudia N. Kontorshchikova in saline solution at 0.9 % ozonized (0.55 mg/L O_3) on average were found 0.004 mM/L of chloride ions, which is equal to nothing. Regarding the analysis of H_2O_2 in samples of 0.9% NaCl done by analytical chemistry methods did not reveal any accumulation of H_2O_2 in concentrations exceeding 0.002 % in any of the ozonation schemes, although it was found even much lower concentrations, in the order of 0.0004 %.^{25 26}

¹⁹ A. Larini, V. Bocci. “Effects of ozone on mononuclear cells isolated from peripheral blood. Toxicol in vitro”. February 2005; 19 (1): 55-61.

²⁰ Qu DD, Peng FJ, Liu L, Yang SL, Guo YB. [Effect of ozonized saline on signaling passway of Keap1-Nrf2-ARE in rat hepatocytes]. Zhonghua Gan Zang Bing Za Zhi. May 2011;19(5):367-371.

²¹ Pecorelli A, Bocci V, Acquaviva A, et al. NRF2 activation is involved in ozonated human serum upregulation of HO-1 in endothelial cells. Toxicol Appl Pharmacol. Feb 15 2013;267(1):30-40.

²² Cho HY, Gladwell W, Yamamoto M, Kleeberger SR. Exacerbated airway toxicity of environmental oxidant ozone in mice deficient in Nrf2. Oxid Med Cell Longev. 2013;2013:254069.

²³ Re L, Martinez-Sanchez G, Bordicchia M, et al. Is ozone pre-conditioning effect linked to Nrf2/EpRE activation pathway in vivo? A preliminary result. Eur J Pharmacol. Nov 5 2014;742:158-162.

²⁴ Kim MY, Song KS, Park GH, et al. B6C3F1 mice exposed to ozone with 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and/or dibutyl phthalate showed toxicities through alterations of NF-kappaB, AP-1, Nrf2, and osteopontin. J Vet Sci. Jun 2004;5(2):131-137.

²⁵ Kontorshchikova KN, Solopaeva IM, Peretiagin SP. [Effect of ozone on the liver state in experimental chronic hepatitis]. Biull Eksp Biol Med. Aug 1996;122(8):238-240.

²⁶ P.V. Peretiagin, A.K. Martusevich, S.P. Peretiagin. A study of dose-dependent in microcirculatory response on prolonged course of ozonized saline infusions.

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At the same time, a research team led by Professor S. Razumovski verifies through research that the ozone decomposition processes in NaCl 0.9 % aqueous solutions is not accompanied by formation of products other than oxygen. In particular, no noticeable amounts of HOCl and chlorates were observed.²⁷ This is particularly significant for the medicinal application of ozonized isotonic solutions. In addition, professor Sergey Peritiagyn demonstrated that the concentration of sodium hypochlorite in the O₃SS was less than 0.001 g/mL.²⁸ It is clear that the concentration of hydrogen peroxide and HOCL is not visible or even noticeable.

In contrast, Levanov A.V. (2008, 2012) proved the presence of HOCL during the reaction of Ozone with a solution of NaCl.²⁹ However, the conditions under which Levanov did his experiment (pH, NaCl concentration, ozone concentration) were out of range of the conditions that apply during therapeutic O₃SS applications (Table 1).

Table 1. Different experimental conditions in which the presence of HOCL was measured during the reaction of O₃ with sodium saline solutions.

	NaCL (Conc.)	pH	O ₃ (µg/mL)	Flow (L / min)
Levanov <i>et al.</i>	1 M= 5,85 %	8,4-9,8	+ 30	21
Razumovskii	0,154 M= 0,9%	6,3	3-20	50, 15 min
O ₃ SS Therapeutic formula in use.	0,154 M= 0,9 %	5,5-5,7	1-2	200 mL/min Ozonation time: 10- 15 min

Legend: O₃SS, ozonized saline solution; Conc., concentration of NaCl in Molar or percent w/v. The criticisms of O₃SS toxicity by Levanov et al.³⁰ are extrapolated beyond their own experimental results, even suggesting the formation of chlorates and referring to their toxicity citing studies that show the formation of these components during the reaction of the fuel of the space shuttle with the sodium chloride present in the desert sand of Cape Canaveral in Florida-USA or in other environmental conditions.³¹ Nothing is further from the conditions of the therapeutic use of the O₃SS.

²⁷Razumovskii SD, Konstantinova ML, Grinevich TV, Korovina GV, Zaitsev VY. Mechanism and kinetics of the reaction of ozone with sodium chloride in aqueous solutions. *Kinetics and Catalysis*. 2010;51(4):492-496.

²⁸ Peretiagyn SP, Struchkov AA, Peretiagyn NC, Kulechina NB, Inventors; 2289413, assignee. *Ozonization Method of Saline Solution*. 2006.

²⁹ Levanov A, Kuskov I, Antipenko E, Lunin V. The Solubility of Ozone and Kinetics of Its Chemical Reactions in Aqueous Solutions of Sodium Chloride. *Russian Journal of Physical Chemistry*. 2008;82(12): 2045-2050.

³⁰ Levanov AV, Antipenko EE, Lunin VV. Primary stage of the reaction between ozone and chloride ions in aqueous solution: Oxidation of chloride ions with ozone through the mechanism of oxygen atom transfer. *Russian Journal of Physical Chemistry A*. 2012;86.

³¹ Suquet C, Warren JJ, Seth N, Hurst JK. Comparative study of HOCl-inflicted damage to bacterial DNA ex vivo and within cells. *Arch Biochem Biophys*. Jan 15 2010;493(2):135-142.

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Despite the work of Razumovskii, being criticized by Levanov *et al.*³² on the grounds that the analytical method used by Razumovskii,³³ is not sensitive enough to detect the concentrations of HOCL that are formed. According to the work of Levanov, the concentrations of HOCL that would form under the conditions of the O₃SS would be around ~ 2 µM, which would not have great relevance, because under physiological conditions, during the activation of neutrophils the concentrations of HOCL reach ~ 15 µM and these are the ones that manage to damage the bacterial DNA.^{34 35}

The concentration of other oxidants such as H₂O₂ determined under the conditions of the O₃SS is of the order of 0.11 mM. Levanov hypothesizes that the major risk of O₃SS is not the formation of reactive chlorine species, but, bromine. This hypothesis, according to the author, is based on the fact that bromine accompanies NaCl, the high reactivity of Br with O₃ and the possibility that hypochromic acid and bromate BrO₃⁻ are formed. However, the presence of these species has never been demonstrated under the conditions in which the O₃SS is performed. On the contrary, in the study carried out in China in 2019,³⁶ it was demonstrated that ozonation of the saline solution eliminates traces of Bromine that exist in the normal pharmacological formulation of the saline.

The study of the formation of other possible intermediaries such as sodium hypochlorite, hypochlorous acid, chlorates (ClO⁻, ClO₂⁻, ClO₃⁻), nitrites and nitrates (NO₂⁻ and NO₃⁻) indicate their absence in the reaction medium.^{37 38 39}

In regards to the mutagenicity and toxicity of ozone, studies in any of its administration methods show that the body can deal with it perfectly and trigger Nrf2 response, as long as repetitive

³² Levanov A, Kuskov I, Antipenko E, Lunin V. The Solubility of Ozone and Kinetics of Its Chemical Reactions in Aqueous Solutions of Sodium Chloride. Russian Journal of Physical Chemistry. 2008;82(12): 2045-2050.

³³ Razumovskii SD, Konstantinova ML, Grinevich TV, Korovina GV, Zaitsev VY. Mechanism and kinetics of the reaction of ozone with sodium chloride in aqueous solutions. Kinetics and Catalysis. 2010;51(4):492-496.

³⁴ Levanov AV, Antipenko EE, Lunin VV. Primary stage of the reaction between ozone and chloride ions in aqueous solution: Oxidation of chloride ions with ozone through the mechanism of oxygen atom transfer. Russian Journal of Physical Chemistry A. 2012;86.

³⁵ Suquet C, Warren JJ, Seth N, Hurst JK. Comparative study of HOCl-inflicted damage to bacterial DNA ex vivo and within cells. Arch Biochem Biophys. Jan 15 2010;493(2):135-142.

³⁶ Detection of chlorite, chlorate and perchlorate in ozonated saline. Experimental and Therapeutic Medicine 2019. dr. Ying Li or dr. Song Cao, Department of Pain Medicine, affiliated Hospital of Zunyi Medical University, 149 Dalian road, Zunyi, Guizhou 563003, P.R. China.

³⁷ Rao B, Anderson TA, Redder A, Jackson WA. Perchlorate formation by ozone oxidation of aqueous chlorine/oxy-chlorine species: role of ClxOy radicals. Environ Sci Technol. Apr 15 2010;44(8):2961-2967.

³⁸ Kang N, Jackson WA, Dasgupta PK, Anderson TA. Perchlorate production by ozone oxidation of chloride in aqueous and dry systems. Sci Total Environ. Nov 1 2008;405(1-3):301-309.

³⁹ Hypochlorous Acid Chemistry in Mammalian Cells—Influence on Infection and Role in Various Pathologies. Celia María Curieses Andrés 1, José Manuel Pérez de la Lastra 2,*, Celia Andrés Juan 3, Francisco J Plou 4, Eduardo Pérez-Lebeña 5. PMID: PMC9504810 PMID: 36142645. Int J Mol Sci. 2022 Sep 14;23(18):10735. doi: 10.3390/ijms231810735. International Journal of Molecular Sciences.

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stimuli of low doses of ozone are used.^{40 41 42} Strong evidence has been provided that the induced damage to DNA by the O₃ (chain breakage) in human leukocytes of the peripheral blood has a reversible effect; this indicates that the cells quickly recover from the genotoxic effect induced by treatment with the gas.^{43 44} The genomic mechanism of action of ozone was described much earlier in O₃SS than in major autohemotherapy, as described in Korean and Russian studies published in 2004-2011-2013.⁴⁵ These studies have demonstrated that the response is dependent on the activation of the transduction mechanisms of nuclear signals Nrf2 (nuclear factor erythroid 2), which is a powerful protein located within each cell in the body and which is driven by the activator Nrf2 inducing protein synthesis, such as SOD (superoxide dismutase), CAT (catalase), and HO1 (heme oxygenase 1) among others.^{46 47 48}

Ozone therapeutic indications are based on the knowledge that the use of low physiological doses of ozone plays an important role within the cell.⁴⁹ In 2013 and 2014 the same experiment as performed with O₃SS was done *in vitro* and *in vivo* with the MAHT. These studies demonstrated that the results were exactly the same as those that were found in the O₃SS. These data point for the first time to the activation of the Nrf2 pathway by low doses of ozone with the consequent promotion of feedback mechanisms which induce protein synthesis thus favouring collectively cell survival. Thus, Nrf2 system contributes to the protection against various pathologies,

⁴⁰ Boyarinov GA, Gordetsov AS, Peretyagin SP, Matusyak KS, Ovchinnikov YV, Boyarinnova LV. The analysis of interaction of ozone and sodium chloride in Aqueous solution. Rev Esp de Ozonoterapia. 2016;6(Supp 1):77.

⁴¹ Viviana C, Gabriele T. Exposure to low ozone concentrations induces cytoskeletal reorganization, mitochondrial activity and nuclear transcription in epithelial human cells. Paper presented at: European Cooperation of Medical Ozone Societies Congress2014; Zurich.

⁴² Viebahn-Hänsler R, Fernández OSL, Fahmy Z. Ozone in Medicine: The Low- Dose Ozone Concept. Guidelines and Treatment Strategies. Ozone Science & Engineering. 2012;34(6):408-424.

⁴³ Bocci V. Is it true that ozone is always toxic? The end of a dogma. Toxicol Appl Pharmacol. Nov 1 2006;216(3):493-504.

⁴⁴ Diaz-Llera S, Gonzalez-Hernandez Y, Prieto-Gonzalez EA, Azoy A. Genotoxic effect of ozone in human peripheral blood leukocytes. Mutat Res. May 27 2002;517(1-2):13-20.

⁴⁵ Kim MY, Song KS, Park GH, et al. B6C3F1 mice exposed to ozone with 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and/or dibutyl phthalate showed toxicities through alterations of NF-kappaB, AP-1, Nrf2, and osteopontin. J Vet Sci. Jun 2004;5(2):131-137.

⁴⁶ Qu DD, Peng FJ, Liu L, Yang SL, Guo YB. [Effect of ozonized saline on signaling passway of Keap1-Nrf2-ARE in rat hepatocytes]. Zhonghua Gan Zang Bing Za Zhi. May 2011;19(5):367-371.

⁴⁷ Kim MY, Song KS, Park GH, et al. B6C3F1 mice exposed to ozone with 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and/or dibutyl phthalate showed toxicities through alterations of NF-kappaB, AP-1, Nrf2, and osteopontin. J Vet Sci. Jun 2004;5(2):131-137.

⁴⁸ Cho HY, Gladwell W, Yamamoto M, Kleeberger SR. Exacerbated airway toxicity of environmental oxidant ozone in mice deficient in Nrf2. Oxid Med Cell Longev. 2013;2013:254069.

⁴⁹ Viviana C, Gabriele T. Exposure to low ozone concentrations induces cytoskeletal reorganization, mitochondrial activity and nuclear transcription in epithelial human cells. Paper presented at: European Cooperation of Medical Ozone Societies Congress2014; Zurich.

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including carcinogenesis, liver toxicity, respiratory and chronic inflammatory diseases, neuronal ischemia and renal problems.^{50 51 52}

Currently, the use of O₃SS has been heavily manipulated,⁵³ and pseudo-scientific arguments have attempted to hinder the use of this therapy. But the real interest is probably commercial, with the assumption that those who practice O₃SS will stop using the Major Autohemotherapy (MAHT) method and consequently stop buying the MAHT kit. Actually, the O₃SS and MAHT are two systemic methods of ozone administration, each with its advantages and disadvantages.

⁵⁰ Delgado-Roche L, Riera-Romo M, Mesta F, Hernández-Matos Y, Barrios JM, Martínez- Sánchez G. Medical Ozone Promotes Nrf2 Phosphorylation Reducing Oxidative Stress and Proinflammatory Cytokines in Multiple Sclerosis Patients. Rev Esp Ozonoterapia. 2018 2018;8(2 Supp 1):48-49.

⁵¹ Huth KC, Saugel B, Jakob FM, et al. Effect of aqueous ozone on the NF-kappaB system. J Dent Res. May 2007;86(5):451-456.

⁵² Yu G, Liu X, Chen Z, et al. Ozone therapy could attenuate tubulointerstitial injury in adenine-induced CKD rats by mediating Nrf2 and NF-κB. Iranian Journal of Basic Medical Sciences. Oct 2016 2016;19(10):1136-1143.

⁵³ WFOT. Study on the scientific basis of ozonized saline solution. <https://www.wfoot.org/wp-content/uploads/2015/11/WFOT-about-ozonized-saline-FINAL-signed.pdf>. 2017.

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1.1.3 Ozonized Saline Solution in Covid-19

During the peak periods of the pandemic (April, May 2020) in Madrid, Dr. Adriana Schwartz conducted a study on 600 patients at the Virgen de la Paloma hospital in Madrid. ⁵⁴

The study was approved by the ethics committee of the Community of Madrid and the hospital Viamed Virgen de la Paloma.

The study was based on the following mechanisms of ozonated solution action:

- as an inducer of adaptation to oxidative stress.
- as a modulator of pro-inflammatory cytokines.
- as an improvement of tissue oxygenation.
- as an anti-platelet aggregant.
- as a virucidal.

Results.

The main results of O₃SS treatment were a tendency to improve clinical symptoms without side effects.

- Within 24 hours, PCR curves plummeted.
- The fever returned to normal quickly, while the dyspnea and fatigue decreased significantly.
- Patients with COVID-19 with mild to severe symptoms who received intravenous O₃SS as an adjunct treatment experienced no side effects.
- At 72 hours the PO₂ saturation in all patients improved markedly to 96-98%.
- On the 5th day of treatment, laboratory tests (Dimer-D, Fibrinogen, PCR, LDH, Ferritin) were at low risk and the patients began to be discharged from the hospital.
- On the 10th day, the entire COVID-19 unit of the hospital was discharged.
- No deaths were recorded.

1.1.4 Ozonized Saline Solution Under Micro bubbling

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The size of an ozone gas bubble in O₃SS is a determining characteristic to understand its properties, since a micro/nanometric scale size distribution is associated with better stability, mass transfer, significantly influencing its behavior and others physicochemical and electrical characteristics within a liquid such as saline solution.⁵⁵

The micro/nano bubbles remain in the water or saline solution for a long time and act like a battery that continuously supplies ozone to the water. When the ozone is consumed, they spread more quantity, maintaining the dissolved ozone level (the concentration). The micro/nano bubbles do not go to the surface, but are distributed evenly within the body of water. Larger bubbles tend to emerge showing greater buoyant force, while smaller bubbles remain in the liquid medium more easily and longer due to a pattern of random motion or Brownian motion.

Inhibition of bubble coalescence under the microbubble leads to greater ozone exposure to the treated tissue surface, therefore optimizing and accelerating treatments. Reduces solution saturation time to 5 min at a low flow rate of 200 mL/min, which significantly reduces preparation times, saving time, oxygen and generator wear. Ozone concentration in the solution is maintained for more than 30 minutes, which means the solution can be transfused to the patient without bubbling.⁵⁶

The ASSO3® micro bubbling device was designed by Dr. Adriana Schwartz and it has been patented in Spain, Europe and in more than 150 countries. It is made of reusable medicinal glass, meaning it can be sterilized in an autoclave, preserving the ecological environment. In addition, it lowers the cost for the doctor and for the patient.

Plexiglas devices are less resistant to sustained exposure to ozone.

⁵⁴ Schwartz et al. Complementary application of the ozonized saline solution in mild and severe patients with pneumonia COVID-19: A non-randomized pilot study. *Journal of Pharmacy & Pharmacognosy Research*, 9 (2), 126-142, 2021 ISSN 0719-4250 <http://jppres.com/jppres>

⁵⁵ . Schwartz, Adriana (June 2023). Study on Ozonated Saline Solution (O3SS) Under Microbubbling in a Glass Device (ASSO3). Basis, Advantages and Clinical Applications. *Ozone Therapy Global Journal* Vol. 13, n° 1, pp 29-45

⁵⁶ . Schwartz, Adriana (June 2023). Study on Ozonated Saline Solution (O3SS) Under Microbubbling in a Glass Device (ASSO3). Basis, Advantages and Clinical Applications. *Ozone Therapy Global Journal* Vol. 13, n° 1, pp 29-45

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Recent comparative studies of micro bubbling versus conventional bubbling in patients with kidney failure undergoing dialysis (Dr. Estoneck Guevara, 2025)⁵⁷ showed:

- That the micro bubbling technique generates 30-50% less H₂O₂ than conventional bubbling.
- That both O₃SS methods increase mitochondrial activity and cell apoptosis in patients with chronic kidney disease, although the increase was more evident in micro bubbling. This finding implies an increase in mitochondrial membrane potential, which implies greater activity in the organelle, which could be associated with recovery and therefore greater ATP synthesis.
- That the O₃SS solution under micro bubbling increases antioxidant defense (CAT, SOD, GSH/GSSG) and decreases cellular catabolism and organ deterioration in patients with chronic kidney disease.
- In patients treated with O₃SS using the traditional bubbling method, although an increase in GFR (glomerular filtration rate) is observed, it is not statistically significant. Meanwhile, in patients treated with SSO₃ using micro bubbling, their glomerular filtration rate increased by 80%.

Reflection: If these encouraging data are observed in patients as severely ill as those with kidney failure undergoing hemodialysis, then in patients with early-stage kidney failure, we can infer that these inflammatory processes would possibly be reversed, leading to greater life expectancy and quality of life.

The properties of O₃SS under micro bubbling can be summarized:

1. It is highly virucidal.
2. Modulates the Nrf2 / NF-kB balance
3. Undetectable levels of hypochlorous acid (HOCL).
4. Does not produce chlorates. Does not produce bromates.
5. It is not carcinogenic.
6. Modulates and decreases inflammatory cytokines TNF alpha, IL-1 Beta and IL-6.
7. Modulates oxygen metabolism. Improves hypoxia in 24/48 h.
8. At low doses it increases mitochondrial activity and cellular apoptosis in patients with chronic kidney disease.
9. Increases antioxidant defense (CAT, SOD, GSH/GSSG), decreases cellular catabolism and organic deterioration in patients with chronic kidney disease.
8. Increases prostacyclin levels.
9. Modulates the release of nitric oxide.
10. Decreases the NLRP3 inflammasome
11. Modulates oxidative stress with cytoprotective effect.
12. Reverses chronic oxidative stress by modulating the TH1, TH17 and TH2 cytokine pattern.
13. Modulates PLA-2 (phospholipase A2).
14. Decreases ICAM (intercellular adhesion molecule).
15. Fewer sessions are needed to obtain results.

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16. It can be applied without distinction to any religious group.
17. Broad scientific basis and use for more than 40 years.
18. By not manipulating blood, it is a much more user-friendly procedure for health authorities and the patient.

1.2. Purpose

The purpose of this SOP is to describe the procedure for O₃SS, in particular the micro bubbling system.

1.3. Scope

This procedure specifies the technique, doses, volume of gas, volume of saline solution, ozone flow, ozone bubbling time and frequency of application of O₃SS by I.V. infusion.

1.4. Acronyms, abbreviations and definitions

G6PD	Glucose 6 phosphate dehydrogenase
O ₃ SS	Ozonized Saline Solution
HOCL	Hypochlorous Acid
NaCl	Sodium Chloride
MAHT	Major autohemotherapy
RIO ₃	Rectal Insufflations of Ozone
ROS	Reactive Oxygen Species
SOP	Standard Operating Procedure
Total doses	Total amount, in micrograms, of ozone given per session, calculated as volume in mL multiplied by concentration in µg/mL

2. Responsibility

Physician	Patients' Clinical records registration Assessment of the indication, contraindications Request the informed consent (ISCO3/QAU/00/21) and the privacy consent Applications and monitoring Recording all data on medical records Prescription of investigations to assess the effectiveness of the treatment (e.g. biochemical or immunological assays) Reporting any late complications
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⁵⁷ Guevara-Aguilar, E., Moroni-González, D. Jiménez-Ortega, J. C., Treviño, S., & Sarmiento-Ortega, V.E. (2025). Comparison of Microbubbling and Conventional Bubbling Methods for Ozonated Saline Solution in CDK patients: Pilot Study. Free Radical Research, 1-13.

[https://www.tandfonline.com/doi/full/10.1080/10715762.2025.2483454?src=#:~:text=In%20vivo%2C%20both%20methods%20increased, ratio\)%20compared%20to%20conventional%20bubbling.](https://www.tandfonline.com/doi/full/10.1080/10715762.2025.2483454?src=#:~:text=In%20vivo%2C%20both%20methods%20increased, ratio)%20compared%20to%20conventional%20bubbling.)

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Patient follow-up

Nurses	Accommodate the patients Preparation of the clinical procedure Supervision of patients, and vital signs control (temperature and pressure) Detects and alerts the doctor to anomalies due to possible reactions Notification of possible complications.
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A O₃SS session should be done by a physician, adequately trained in ozone therapy. Also, a trustworthy assistant, nurse, or paramedical professional, may do the procedure, provided this person is adequately trained for this work. It is the physician's responsibility to see that all steps of the procedure are done in the correct manner, in order to always avoid errors, accidents, and to prevent incidents.

3. Procedure

3.1 Doses and Indications.^{58 59}

The ozonation is carried out with very low ozone concentrations which are calculated according to the weight of the patient, which makes the method very personalized.

Low doses 1.0 µg/kg are used to stimulate the immune system for diseases of the cardiovascular system, and for obstetrics, to prevent toxicity in the first trimester of pregnancy and foetal hypoxia in the third trimester. Cancer.

Low/Medium doses 2-3 µg/kg are used for detoxification in endo-toxemia, chronic inflammatory diseases of different aetiology, viral diseases and neurodegenerative diseases.

High doses 4-5 µg/kg are used in the treatment of sepsis, infectious diseases, as well as in skin and burn diseases.

3.2 Contraindications.⁶⁰

- ✓ Absolute contraindication: Favism: Glucose-6-phosphate dehydrogenase deficiency (favism).*
- ✓ Acute myocardial infarction

⁵⁸ Schwartz et al. Complementary application of the ozonized saline solution in mild and severe patients with pneumonia COVID-19: A non-randomized pilot study. Journal of Pharmacy & Pharmacognosy Research, 9 (2), 126-142, 2021 ISSN 0719-4250 <http://jppres.com/jppres>

⁵⁹ Schwartz, Adriana (June 2023). Study on Ozonated Saline Solution (O3SS) Under Microbubbling in a Glass Device (ASSO3). Basis, Advantages and Clinical Applications. Ozone Therapy Global Journal Vol. 13, n° 1, pp 29-45

⁶⁰ Schwartz A. Ozone Therapy Clinical Manual. Madrid, Spain: Medizeus Soluciones Médicas S.L.; 2020

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- ✓ Pregnancy in the first 3 months
- ✓ Uncompensated toxic hyperthyroidism - Basedow Graves status
- ✓ Thrombocytopenia less than 50.000 and serious coagulation disorders
- ✓ Severe Cardiovascular instability
- ✓ Acute alcohol intoxication
- ✓ Acute infarction of myocardium
- ✓ Massive and acute hemorrhage
- ✓ During convulsive states
- ✓ Hemochromatosis
- ✓ Patients receiving treatment with copper or iron.

* The prevalence of Glucose 6 phosphate dehydrogenase (G6PD) deficiency varies among ethnic groups with overall lower frequency in the Americas (3.4%), Europe (3.9%), and the Pacific (2.9%) as compared to sub-Saharan Africa (7.5%), the Middle East (6.0%), and Asia (4.7%).^{61 62} Test of G6PD is recommended prior to O₃ therapy in order to avoid complications).

3.3 O₃SS Dose Calculation. ^{63 64 65}

Calculation of the ozone gas concentration to prepare ozonated saline:

Please note that the dissolved ozone concentration is 25% of the ozone gas concentration, then the dissolved ozone concentration in saline, should be multiplied by 4 to get the ozone concentration for bubbling the saline solution.

Dose Formula:

Dose (μ g) = dissolved ozone concentration (μ g/mL) · Volume (mL) saline solution.

Low Dose 1.0 μ g/kg in 200 mL

A patient weighing 80 kg will receive **80 mg Total Concentration**.

When administering 200 mL, the ozone concentration in the bottle should be:

80 mg x (1.0 L/0.20 L) = 400 mg /L Concentration in the bottle.

(1.0/0.2 = 5) 5 x 80= 400 μ g/L

400 mg/L x 4 = 1600/1000= 1.6 μ g/NmL Saturation concentration (ozone generator output)

1.6 μ g/NmL / 4 = 0.4 μ g/NmL Dose that the patient receives

⁶¹ Nkhoma ET, Poole C, Vannappagari V, Hall SA, Beutler E. The global prevalence of glucose- 6-phosphate dehydrogenase deficiency: a systematic review and meta-analysis. Blood Cells Mol Dis. May-Jun 2009;42(3):267-

⁶² González Sánchez, Esteban et al.. (2019). Anemia hemolítica por déficit de G6PDH. a propósito de la ozonoterapia. Ozone Therapy Global Journal vol. 9, nº 1, pp 87-102

⁶³ Schwartz A. Ozone Therapy Clinical Manual. Madrid, Spain: Medizeus Soluciones Médicas S.L.; 2020

⁶⁴ Schwartz et al. Complementary application of the ozonized saline solution in mild and severe patients with pneumonia COVID-19: A non-randomized pilot study. Journal of Pharmacy & Pharmacognosy Research, 9 (2), 126-142, 2021 ISSN 0719-4250 <http://jppres.com/jppres>

⁶⁵ Schwartz, Adriana (June 2023). Study on Ozonated Saline Solution (O3SS) Under Microbubbling in a Glass Device (ASSO3). Basis, Advantages and Clinical Applications. Ozone Therapy Global Journal Vol. 13, nº 1, pp 29-45

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Low Dose 1.0 µg/kg in 250 mL

A patient weighing 80 kg will receive **80 mg Total Concentration**.

When administering 250 mL, the ozone concentration in the bottle should be:

$80 \text{ mg} \times (1.0 \text{ L} / 0.250 \text{ L}) = 320 \text{ mg/L}$

$(320 \text{ mg/L} / 4) \times 80 = 6400 / 1000 = 6.4 \text{ µg/NmL}$ Saturation concentration (ozone generator output)

$6.4 \text{ µg/NmL} / 4 = 1.6 \text{ µg/NmL}$ Dose that the patient receives.

Low/Medium Dose 2.0 µg/kg in 200 mL

A patient weighing 80 kg will receive **160 mg Total concentration**.

$160 \text{ mg} \times (1.0 \text{ L} / 0.20 \text{ L}) = 800 \text{ mg/L}$ Concentration in the bottle.

$800 \text{ mg/L} \times 4 = 3200 / 1000 = 3.2 \text{ µg/NmL}$ Saturation concentration (ozone generator output).

$3.2 \text{ µg/NmL} / 4 = 0.8 \text{ µg/NmL}$ Dose that the patient receives.

Low/Medium Dose 2.0 µg/kg in 250 mL

A patient weighing 80 kg will receive **160 mg Total concentration**.

$160 \text{ mg} \times (1.0 \text{ L} / 0.25 \text{ L}) = 640 \text{ mg/L}$ Concentration in the bottle.

$640 \text{ mg/L} \times 4 = 2560 / 1000 = 2.56 \text{ µg/NmL}$ Saturation concentration (ozone generator output).

$2.56 \text{ µg/NmL} / 4 = 0.64 \text{ µg/NmL}$ Dose that the patient receives.

Medium Dose 3 µg/kg in 200 mL

A patient weighing 80 kg will receive: $80 \times 3 = 240 \text{ mg Total concentration}$.

$240 \text{ mg} \times (1 \text{ L} / 0.2 = 5) = 1200 \text{ mg/L}$ Concentration in the bottle.

$1200 \text{ mg/L} \times 4 = 4800 / 1000 = 4.8 \text{ µg/NmL}$ Saturation concentration (ozone generator output).

$4.8 \text{ µg/NmL} / 4 = 1.2 \text{ µg/NmL}$ Dose that the patient receives.

Medium Dose 3 µg/kg in 250 mL

A patient weighing 80 kg will receive: $80 \times 3 = 240 \text{ mg Total concentration}$.

$240 \text{ mg} \times (1 \text{ L} / 0.25 \text{ L} = 4) = 960 \text{ mg/L}$ Concentration in the bottle.

$960 \text{ mg/L} \times 4 = 3840 / 1000 = 3.84 \text{ µg/NmL}$ Saturation concentration (ozone generator output).

$3.84 \text{ µg/NmL} / 4 = 0.96 \text{ µg/NmL}$ Dose that the patient receives

Medium/High Dose 4 µg/kg in 200 mL

A patient weighing 80 kg will receive: $80 \times 4 = 320 \text{ mg Total concentration}$.

$320 \text{ mg} \times (1 \text{ L} / 0.2 = 5) = 1600 \text{ mg/L}$ Concentration in the bottle.

$1600 \text{ mg/L} \times 4 = 6400 / 1000 = 6.4 \text{ µg/NmL}$ Saturation concentration (generator output)

$6.4 \text{ µg/NmL} / 4 = 1.6 \text{ µg/NmL}$ Dose that the patient receives.

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Medium/High Dose 4 µg/kg in 250 mL

A patient weighing 80 kg will receive: $80 \times 4 = 320 \text{ mg}$ **Total concentration.**

$320 \text{ mg} \times (1 \text{ L}/0.25 \text{ L} = 4) 4 \times 320 = 1280 \text{ mg/L}$ **Concentration in the bottle.**

$1280 \text{ mg/L} \times 4 = 5100/1000 = 5.1 \text{ µg/NmL}$ **Saturation concentration (ozone generator output).**

$5.1 \text{ µg/NmL}/4 = 1.2 \text{ µg/NmL}$ **Dose that the patient receives.**

High Dose 5 µg/kg in 200 mL

A patient weighing 80 kg will receive: $80 \text{ kg} \times 5 \text{ µg/kg} = 400 \text{ mg}$ **Total concentration.**

$400 \text{ mg} \times (1 \text{ L}/0.2 = 5) 5 \times 400 = 2000 \text{ mg/L}$ **Concentration in the bottle.**

$2000 \text{ mg/L} \times 4 = 8000/1000 = 8.0 \text{ µg/NmL}$ **Saturation concentration (generator output)**

$8.0 \text{ µg/NmL}/4 = 2.0 \text{ µg/NmL}$ **Dose that the patient receives.**

High Dose 5 µg/kg in 250 mL

A patient weighing 80 kg will receive: $80 \text{ kg} \times 5 \text{ µg/kg} = 400 \text{ mg}$ **Total concentration.**

$400 \text{ mg} \times (1 \text{ L}/0.25 = 4) 4 \times 400 = 1600 \text{ mg/L}$ **Concentration in the bottle.**

$1600 \text{ mg/L} \times 4 = 6400/1000 = 6.4 \text{ µg/NmL}$ **Saturation concentration (generator output).**

$6.4 \text{ µg/NmL}/4 = 1.6 \text{ µg/NmL}$ **Dose that the patient receives.**

Bubbling Saturation time of the ordinary saline solution is of 10-15 min.

The transfusion of the ozonated saline solution is done under constant bubbling at the same parameters.

The upper limit of the dose of ozone in the ozonized saline solution the patient receives is 2 µg/L ; exceeding this limit is dangerous and can cause phlebitis. The exceptional cases are severe sepsis and severe viral infections. In such cases, the dose may be increased up to 5 µg/kg *The patient receives 1.6 µg/NmL $µ\text{g/NmL}$.*

In the case of micro bubbling, the saturation time of the O_3SS is just 5 minutes and the patient is not reinfused under bubbling. Ozone concentration in the solution is maintained for more than 30 minutes, which means the solution can be transfused to the patient without bubbling, releasing the generator. The generator flow must be variable, to adjust it to 200 mL/min

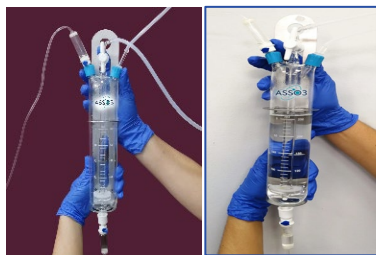


Fig.1 ASSO3® Micro bubbling medical device.

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Note: The volume of saline solution used for one procedure is (200-250) mL, regularly 250 mL. The number of procedures for one course of treatment is 6 to 10. Procedures are conducted daily, every two days or once a week. It will depend on the severity of the patient's pathology. Very important. The maximum weight to consider for dose calculation is 90 kg regardless of whether the patient weighs more.

3.4 Clinical evaluation

A clinical and/or laboratory evaluation is necessary to establish a precise diagnosis and to permit comparisons between the patient's status before, during and after ozone therapy.

3.5 Preliminary operations

The patient must be fully informed in advance about the method itself, about all the steps of the procedure, about the desired effect(s) and also about the possible unwanted side effects. Also, a written Term of Informed Consent should be read, understood and signed by the patient or the person responsible for the patient.

Device: Generator used should be in line with the recommendations of ISCO3 ISCO3/DEV/00/01.⁶⁶ and the oxygen-ozone gas mixture must pass an antimicrobial sterile filter ($< 20 \mu m$) before being used. Generator should be generating continuous and variable flow. In the case of O₃SS the flow should be low (100-200 mL/min), generate precise low normalized ozone concentration ranges (1-10) $\mu g/NmL$, and have a dynamic control that standardizes the concentrations.

All materials used must be disposable and ozone resistant: glass, silicone probes, catheters and silicone tubes, connections of Kynar or stainless steel 316, and siliconized syringes. Saline solution should always be administered in a glass bottle, which is the only material that guarantees the non-production of phthalates.^{67 68 69}

Syringes: Plastic-based devices, must meet the ISO 15747 standard: 2005 (This is the European Union regulation). All containers and devices used in O₃ therapy must be ozone-resistant and must not release phthalates.

⁶⁶ISCO3. Guidelines and Recommendations for Medical Professionals Planning to Acquire a Medical Ozone Generator. <http://www.isco3.org/offdocs.html>. 2014.

⁶⁷ Schwartz A. Solución Salina Ozonizada (SSO3): Fundamentos Científicos. Revista Española de Ozonoterapia. 2016;6(1):111-120.

⁶⁸ Schwartz A. Ozone Therapy Clinical Manual. Madrid, Spain: Medizeus Soluciones Médicas S.L.; 2020. In English.

⁶⁹ . Schwartz, Adriana (June 2023). Study on Ozonated Saline Solution (O3SS) Under Microbubbling in a Glass Device (ASSO3). Basis, Advantages and Clinical Applications. Ozone Therapy Global Journal Vol. 13, n° 1, pp 29-45

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3.6 Main procedure

Three needles technique.

The nurse should wash his/her hands and put on gloves.

Physicians should establish the dose (low, medium or high).

Weigh the patient and multiply the weight by the conversion factor according to the dose level selected.

Take a bottle of volume ~250 mL saline solution 0.9% sterile for intra venous infusion, insert a mono-use infusion system for intravenous administration (in the saturation process it remains closed) and two needles, one long (e.g. 1.3 mm · 75 mm /18 G · 3”) and one shorter (e.g. 1.2 mm · 38 mm / 18G · 1 ½”). Place the bottle with the cap on the bottom of the holder.

Saturation of the saline solution: Put a sterilizing filter in the outflow nozzle of the ozone generator and connect this line to the shorter needle. At the exit of the longest needle an ozone destroyer is placed. Bubble saline solution with ozone for 10 min.

After bubbling, using the infusion set, the solution is administered i.v during (15 to 30) min. When using this method, the drip should be monitored very carefully, since it increases the risk of a gas embolism if the patient is not separated from the intravenous administration system in time. Therefore, ozonation should be completed when about 50 mL of liquid remaining in the bottle (interrupt the arrival of the mixture of ozone and oxygen to the bottle).

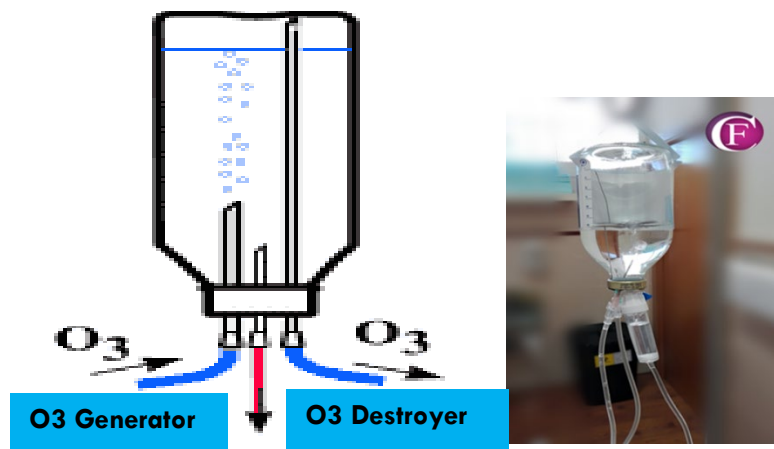


Fig.2 O₃SS, Ozonized Saline Solution. Three needle method

3.7 Side effects

Most adverse effects are due to malpractice or lack of knowledge of the therapy.

- Phlebitis. This is in the case of using very high nor therapeutic doses.
- Accidental gas embolism. This happens in conventional bubbling, when the therapist doesn't stop bubbling at 50 mL and the procedure is not being checked.
- Using plastic bags for bubbling leads to the production of a huge amount of phthalates, which are toxic to the body and increase inflammatory processes.
- In case of side effects follow the instructions of ISCO3/CLI/00/01 "First Aid in ozone therapy (Inhalation exposure and accidental over dose)" and report the side effect using

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ISCO3/REC/00/03 "The ISCO3 Safety Information and Adverse Event Reporting Program Form".

3.8 Patients Follow-up

Patients may be followed and re-evaluated from time to time, from the clinical / subjective point of view and/or with laboratory and/or image examinations.

3.9 Informed consent

Written informed consents are mandatory.

4.0 Mechanism of action of O₃SS

At low doses, systemically applied ozone in the form of O₃SS acts as a bioregulator, ozone intermediary (H₂O₂, 4-hydroynonenal, etc.)⁷⁰ induce a signal transduction via the oxidation of glutathione or cysteine residues and the corresponding nuclear factors, resulting in a regulation of the antioxidants via Nrf2 information, or an immunomodulation via NFkB. In fact, Nrf2 signaling was discovered in the O₃SS.⁷¹

Table1. Pre-clinical, clinical and side effects described for Ozone Saline Solution.

Method	Support by Pre-clinic research	Support by clinical trials	Side effects
O ₃ SS	Biochemical, Molecular and pre-clinical.	>500 Russian doctoral theses. See, table 2	No side effects reported

⁷⁰ Schwartz A. Ozone Therapy Clinical Manual. Madrid, Spain: Medizeus Soluciones Médicas S.L.; 2020

⁷¹ Qu DD, Peng FJ, Liu L, Yang SL, Guo YB. [Effect of ozonized saline on signaling passway of Keap1-Nrf2-ARE in rat hepatocytes]. Zhonghua Gan Zang Bing Za Zhi. May 2011;19(5):367-371.

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Table 2. Examples of clinical trial using O₃SS.

Pathology / Population studied	Dosage	Results	Reference
Acute appendicitis 100 children from 1 to 14 years old (70 treated, 30 control).	Concentration of O ₃ in the O ₃ SS 1.0 mg / L. Dosage 5-8 mL / kg b.w. in children under 5 years old and 8-10 mL / kg b.w. in children over 5 years old. O ₃ SS via i.v. and in to wash the surgical wound.	Analgesic effects, avoiding the use of narcotics, appetite normalization, sleep improvement. 82.3% effectiveness compared to control.	Azov N.A. <i>et al.</i> 2016. ⁷²
Brain trauma Concussion (62 patients), mild contusion of the brain (35). 46 controls that received conventional therapy	O ₃ concentration in the O ₃ SS 3.5 mg / L, 0.5 L / min for 15 min. A daily treatment for 10 days.	Improvement of the clinical symptomatology compared to the control group.	Barkalov S.V. and Y.V.Daniel (2016). ⁷³
Diabetic foot 20 control patients and 20 treated patients	Concentration of O ₃ in the O ₃ SS 2.5 mg / L, 400 mL of saline. A daily treatment for 10 days via i.v. plus local treatment with antiseptics.	Improvement of the clinical symptomatology compared to the control group.	Belyaev A. <i>et al.</i> (2016). ⁷⁴
Obstructive jaundice 36 patients treated with O ₃ SS and 44 with conventional therapy	Concentration of O ₃ in the O ₃ SS 2.5 mg / L. A daily treatment for 10 days via i.v. during the post-operative.	Thromboplastin time was recovered 2 times faster than in the control group.	Belyaev A.N. <i>et al.</i> (2016). ⁷⁵
Vulvar leukopathy 155 women, 75 treated with ozone and 80 with conventional therapy.	O ₃ concentration in the O ₃ SS 4.0-4.5 mg / L. From 5 to 6 treatments on alternate days via i.v. Local treatment with O ₃ SS 9-10 mg / L. Subsequent treatment with ozonated oil.	Symptoms resolution by 95%, and pain reduction.	Boyko E.L. and A.I. Malyshkina (2016). ⁷⁶

⁷² Azov NA, Azova EA, Chekalova SA. Ozone, EHF-therapy in complex treatment of children. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):5.

⁷³ Barkalov SV, Y.V.Daniel. Evaluating the effectiveness of the use of ozone therapy in the treatment of combat trauma with brain damage. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):6.

⁷⁴ Belyaev A, Rodin A, Zakhvatov A. Effects of ozone therapy on the course of diabetic wound process. Rev. Española de Ozono Terapia. 2016;6(2):11.

⁷⁵ Belyaev AN, Kozlov SA, Belyaev SA, Kostin SV. Influence of ozone therapy on postoperative hemostasis system dynamics in patients with obstructive jaundice. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):13.

⁷⁶ E.L. B, Malyshkina AI. The use of ozone therapy in the treatment of leukoplakia vulva in women. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):17.

	International Scientific Committee of Ozone Therapy Cell Phone (+34) 636769899 Avenida Juan Andrés 60. Local 1 – Bajo CP 28035, Madrid (Spain) info@isco3.org www.isco3.org	SOP: ISCO3/MET/00/25 Version: Final Date: April.17, 2025 Page 21 of 22 Author: Dr. Adriana Schwartz
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Delayed foetal growth. 24 women treated with O ₃ SS and 80 in the control group.	O ₃ concentration in the O ₃ SS 0.4 mg / L. 5 treatments via i.v.	Significant improvement in labor and in the Apgar index.	Boyko E.L. and P.L. Mileeva (2016). ⁷⁷
Lymphovenous failure of lower limbs. 37 patients	Concentration of O ₃ in the O ₃ SS (0.8-1.0) mg / L. 10- 12 treatments via i.v. Local treatment with O ₃ SS	Decreased pain and microbial count, increase quality of life.	Knyazev V.N. and E.S. Fattyakhudinova (2016). ⁷⁸

Legend: O₃SS, Ozonized Saline Solution; b.w., body weight; i.v., intravenous.

⁷⁷ Boyko EL, Mileeva PL. The use of medical ozone in complex treatment women with fetal growth retardation. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):18.

⁷⁸ Knyazev VN, Fattyakhudinova ES. Phlebotropic adjuvant regional ozone therapy of lower limb lymphovenous failure. Rev. Española de Ozono Terapia. 2016;6(2 Suppl. 1):34.

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5.0 SOP References

ISCO3/QAU/00/21. Informed Consent Form in Ozone Therapy.
 ISCO3/DEV/00/01 Guidelines and Recommendations for Medical Professionals Planning to Acquire a Medical Ozone Generator.
 ISCO3/CLI/00/01. First Aid in ozone therapy (Inhalation exposure and accidental over dose)
 ISCO3/REC/00/03 The ISCO3 Safety Information and Adverse Event Reporting Program Form.
 ISCO3/QAU/01/03. **Madrid Declaration on Ozone Therapy** 3th ed. ISCO3 2020. Schwartz-Tapia A. *et al.*; ISBN 978-84-606-8312-4

6.0 Documentation and Attachments

6.1 List of recommended medical devices.

ASSO3® medical device. Dual Kit®, Siliconated Luer lock syringe of 100 mL, 50 mL or 60 mL Gloves and disinfectant solution.

6.2. Change History

SOP N°	Effective Date	Significant Changes	Previous SOP no.
ISCO3/MET/00/21	Dec. 4 th 2024	First Draft.	First version
ISCO3/MET/00/21	Dec. 17 th 2024	English Review done by Dr. Wayne McCarthy	Second version
	April 17 2025	Final version	Final version

7.0 Document Records

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Author	Adriana Schwartz, M.D. Gynecologist E-mail: adrianaschwartztapia@gmmail.com	ISCO3 president
Authoriser / Approved	ISCO3 Board and members	All members April 17 th 2025