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## Original paper

# Study on Ozonated Saline Solution (O3SS) Under Microbubbling in a Glass Device (ASSO3). Basis, Advantages and Clinical Applications.

**Schwartz, Adriana**
Director and Scientific Advisor of Clínica Fiorela, President of Aepromo, ISCO3 Scientific Secretary

## Keywords

*micro bubbling, nano bubbling, ozonated saline solution (O3SS), ozone, advanced ozonated saline solution (ASSO3®), antibacterial agent, anti-inflammatory agent, ozone in dental plaque, ozone in medicine. ozone therapy..*

## Abstract

*Ozone therapy is a technology that is currently being used in the treatment of a wide variety of diseases, with increasing diffusion in the medical field.*
*One of the most widely used techniques to transfer ozone to a patient consists of bubbling ozone at low concentrations into a saline solution and injecting it into the patient. Objective: to analyze the efficiency of the ozonation technique of a saline solution under micro-bubbling/nano-bubbling in a closed glass device system: its advantages compared to the ozone dose that is finally transferred to the patient intravenously and its benefits in applications in medicine and dentistry. Method: For the study, ozone was micro-bubbled at different standardized concentrations in a closed glass device, specially designed for this purpose, with saline solution (NaCl 0.9%) and then passed through an ozone concentration analyzer in liquids. brand BMT964 AQ-LC, Messtechnick GmbH, (Germany), for an Anseros® spectrophotometer model OZONE MONITOR GM-RTI® (Germany) and a Mass Spectrophotometer, (GCMS). Two ozone generators were used: the Ozonobaric P® Sedecal® (Spain) and the Ozonette® Sedecal® (Spain) The first and second parts of the study were carried out at CSI ANALITICA SL, Tres Cantos, Madrid, Spain. https://www.csianalitica.com*
*Results: After 5 min of ozone micro-bubbling, the solution became saturated. 25% ozone was dissolved in the saline solution. Post saturation, the ozone concentration in the solution remained stable for 30 minutes without bubbling. Technical conclusions: The preparation of the micro bubbled solution is carried out more quickly, saving time, oxygen and equipment wear. By keeping the ozone concentration stable in the post-saturation solution under micro-bubbling, for 30 min without bubbling, the administration to the patient can be carried out without bubbling. What allows to release the generator, save time, oxygen and time to the operator.*
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Autho mail Adriana Schwartz. E-mail: adrianaschwartztapia@gmail.com

## Introduction

Ozone therapy has been used for therapeutic purposes since the end of the 19th century, in different modalities with good therapeutic results. The different ozone therapies have shown benefits such as the modulation of immunocompetent cells and the synthesis of immunoglobulins, interleukins and prostaglandins that benefit the inflammation and healing processes; likewise, stimulation of circulation and oxygen transport, promoting the secretion of vasodilators and inducing the production of antioxidant mechanisms thanks to the overexpression of Nrf2 in specific pathologies. [1]

Ozone therapy is framed as a therapy/technique of new technologies that is currently being used in the treatment of a wide variety of diseases, with increasing diffusion in the medical field. Currently, the number of publications on ozone therapy is increasing. This is demonstrated by the Pub Med Central database of the United States National Library of Medicine, (https://pubmed.ncbi.nim.nih.gov) with an increasing trend in the number of molecular, preclinical and clinical clinical papers. The database (05/02/2023) accumulates a total of 14,208 records. In 2020/2021, the trend was four daily articles, in 2022 it was five daily articles on the subject. This indicates that ozone therapy has excellent scientific support. For example, the Ozonated Saline solution (O3SS) has been used successfully in the treatment of Covid-19 in the midst of a pandemic at the Virgen de la Paloma hospital in Madrid, certifying its safety and efficacy.[2]

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.[3]

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.[4]

The International Organization for Standardization (ISO) has made different definitions regarding bubbles, determining that a bubble is a gas contained in a medium surrounded by an interface (ISO 20480-1:2017). According to their diameter, the bubbles can be classified as fine (less than 100 μm), micro bubbles (greater than 1 μm, between 10 and 50 μm) and finally nano bubbles (less than 1 μm). The diameter of the bubble and the viscosity of the liquid condition the rate of rise, being higher for macro bubbles and lower for micro bubbles and almost negligible for nano bubbles.[5]

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.

There are several factors that affect the mass transfer and solubility of ozone gas in water, mainly: water temperature, pH, and flow rate. The lower the ozone gas output flow from the generator, the higher the concentration and vice versa, that is, the flow is inversely proportional to the concentration. But in a liquid, the greater the flow of gas that enters with a given concentration, the greater the concentration of ozone will interact and it will be able to dissolve. The relationship is then directly proportional.

Within the chemical characteristics of micro/nano bubbles, it is known that they are also affected by the pH of the medium in which they are found.

Whether or not a substance (such as ozone) remains stable over time in solution can be crucial for its functionality to be correct. In these cases, the study of the Zeta Potential allows us to understand the stability of the ozone in the suspension. The zeta potential of the micro/nano bubbles will change depending on the gas they contain, being higher for gases that present greater solubility such as ozone and oxygen because they generate free radicals from hydroxyl groups and thus increase the values of negative charges in the solution. A particle with good colloidal stability is characterized by zeta potential values close to -30 mV (millivolts), values very similar to those reported in ozone micro/nano bubbles between 20 mV and 27 mV.[6]

Different reports observed that changes in pH do not cause significant changes in their size and distribution, but they do influence their stability. At alkaline pH, the micro/nano bubbles increase their stability thanks to their surface charge, becoming more negative and therefore more stable, while in a solution at acidic pH, their stability decreases as the ionic strength of the solution is compromised. which they meet.[7]

The antimicrobial action of the micro/nano bubbles of ozone is exerted from the free radicals that are generated at the moment of the cavitation of the micro/nano bubbles. Likewise, the pH of the solutions where they are found significantly affects the amount of free radicals that are generated, where a lower pH increases the number of free radicals from hydroxyl groups. The saline solution has a pH of 5.5-5.7 ideal for the generation of these free radicals.[8]

Studies of ozonation of salts in aqueous solutions, particularly NaCl, describe two possible mechanisms: chemical and physical. The Russian research by Boyarinov et al,[9] concluded that ozone does not react with sodium or chloride in physiological saline solution. The same conclusions were reached by the Russian chemist Razumovski.[10] These investigations showed that the concentration of sodium hypochlorite in the ozonated saline solution was less than 0.001 µg/mL.

Christenson et al. described the physical effect of salt solutions containing a critical range of electrolyte concentrations, called "transition concentration", which greatly inhibit the melting bubble.[11] Among the salts studied, only CaCl2 and NaCl can have this property. which implies a change in surface tension, generating a smaller bubble size and an increase in gas mass transfer rates.[12]

On the other hand, the dissolution of ozone and oxygen in the saline solution depends on the exposure/bubbling time. But the requirement of long exposure/bubbling times is linked to the occupation of the ozone generator, oxygen consumption, equipment wear, risk of embolism due to carelessness by bubbling the O3SS while infusing the patient.
The objectives of this study are: to analyze the efficiency of the ozonation technique of a saline solution under microbubbling/nanobubbling in a closed glass device system, to assess its advantages compared to the ozone dose that is finally transferred to the patient intravenously. and its benefits in applications in medicine and dentistry.

**METHOD:**

The first and second parts of the study were carried out at CSI ANALITICA SL, Tres Cantos, Madrid, Spain. https://www.csianalitica.com The CSI is a Spanish, independent, comprehensive research company based on Analytical Chemistry with experience and training in analytical techniques of a diverse nature such as mass spectrometry techniques (with or without chromatography, LC&GC-MS, MS/MS), solid phase microextraction (SPME), infrared (IR), electron microscopy techniques (SEM, TEM, etc.), AFM and a wide etcetera.

As ozone generation and measurement equipment, the following were used: an Ozonette® ozone generator from the Sedecal® company (Figure 1), an ozone concentration meter for liquids from the German company (BMT 964 AQ-LC, Messtechnick GmbH, Germany) with ultraviolet detector, detection interval between 0.5 to 15 g/m3 and precision of 0.5% and a Mass Spectrophotometer (GCMS).


**Figure 1. Ozonette® from the company Sedecal®**

The main objective was the study of the ascending ozone content generated in situ in the glass device “Advanced Ozonated Saline Solution” (ASSO3®) (Figure 2) with a known volume of saline serum by means of GCMS analysis.

A 

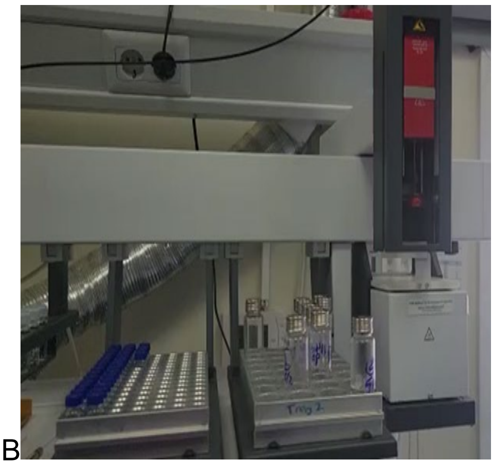
B 

**Fig. 2. A Glass device, closed system with micro-sparring plate (ASSO3®). 2. B Measurement with GCMS.**

In order to optimize the method for the detection and separation of ozone, a sample of known concentration of ozone dissolved in 5 mL of saline was analyzed, obtained directly inside a vial of SPME. Figure 3 shows the chromatogram with the extraction of ion 48, which corresponds to the mass of ozone, which elutes (“extract, using an appropriate liquid, a substance from the solid medium that has adsorbed it”. Dictionary, Royal Spanish Academy ) at a retention time of 1,358 minutes.

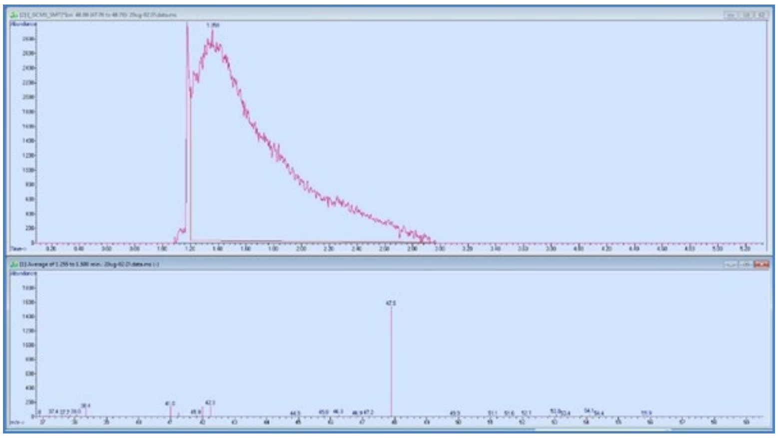


**Figure 3. Extraction of the 48 ion that corresponds to the ozone generated in a saline solution sample obtained by the ASSO3® device.**

Using the same method developed for this purpose, samples of increasing ozone concentration (1, 2 and 3 µg/NmL) were prepared in the same volume of saline, obtaining area values that are described in **Table 1**.

| TEST | Concentration O3, µg/mL | Vol.Syringe, mL | Ozone mass in µg | *Vol, mL | **CC µg/mL | ***Area, U.A. |
|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 5 | 0,2 | 93.379 |
| 2 | 1 | 10 | 10 | 5 | 2 | 664.836 |
| 3 | 1 | 20 | 20 | 5 | 4 | 1.156.052 |

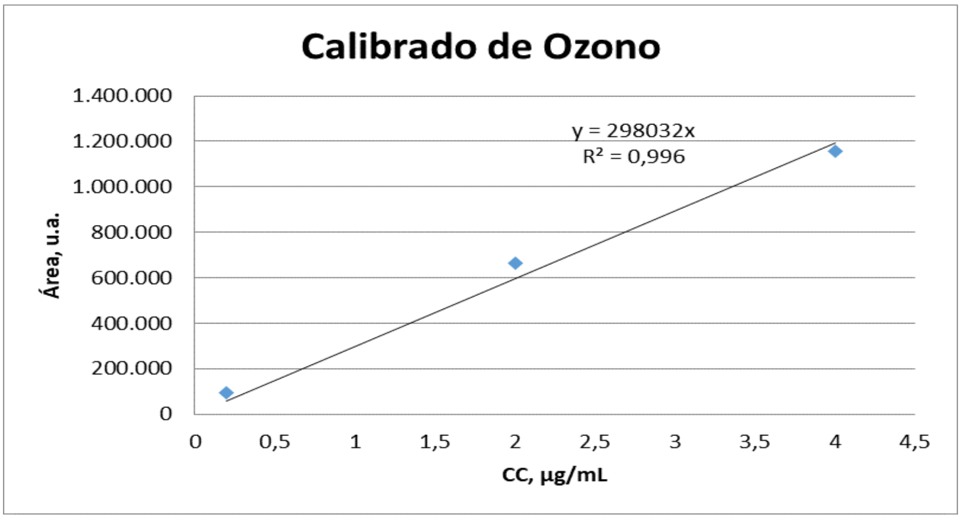
**Table 1. Area results in the linearity study.**

* Volume of distilled water to which was added by injecting various volumes of ozone.

** Ozone concentration in the vial analyzed.

*** Area of the chromatographic peak corresponding to ozone. Arbitrary unit.

In order to study the linear behavior, these results are represented, and the adjustment by least squares is applied, obtaining a linearity with $R2=0.996$ (Figure 4). As can be seen, at the analytical level, the linear behavior of the ozone generated by the ASSO3® device is considered satisfactory.




**OZONE CALIBRATION**

**Figure 4. Linearity study.**

In order to study ozone saturation in a given volume of saline solution, three samples with increasing ozone concentrations were prepared, using the Advanced Ozonazed Saline Solution (ASSO3®) device. The results obtained are described in Table 2.

| | | | | Added The concentration resulting from passing the ozone microbubbles in 250 ml of serum | Found |
|---|---|---|---|---|---|
| Flow, mL/min | CC, µg/mL | During, min | Vol, mL | CC, µg/mL | |
| 200 | 1 | 10 | 250 | 8 | 0,20 |
| 200 | 3 | 10 | 220 | 27,3 | 0,19 |
| 200 | 5 | 10 | 250 | 40 | 0,20 |
| | | | | Average | 0,20 |
| | | | | S (Standard deviation) | 0,0046 |
| | | | | DER (Relative standard deviation.) | 2,3 |

Table 2. Saturation study.

**Fig. 5 ASSO3® micro bubbling.**

As can be seen in Table 2, a saturation is observed in the saline solution where, although the amount of ozone bubbled in the saline solution increases, an increase in ozone concentration in the saline solution is not observed. The relative standard deviation, DER of all three assays is below 3%, indicating satisfactory assay precision.

**SECOND PART OF THE STUDY**

In a second phase of the study, a measurement of the ozone concentration in SSO3 was carried out under micro bubbling with the ASSO3® device. For this, an ozone concentration meter for liquids from the German company (BMT 964 AQ-LC, Messtechnick GmbH, Germany) with an ultraviolet detector, detection range between 0.5 to 15 g/m3 and precision of 0 was used. ,5%. The generator used was an Ozonobaric P® from the company Sedecal® (Figure 6).


**Figure 6. Ozonobaric P® from Sedecal®**

Measurements were made for each medium (water and saline solution) and for each of the conditions studied without ozonation or ozonation at (2 or 5) µg/NmL. The values of the kinetic behavior were represented and calculated eliminating the background interference.

## RESULTS

The results of the measurements of the ozone concentration in the different solutions are represented in figure 7. The result of the calculation of the concentration values eliminating the blank values (concentration value determined in the non-ozonized solutions) are shown in the table 3.

| Normalized concentration applied in gas | Standardized concentration measured in H2O | Standardized concentration measured in saline 0,9 % NaCl |
|---|---|---|
| 2 mg/L | 0,56 mg/L | 0,49 mg/L |
| 5 mg/L | 1,35 mg/L | 1,20 mg/L |

**Table 3. Mean ozone concentration values subtracting the blank values from the corresponding non-ozonated solutions.**

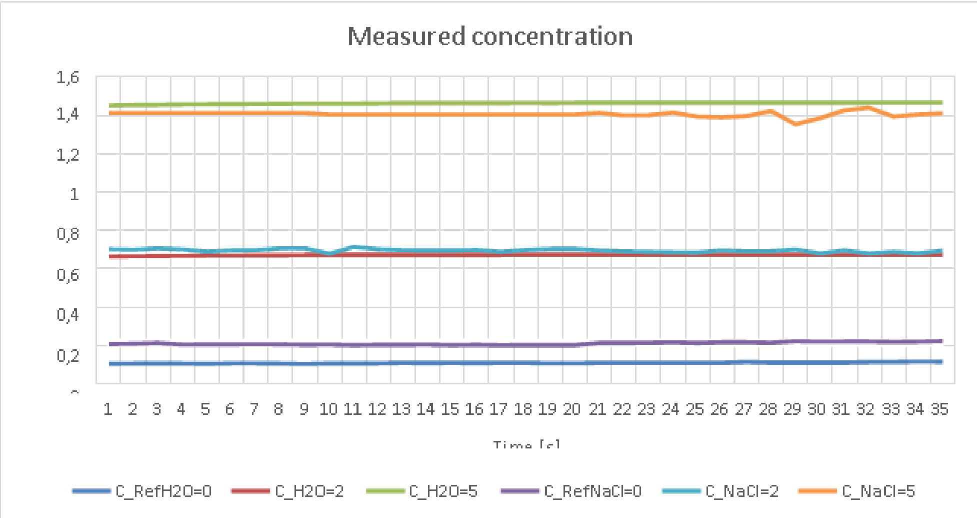



**Figure 7. Kinetics of the behavior of ozone concentrations in each of the conditions analyzed.**

<u>Reference Measurements.</u>

C_RefH2O=0: distilled water reference measure, deionized without ozone.

C_H2O=2: measure of distilled, deionized ozonated water with 2 mg/L.

C_H2O=5: measure of distilled, deionized ozonated water with 5 mg/L.

C_RefNaCl=0: reference measurement of saline solution without ozone.

C_NaCl=2: measurement of ozonated saline solution with 2 mg/L.

C_NaCl=5: measurement of ozonated saline solution with 5 mg/L.

As described, several factors affect the rate of ozone transfer to the liquid. However, after a saturation time of 5 min under a constant flow of 20 L/H at constant concentration and under micro sparging using the ASSO3 device. , an equilibrium has been reached in which the amount of dissolved ozone is equal to the amount of released ozone. By micro bubbling equilibrium is reached in 5 min.

In a balanced situation we can consider Henry's Law to calculate the amount of ozone dissolved in the water:[13,14]

$H = Ca/Pr$ [mol/m³/Pa](Equation 1)

Ca: concentration in liquid phase [mol/m³] Pr: partial pressure of the gas in equilibrium [Pa] The partial pressure of ozone is given by:

$Pr = Cg \, . \, R \, . \, T$ (Equation 2)

Cg: concentration in the gas [mol/m³]

The most agreed parameter for ozone is:

$Hc = 0{,}00011$ mol/m³/Pa @ $Tc = 298.15$ K

This parameter is very sensitive to temperature, but it follows the Van't Hoff equation [14]: $H(T) = Hc \, . \, \exp (d \ln H/d (1 / T) \, . \, (1/T – 1/Tc)$

where: $d \ln H/d (1/T) = 2400$ K

Although, for the calculations, the working temperature of 298.15 K is directly considered.[15]

The measurement system provides the data under normalized conditions at: $P0 = 1013.25$ h Pa $T0 = 273.15$K

So to calculate the concentration at room temperature:15

$Pc = 950$ hPa $Tc = 298.15$ K

According to the equation:

$Cgc = Cg0 \, . \, T0/Tc \, . \, Pc/P0 = Cg0 \, . \, 0.977$ (Eq. 3)

Taking these equations into account, the resulting ozone concentration in the liquid (Ca) can finally be obtained as a function of the normalized ozone concentration (Cg0) in the gas:

$Ca = Cg0 \, . \, 0{,}977 \, . \, 0{,}011 \, . \, 0{,}08314459848 \, . \, 298{,}15 = Cg0 \, . \, 0{,}2664$

That is, the solubility of the ozone concentration is 26.64% with respect to the normalized. To have a coherent reference, the concentration in the liquid will also be considered normalized, that is:

$Ca0 = Ca \, . \, Tc/T0 \, . \, P0/Pc = Ca/0{,}977$

With what is finally left:

$Ca0 = Cg0 \, . \, 0.272685$ indicating a solubility of 27.27%. The values are indicated in Table 4.

| Normalized concentration applied in gas. | Concentration applied in gas at 950 hPa and 25º C | Theoretical concentration dissolved in H2O at 950 hPa and 25º C | Theoretical normalized concentration dissolved in H2O |
|---|---|---|---|
| 2 mg/L* | 1,95 mg/L | 0,53 mg/L | 0,55 mg/L* |
| 5 mg/L* | 4,9 mg/L | 1,33 mg/L | 1,36 mg/L* |
| 15 mg/L* | 14,7 mg/L | 4,00 mg/L | 4,1 mg/L* |
| 40 mg/L* | 39,1 mg/L | 10,7 mg/L | 10,9 mg/L* |
| 60 mg/L* | 58,6 mg/L | 16,0 mg/L | 16,4 mg/L* |
| 80 mg/L* | 78,2 mg/L | 21,3 mg/L | 21,8 mg/L* |

**Table 4. Theoretical values of dissolved ozone concentrations in liquids.**

Legend: * Normalized ozone concentration.

Taking the actual measured data from Table 3 and comparing them with the theoretical results from Table 4, it can be seen that the deviation between them is in accordance with the theoretical reasoning presented in Table 5.

| Normalized concentration applied in gas. | Concentration deviation in H2O (measured – theoretical) | Concentration drift in 0.9% NaCl Saline Solution (measured – theoretical) |
|---|---|---|
| 2 mg/L | +0,01 mg/L => +1,8% | -0,06 mg/L => -11% |
| 5 mg/L | -0,01 mg/L => -0,7% | -0,16 mg/L => -12% |

Table 5. Deviation between the measurements and the theoretical results.

Taking table 3 again and presenting the measured data as a percentage with respect to the gas concentration entered, the actual amount of ozone absorbed by the liquids can be assessed as indicated in table 6.

| Normalized concentration applied in gas. | Standardized concentration measured in $H_2O$ in % | Standardized concentration measured in 0.9% NaCl saline solution in % |
|---|---|---|
| 2 mg/L | 28 % | 24,5 % |
| 5 mg/L | 27 % | 24 % |

**Table 6. Percentage relationship between bubbled and dissolved ozone.**

## DISCUSSION AND CLINICAL APPLICATIONS

**FIRST PHASE OF THE STUDY. ANALYSIS OF RESULTS.** A method has been optimized by GCMS capable of studying the ozone content in saline generated by state-of-the-art ozone generators (Ozonobaric P® and Ozonette®) using a glass microbubble plate device, ASSO3®.
It is observed that, from a concentration, ozone saturation is reached in a volume of 250 mL of saline in less than 5 minutes.

It should be noted that the samples prepared and described in Table 2 were reanalyzed 30 minutes after their preparation, obtaining the same results. Consequently, the ozone concentration in the solution remained stable (without degrading) for at least 30 minutes without bubbling, which means that (within that 30 minute period) the solution can be transfused into the patient without bubbling, thus avoiding the risk from accidentally passing bubbles to the patient when the solution is bubbling during the transfusion, saving time, oxygen and wear on the generator.

Thanks to the inhibition of the coalescence[11] of the bubbles under micro-bubbling, this leads to a greater exposure of ozone to the surface of the treated tissue, therefore optimizing, speeding up and shortening the treatments.

This is reinforced by the fact that among the properties that bubbles have is their ability to retain gases versus the occupation of dissolved gas in solution.[17] This is explained by the greater surface area conferred by micro/nanobubbles per unit area, which makes them up to 200 times more efficient than a normal bubble.[18]

Shortening the saturation time of the solution to 5 min. with a low flow rate of 200 mL/min, the preparation times (saturation) of the solution are significantly reduced. Reaching the desired concentration in the shortest ozonation time is one of the objectives of the doctor and dentist.

The device, being closed and made of glass, is reusable (it is sterilized in an autoclave) and is ecological. It lowers the economic cost for both the health professional and the patient, in addition to being very easy to use.

**Odontology**. One of the main applications of this technology would be in dentistry. The application of ozone in dentistry has evolved and now becomes safe thanks to the promising effects of intraoral irrigation with ozonated saline solution under micro/nano bubbling.

The intraoral use of ozone in the form of a gas has long been the most critical method of ozone application in dentistry, due to the difficulty of its administration due to inadvertent or accidental aspiration of the ozone gas, due to its close proximity to the upper respiratory tract of the patient. O3SS under micro sparging has solved this big problem. It is much safer to work with ozonated saline irrigation than to apply dry gas.

In dentistry and medicine, micro-bubbling O3SS irrigation is more biocompatible and less irritating to epithelial cells than dry ozone gas.

O3SS under micro/nano bubbling reduces the number of bacterial species associated with the biofilm, mitigating the dependence on the use of antibiotics with their respective resistance that is becoming more evident every day, thus favoring periodontitis and caries. In this way, the appearance of new microorganisms with the capacity to resist antibiotic therapy is avoided,[19] which is why the development of these new technologies is very necessary and useful and, in different studies, their antimicrobial capacity and antimicrobial properties have been demonstrated. cytocompatibility when compared with other types of rinses such as chlorhexidine, which is currently considered the gold standard.[20]

O3SS under micro bubbling remineralizes dental plaque, relieves pain when scraping and smoothing the root, stops bleeding, therefore, the surgeon can work in an aseptic and bleeding-free area.[23] Finally, SSO3 under micro bubbling it allows the dissolution of tartar,[24] which translates into a reduction in working time as well as less pain for the patient.

**Internal Medicine**. The application of O3SS under micro bubbling in internal medicine is wide. O3SS under micro-bubbling ozonizes a greater amount of blood.[21,22] In a single procedure, O3SS ozonates between 5-6 liters of blood, substantially optimizing and shortening treatments, thus saving time, suffering and money for the patient. patient. This is one of the most crucial points of the effectiveness of O3SS under micro bubbling.

**Second phase of the study. Analysis of results**. The approximation of 25% to calculate the amount of ozone that dissolves in the O3SS, is in line with the calculations and measurements carried out and can be considered correct. These data are in absolute agreement with the study by Fernández Yoldi, Carlos published in 2019 in the Ozone Therapy Global Journal.[25] Ozone concentrations remain stable under micro-bubbling in the ASSO3®.

It does not hijack the generator to deliver the solution to the patient as it can be delivered without bubbling. In dentistry, it eliminates the risk of accidental ozone aspiration by the patient. O3SS under micro bubbling has the capacity for greater biocompatibility and penetration into tissues. Eliminate tartar, it is hemostatic. In medicine, it optimizes treatments. It ozonates a greater amount of blood in a single procedure, making it more effective. With fewer sessions (1 to 2) you get results.

By equipping with micro-sparging features, after only 5 min of ozone micro-sparring, the solution was saturated. 25% ozone was dissolved in the saline solution. The ozone concentration in the solution was stable post saturation for 30 minutes without bubbling. In this way, the preparation and administration of the micro bubbled solution can be carried out more quickly, with potential savings in time, oxygen, wear and tear on the equipment.

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