Original paper

Practical aspects in ozone therapy: Study of the ozone concentration in the ozonized saline solution

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Keywords
Ozonized saline solution, ozone therapy, ozone.

Abstract
Ozonized Saline Solution (O₃SS) is one of the systemic routes of ozone administration that has been used for more than 40 years, mainly in Russia. The application protocol must be carried out under established parameters to achieve therapeutic efficacy and prevent side effects. The purpose of this paper is to study the concentrations of ozone in saline solution during the bubbling stage to establish the optimal saturation time and to study the degradation of ozone in it over time, thus defining the need for continued bubbling during reinfusion to the patient. The O₃SS preparation process was simulated using a certified kit, working at the concentrations of 1 and 5 µg / NmL and the variation of the ozone concentrations in solution was determined using the spectrophotometric method. The optimum bubbling time for saturation was estimated to be 15 min and at this time 10% of the initial ozone concentration is reached in the solution. It was found that due to the accelerated degradation of O₃ in saline solution, it is necessary to continue bubbling the ozone until 50 mL of saline solution remain in the container. As the western generators lacked ozone emissions in fractions of µg/NmL, it was estimated that the concentrations of 1, 2 and 5 µg/NmL at the generator outlet would be optimal for applying the low, medium and high doses required by the O₃SS application according to the type of indication.

Suggestion on how to quote this paper:
Introduction

The use of ozonized physiological saline solution (NaCl 0.9%) (O$_3$SS) is a widespread practice in Russia and developed by the ozone therapy school in the city of Nizhny Novgorod (Federal District of Volga). The method constitutes a systemic application route of ozone for different pathologies and consists of the previous saturation of the physiological saline solution with a mixture of oxygen-ozone (O$_2$/O$_3$) at low concentrations and its intravenous infusion to 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 orthopedics, dermatology, gynecology / obstetrics and neonatology. Since then it has been officially implemented in public hospitals. Ukraine made its application official in 2001. In response to different reasons, its use has gradually spread to countries such as Spain and America in general. The original method involves calculating the dose according to the weight of the patient. The methodology has many implementation variants and uses ozone generators graduated in µg/L, also the reported volumes of saline solution to ozonize are variable, as well as the saturation times and the bubbling time once the procedure is started to the patient.

Currently, the use of O$_3$SS is being manipulated, with the use of pseudoscientific arguments to hinder its application as complementary therapy. But the real interest is probably commercial, with the assumption that those who practice it will stop using the Major Autohemotherapy (MAH) method and consequently stop buying the MAH kit. In reality, O$_3$SS and MAH are two systemic forms, each with its advantages and disadvantages. Studies using O$_3$SS are increasingly being disseminated in scientific databases and the retrograde arguments that qualify it from "placebo" to "tumor-generating" are being left behind. In the current pandemic COVID-19, studies have been reported both with the MAH method and with the O$_3$SS method, although in this case the O$_3$SS actually has great advantages over the MAH method because the infectious process mediated by SARS CoV2 transits with disturbances in coagulation that may hinder the use of MAH.

By taking up the O$_3$SS method outside of Russia for different reasons, an attempt was made to harmonize it in a single protocol. A general description of the method was presented in the second and third edition of the Madrid Declaration on Ozone Therapy. However, all the parameters that govern this procedure are complex to implement. The objective of this paper was to quantify ozone concentrations during the O$_3$SS saturation process using a spectrophotometric method and to study the time in which ozone degrades in saline solution. Based on this study, saturation times are proposed based on measurements and also the adjustment of doses to generators that use concentrations in µg/mL in values of 1 unit.
Materials and methods

Ozone was generated by a class IIb CE medical device (Ozonette®, SEDECAL®, Spain). The container for the preparation of the single use saline solution was made of medical grade materials, free of phthalates and totally compatible with ozone that is classified as a Bexozone® class IIb medical device (Bexen medical®, Spain). The outlet connection of the bubble chamber (Fig. 1 point 3) was cut and the clamp 8 (Fig. 1) was kept closed to couple a 5 mL syringe that allowed taking samples of the saline solution present in the chamber at the different sampling times. The sample (5 mL) was taken by aspiration, re-insertion in the chamber and successive aspiration of the sample. Once the absorbance of the sample was determined, it was re-injected into the bubble chamber. Sterile physiological saline solution (0.9% NaCl) from (Lab. S.A.L.F, Italy) was used.

**Description:**
1. Container chamber
2. Top line
3. Two-way bottom line. 3A Downline. 3B Upline
4. Female Luer Lock connector
5. Male Luer Lock connector
6. Clamp
7. Addition point in Y
8. Clamp
9. Clamp roller
10. Addition point Y
11. Male Luer Lock connector
12. Drip chamber with filter
13. Attachment hook to serum holder

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**Figure 1.** Diagram and description of the Bexozone® Dual Kit (Bexen® medical, Spain).
As a first step, the correspondence of the ozone concentration at the generator output preset in the equipment with that determined with the spectrophotometric method was analyzed, for which a Metertech spectrophotometer UV / VIS SP8001 (Taiwan) was used. The absorbance was read at 254 nm in a 1 cm quartz cuvette and the extinction coefficient of 2 987 M-1 cm-1 was used for the gas phase ozone.

The saturation process of 200 mL of saline solution for 15 min was simulated with an ozone flow of 30 L/h in the Bexozone® device at concentrations of 1 and 5 µg/mL and samples were taken from the container every 5 min to measure the absorbance on the spectrophotometer and calculate the ozone concentrations in solution. In this case, the extinction coefficient 3 840 M-1 cm-1 was used.

Concentration calculations were based on the Lambert-Beer formula, where:

\[ C = \frac{D.O.}{\varepsilon \cdot L}, \]  
being: D.O., optical density; \( \varepsilon \), molar extinction coefficient of ozone and L, light passage of the cuvette (1 cm).

The changes that the temperature and pressure exert on the extinction coefficient were considered. \[ C = \left( \frac{D.O.}{\varepsilon \cdot L} \right) \cdot \left( \frac{PNTP}{P} \right) \cdot \left( \frac{T}{TNTP} \right) \]  
where: PNTP, the normal pressure (1 103 · 105 Pa), P the pressure at which the measurements are made; TNTP (normal temperature, 273.15 K), and T the temperature at which the experiment was performed: For the measurement of gaseous ozone 26.3 ºC = 299.45 K, for the measurement of ozone dissolved in saline 24.5 ºC = 297.65 K.

Statistical data processing: Values were obtained in triplicate. Statistical analysis was started with the OUTLIERS preliminary test to detect aberrant values. Different descriptive parameters were calculated for the analyzed variables (mean and coefficient of variation). The ANOVA method (one tail) was then used, followed by the variance homogeneity test (Levene) and the multiple comparison test (Duncan's Ranges). The level of statistical significance used in all cases was at least \( p \leq 0.05 \).

Regression analysis and linear correlation were used to study the relationship between concentration and optical density values. To validate the acceptability of the chosen model, the values obtained for the Durbin-Watson test were taken into account in the residue analysis. Pearson's correlation coefficient was reported. The data was processed using the STATISTICA statistical package (version 6) for WINDOWS.
Results

**Ozonette® Ozone Generator Calibration**

Calibration was carried out in the range of 1 to 20 µg/mL. The results are shown in Table 1. The linear correlation between the predetermined concentration in the equipment and that calculated according to the spectrophotometric determinations was 0.999. The deviations of the calculated concentration from the preset were less than 5%.

**Table 1. Results of the spectrophotometric calibration of the O3 Ozonette® generator.**

<table>
<thead>
<tr>
<th>Concentration preset in the equipment (µg/mL)</th>
<th>D.O. nm</th>
<th>254</th>
<th>Calculated concentration * (µg/mL)</th>
<th>Deviation of the preset concentration from the calculated ** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.056</td>
<td>0.99</td>
<td>-1.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.114</td>
<td>2.01</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.167</td>
<td>2.94</td>
<td>-1.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.237</td>
<td>4.18</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.294</td>
<td>5.18</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.555</td>
<td>9.78</td>
<td>-2.2</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.110</td>
<td>19.55</td>
<td>-2.2</td>
<td></td>
</tr>
</tbody>
</table>

Legend: The results represent the average of three determinations with a coefficient of variation of less than 5%. * Calculations were performed using the extinction coefficient of 2.987 M⁻¹ cm⁻¹ at 254 nm and it was normalized with the correction factor for pressure (1) and temperature (1.096284).

**Study of variations in the concentration of ozone in saline solution over time**

Fig. 2 shows the changes of the concentration of ozone in saline solution. In saturation phase at the concentration of 1 µg/mL the maximum concentration (10% of the initial) corresponding to 0.1 µg/mL is reached after 10 min of bubbling. For the concentration of 5 µg/mL, the maximum concentration (10% of the initial) that corresponds to 0.5 µg/mL is reached after 15 min of bubbling. During the saturation phase, the slope of the logarithmic relationship between the concentration of dissolved ozone at 1 µg/mL is higher (0.056) with respect to that reached at 5 µg/mL (0.027). A similar behavior occurs during the degradation of ozone in solution, for 1 µg/mL the slope of the logarithm line of the concentration vs time is -0.024 while for 5 µg/mL it is -0.041.
Figure 2. Ozone concentration in the saline solution (NaCl 0.9%) over time. Continuous line concentration of 1µg/mL, dashed line concentration of 5µg/mL. A, saturation phase and constant bubbling. B, ozone decomposition phase after stopping the bubbling. The ozone concentrations in solution were calculated using the extinction coefficient of 3840 M\(^{-1}\) cm\(^{-1}\) at 254 nm and normalized with the correction factor for pressure (1) and for temperature (1.089694).
Discussion

The ozone generator used in this experiment (Ozonette®, SEDECAL®, Spain) in the dose range of 1 to 20 µg/mL behaved with a precision level of less than 5% variation between the selected dose and that determined by the spectrophotometric method. The above places it within the established by ISCO3, which recommends that the generators do not differ by at least 10% between the selected dose and the real one. 16

During the saturation process of the saline solution, both the lowest and highest concentrations (1 and 5 µg/mL) reached a maximum concentration that corresponded to 10% of the bubbling concentration. The above corresponded with that reported by Durnovo and Jomutinnikova (2000),17 who after 10 min of bubbling with a concentration of 1.5 µg/mL detect a final concentration in solution that corresponds to 11.4% of the initial and for the 2.5 µg/mL concentration 8.8% of the initial concentration. The results do not correspond to that reported by other authors, who state that the final concentration in the saline solution represents approximately between 22% and 25% of the saturation concentration. 18,19 In one case (Boiarivov et al 2000)18 this difference may be because the author used distilled water, instead of saline solution, where ozone is more stable than in a solution that has dissolved salts.

In the second case, the variation may be due to the fact that the experimental simulation system did not exactly correspond to that used in the clinic. On the other hand, the time to achieve saturation was 10 min for the lowest concentration 1 µg/mL and 15 min for the highest 5 µg/mL. This data can be used to standardize the bubbling time at 15 min, as defined in the westernized variant of the procedure.20

Regarding the time in which ozone degrades to oxygen, once the bubbling is finished, the studies that exist are scarce. The dynamics of ozone degradation under the conditions in which the O3SS runs is important to define whether or not the solution should be kept bubbling with ozone during reinfusion to the patient. According to the results of this study, after 5 min of stopping the bubbling, for both concentrations tested, the ozone concentration that remains in solution is 67% of the initial one, at 10 min the concentration drops to 40% for 5 µg/mL and at 57% for 1 µg / mL.

The foregoing is indicative of the loss of the presence of ozone and the need to continue bubbling during the reinfusion, at least until there are 50 mL of solution left in the container, at which time the bubbling will be removed for safety reasons, just as the methodical indicates.20
Another problem related to the application of O₃SS is related to calculations of ozone concentrations according to the type of pathology, the weight of the patient, and the fact that western generators are not graduated for fractions of µg/mL. Table 2 shows a list of the doses according to their use depending on the pathologies to be treated. The values in column 1 refer to what is described in the 3rd edition of the Madrid Declaration on Ozone Therapy,¹³ which were calculated based on the precedent that the amount of ozone that remains in the solution represents 25% of that used for bubbling. However, if the results of this paper and those reported by Durnovo and Jomutinnikova (2000)¹⁷ are taken into account and the amount of ozone that dissolves in the saline solution after a 15-minute bubbling is considered 10% at 30 L/h, the ozone concentration at the generator outlet and the ozone concentration administered to the patient in 200 mL of saline can be estimated (columns 3 and 4 of table 2). In the western clinical practice of this procedure there are no substantial changes, since the reported ozone concentrations are those that were being used regularly, only it would change the need for body weight-adjusted calculations that still resulted in numbers with fractions of µg/mL that could not be predetermined in Western ozone generating equipment.
Table 2. Proposal of ozone concentrations at the generator outlet to saturate the O₃SS depending on the therapeutic indication.

<table>
<thead>
<tr>
<th>Dose³</th>
<th>Use²</th>
<th>Ozone at the generator outlet³ (µg/NmL)</th>
<th>Ozone concentration in the solution³ (µg/NmL)</th>
<th>200 mL dose of saline (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 µg/NmL Low</td>
<td>To stimulate the immune system and cardiovascular diseases. In obstetrics, to prevent toxicity in the first trimester of pregnancy and fetal hypoxia in the third trimester. In the prevention and adjuvant treatment in cancer.</td>
<td>1</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>0.8 µg/NmL Medium</td>
<td>Detoxification in endotoxemia and chronic inflammatory diseases of different etiologies</td>
<td>2</td>
<td>0.2</td>
<td>40</td>
</tr>
<tr>
<td>2 µg/NmL High</td>
<td>In infectious diseases (bacterial and viral), as well as skin infections and burns. Also, in autoimmune diseases where an immunosuppressive effect is needed.</td>
<td>5</td>
<td>0.5</td>
<td>100</td>
</tr>
</tbody>
</table>

Legend: a, Doses referred to in the Madrid Declaration on Ozone Therapy¹³ on previous experience that considered the concentration of dissolved ozone in the saline as 25% of that used in bubbling (generator output). b, Uses according to the dose referred to in the Madrid Declaration on Ozone Therapy.¹³  c, Proposed concentrations according to the results of this study.
Above the maximum limit of dissolved ozone in solution previously established, in order to avoid phlebitis 2 µg/NmL, according to the current calculation would correspond to 20 µg/NmL. However, according to the results of the application of this complementary therapy, it has been shown that bubbling with 5 µg/NmL is sufficient to treat infectious diseases such as SARS CoV2 infection\(^1\) and it is reiterated that it should not be overcome in any case 8 µg/NmL. Therefore, we consider that for safety reasons the upper limit could be set at 8 µg/NmL. As the clinical study itself shows in COVID-19 patients,\(^1\) the dose can be decreased depending on the evolution of the patient, in that particular study, after initial doses of 5 µg/NmL (ozone at the generator outlet) it was lowered the dose at 3 µg/NmL (ozone at the generator outlet) which would correspond to a medium/high dose.

Regarding the calculation of the dose by body weight, when it must be reported in a study, it can be calculated by relating the amount of ozone administered to the weight of the patient. For example, a patient receiving 200 mL ozonized saline solution of high-dose 5 µg/NmL (ozone at the generator outlet), he has actually received 100 µg of ozone, if his weight was 70 kg, and then, the dose per weight received will be 1.43 µg of ozone per kg of body weight. Taking into account also the variations in the volumes of ozonized saline solution in the different clinical studies (from 200 mL to 1 L) and the fact that it has been shown that the main mechanism by which ozone acts does not require large doses, but rather that the oxidative preconditioning signal is transmitted from exposed to unexposed cells.\(^2\) We recommend keeping solution volumes low at 200 to 250 mL as the total volume to administer.

Finally, we estimate to propose a working protocol for the preparation of the saline solution using the C.E. Bexozone® (Bexen® medical, Spain), because it is the most widely used device for these purposes and that the manufacturer does not offer a clear guide for its use in this modality (Annex 1).
Conclusions

The study of the ozone concentrations present during the saturation process of the saline solution with ozone made it possible to determine that the ozone concentration corresponds to 10% of the ozone concentration at the generator outlet. On the other hand, the study of the ozone degradation in the saline solution, allowed to corroborate that its degradation is sufficiently accelerated as to require constant bubbling at the time of reinfusion of the solution into the patient. After analyzing these results, it is proposed to adjust the bubbling time to 15 min, to maintain the bubbling until there are 50 mL of solution to infuse, to use the ozone concentrations at the outlet of the ozonator of 1, 2 and 5 µg/NmL to the low, medium and high doses according to the field of application.

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Conflict of interest: The author is president of ISCO3 (International Scientific Committee on Ozone Therapy) and member of the editorial committee of Ozone Therapy Global Journal.

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BIBLIOGRAPHY


**Annex 1.** Procedure guide to practice O₃SS with the Bexozone® device (Bexen medical®, Spain)

To see the references of the parts of the device, use Fig. 1 as a guide. Before proceeding to vein channeling, check: The correct condition of the equipment and its connections.

**Preliminary operations. Filling the chamber:**
1) Hold the camera by hook 13 to the serum holder. Close roller 9 and clamp 5.
2) Through the female Luer Lock connector 4, generate a vacuum until reaching 0.5-0.6 bar, connecting it to the equipment vacuum pump, or aspirating with a Luer Lock syringe, in this case aspirate 300 mL of air. In both cases, use an ozone resistant antibacterial filter (0.22 µm).
3) Aspirate through point 11, 200 mL of sterile physiological saline solution (NaCl 0.9%). For aspiration, place a sterile needle (18 G · 1 ½; 1.2 mm · 38 mm) on terminal 11. If the vacuum is not enough to reach 200 mL, suck in more air from terminal 4. Close clamp 8.

**Saline solution saturation:**
4) Place the serum holder at the same level as the generator (e.g. Ozonette, SEDECAL, Spain). Open clamp 4 and connect it to an O₃ destruction system, through an ozone resistant filter (0.22 µm). Place one terminal of the ozonation line (Line not included in the set, sterile male male Luer Lock) in the generator, use an ozone resistant sterile filter (0.22 µm) at the generator outlet. At the other end of the ozonation line, place a needle (18 G · 1 ½; 1.2 mm · 38 mm). Run the generator in continuous mode with the following specifications: Concentration: depending on the dose (see Table 2), do not exceed 8 µg/mL. Flow: 30 L/min. Time: 30 min of which 15 will correspond to the saturation phase of the saline solution and the rest will be established depending on the time it takes for 50 mL of saline solution to remain in the chamber. Once the O₃ generation is activated insert the needle into port 7. Clamp 5 will remain closed. Let the solution saturate for 15 min.
Application to the patient:

5) Place a tourniquet on the patient's arm, clean the area where the vein will be taken with disinfectant. Connect the epicranian needle (butterfly needle 21G to connector 11) and proceed to the vein (basilic, cephalic or medial). Once channeled, fix the butterfly needle with hypoallergenic adhesive tape at two points. Remove the tourniquet. Raise the serum holder to allow the solution to go down by gravity. Open roller 9 and clamp 8 and allow the saline solution to flow from inside the chamber. Continue ozonating (bubbling) until 50 mL of saline remains in the chamber, at which point stop the ozone generation and destruction system. Do not abandon the patient.

6) For dosage see the Madrid Declaration on Ozone Therapy. 13

7) After re-infusion is complete, close all clamps and roller 9 and discard the set in the special waste container.