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Determination of ozone concentration in different types of aqueous solutions for use in clinical practice

Determinación de la concentración de ozono en diferentes tipos de soluciones acuosas para uso en la práctica clínica

Determinação da concentração de ozônio em diferentes tipos de soluções aquosas para uso na prática clínica

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Abstract

The aim of this study was to determine the ozone concentrations in non-sterile double-distilled water (ABDNE), non-sterile monodistilled water (AMDNE), water for sterile injection (IEA) and 0.9% saline solution (SF 0.9%), using if the DPD method. The ozonized solutions were obtained by micro bubbling the ozone gas for 15 minutes, caused by a low flow of medicinal oxygen (¼L / min), at a concentration of 56mg / L. The determination of ozone concentrations was based on the oxidative staining reaction of N, N'-diethyl-p-phenylenediamine (DPD). EIA presented higher ozone value (9.94 ppm). ABDNE showed concentration in the 5, 10 and 15 minutes of ozonation, respectively, of 6.14 ppm, 7.84 ppm and 8.34 ppm. In the subsequent times, there was instability and alternation of concentration. Ozone measurement tests in SF were performed using the same method, with results of 8.08 ppm, 9.36 ppm and 9.08 ppm, at 5, 10 and 15 minutes, respectively. The EIA proved to be the most suitable for reaching a concentration close to 10 ppm. Studies on ozone dosage and concentrations, as well as on their by-products, should be carried out to support decision-making about safe doses for the application of this therapeutic ozone modality.

Descriptors: Ozone; Complementary Therapies; Evidence-Based Nursing.

Resumén

El objetivo de este estudio fue determinar las concentraciones de ozono en agua bidestilada no estéril (ABDNE), agua monoddestilada no estéril (AMDNE), agua para inyección estéril (IEA) y solución salina al 0,9% (SF 0,9%), utilizando si el método DPD. Las soluciones ozonizadas se obtuvieron microburbujeando el gas ozono durante 15 minutos, provocado por un bajo flujo de oxígeno medicinal (¼L / min), a una concentración de 56mg / L. La determinación de las concentraciones de ozono se basó en la reacción de tinción oxidativa de N, N'-dietil-p-fenilendiamina (DPD). El EIA presentó mayor valor de ozono (9,94 ppm). ABDNE mostró concentración en los 5, 10 y 15 minutos de ozonización, respectivamente, de 6.14 ppm, 7.84 ppm y 8.34 ppm. En los tiempos posteriores, hubo inestabilidad y alternancia de concentración. Las pruebas de medición de ozono en SF se realizaron utilizando el mismo método, con resultados de: 8.08 ppm; 9,36 ppm y 9,08 ppm, a los 5, 10 y 15 minutos, respectivamente. El EIA resultó ser el más adecuado para alcanzar una concentración cercana a las 10 ppm. Se deben realizar estudios sobre dosis y concentraciones de ozono, así como sobre sus subproductos, con el fin de apoyar la toma de decisiones sobre dosis seguras para la aplicación de esta modalidad terapéutica de ozono.

Descriptores: Ozono; Terapias Complementarias; Enfermería Basada en Evidencias.

Resumo

Objetivou-se determinar as concentrações de ozônio em água bidestilada não estéril (ABDNE), água monodestilada não estéril (AMDNE), água para injeção estéril (AIE) e solução fisiológica a 0,9% (SF 0,9%), utilizando-se o método DPD. As soluções ozonizadas foram obtidas por micro borbulhamento do gás ozônio, durante 15min, provocado a partir de baixo fluxo de oxigênio medicinal (¼L/min), a uma concentração de 56mg/L. A determinação das concentrações de ozônio foi baseada na reação de coloração oxidativa de *N,N'*-dietil-p-fenilediamina (DPD). AIE apresentou maior valor de ozônio (9,94 ppm). ABDNE mostrou concentração nos 5, 10 e 15 minutos de ozonização, respectivamente, de 6,14 ppm, 7,84 ppm e 8,34 ppm. Nos tempos subsequentes, houve instabilidade e alternância da concentração. Ensaios de medição de ozônio na SF foram realizados utilizando o mesmo método, com resultados de: 8,08 ppm; 9,36 ppm e 9,08 ppm, aos 5, 10 e 15 minutos, respectivamente. A AIE mostrou-se a mais adequada para o alcance de concentração próxima a 10 ppm. Estudos acerca de dosagem e concentrações do ozônio, bem como, de seus subprodutos, devem ser realizados a fim de embasar a tomada de decisão acerca das doses seguras para aplicação desta modalidade do ozônio terapêutico.

Descritores: Ozônio; Terapias Complementares; Enfermagem Baseada em Evidências.

Introduction

Ozone (O_3) is an oxygen allotropic molecule (O_2) and is found in the atmosphere in a gaseous state, has a characteristic odor, at room temperature it is colorless, in the liquid state it is dark blue in color, and the melting temperature is - 192.5º C. It is produced from O_2 molecules that undergo electrical discharges, forming in nature the Ozone Layer, whose function is the protection of life against solar electromagnetic radiation, functioning as a filter[1-2].

In 1896, the first ozone generator was created, by Nikola Tesla (1856 - 1943), using a high voltage electrical discharge that transforms the Oxygen molecule (O_2) into O_3. He patented and formed the first medical ozone generator company. In 1957, the German doctor Dr. Hansler, built a medical ozone generator with the possibility of obtaining precise doses of the oxygen-ozone mixture, on which the current generators are based[1-2].

Because it is a gaseous molecule, it quickly dissolves in water, plasma, and extracellular fluids, depending on the temperature and water quality, due to impurities, organic and inorganic, causing changes in the hydrogen potential (pH) of the medium and the decomposition of ozone[2]. The mechanism of action of O_3 is sustained in the action of its by-products (hydrogen peroxide, nitric oxide, hydroxyl radical, among others) that are formed after contact with biomolecules[1].

The great solubility of ozone in water allows its immediate reaction with any soluble compounds and biomolecules present in the fluids, which is explained by the fact that it is the third strongest oxidizing agent, after fluorine and persulfate. The high solubility and instability of O3 guarantees its total consumption, without causing toxic products to the living organism[3].

A relatively new approach is represented by the topical use of O3 and its derivatives as mediators of reactive oxygen species (ROS)[4]. As O_3 reacts, products derived from contact with organic matter cause acute and transient oxidative stress capable of triggering intra and extracellular pathways that lead to positive biological responses[1]. In addition to having direct action on transcription factors that stimulate the production of antioxidant enzymes, O_3 damages the bacterial cell wall and the cytoplasmic membrane, causing a bactericidal, germicidal, fungicidal effect, without triggering resistance mechanisms[5], in view of these properties it has been increasingly applied in integrative clinical practice, for both humans and animals.

Study developed by European researchers, reinforces the mechanisms of action of O_3 already elucidated and shows to be highly effective in reducing tissue damage mediated by inflammation and oxidative stress[6].

In many countries of the world, Ozone Therapy or Oxygen-Ozone Therapy, has been used on a large scale by veterinary, human medicine and dentistry. In the area of dentistry, successes have been demonstrated with the management of wound healing, dental caries, oral lichen planus, gingivitis and periodontitis, halitosis, osteonecrosis of the jaw, post-surgical pain, plaque and biofilms, root canals, hypersensitivity to dentine, disorders of the temporomandibular joint and tooth whitening[7-9].

For a therapeutic process with ozone, several forms and routes of administration are included, and aqueous solutions have great therapeutic potential, however, they present the challenge of O_3 instability in solutions. Ozone has a longer half-life in the gaseous state than in the aqueous solution. For this reason, it must be prepared immediately before use and cannot be stored for long periods of time[10], in addition to requiring attention to the temperature of the preparation environment, because the lower the temperature the longer the life of the O_3 gas[2].

Exposed to a temperature between 0 to 30° C, the solubility of O_3 in water is 13 times greater than that of O_2 and, it is progressively more soluble in water at lower temperatures. Ozone decomposition is more accelerated at higher temperatures. Aqueous solutions with impurities, organic and / or inorganic, cause variation in pH and, therefore, the final saturation of O_3 will depend on the quality of the water[11-13].

Even with the global expansion of Ozone Therapy, especially in Brazilian territory, there is a knowledge gap between the evidence generated by research and practice. And it is considered that for the development of clinical research related to the treatment of lesions of human skin and mucous membranes, it is necessary to obtain safe concentrations of O_3 in aqueous media, so that the results are reliable and applicable to clinical practice.

Thus, the guiding question of this study is “What is the best ozonized aqueous solution for clinical use?”, Outlining the objective: to determine ozone concentrations in non-sterile distilled water, non-sterile double-distilled water, water for sterile injection and physiological solution a 0.9%, using the N, N’-diethyl-p-phenylediamine (DPD).

Methodology

The procedures of this study were carried out in an environment of the ozone generator industry used, during the month of August 2020, in the city of São José dos Campos, interior of the state of São Paulo, Brazil. This article is part of the preclinical phase of a randomized clinical study, which aims to evaluate the effectiveness of ozonized water for oral mucositis in post-bone marrow transplant patients. The study was approved by the Human Research Ethics Committee, under opinion No. 4,018,509, of the public teaching hospital for the research setting, which is a national reference for bone marrow transplants.

Materials

The solutions used were Sterile Injection Water - IEA (JP Indústria Farmacêutica®), Non-Sterile Monodistilled Water - AMDNE (Asfer®), Non-Sterile Bidistilled Water - ABDNE (Quantica W®) and 0.9% Saline Solution - SF 0.9% (JP Indústria Farmacêutica®). The different aqueous solutions were used to determine the concentration of ozone.

Equipments

The set designed to obtain ozonized solutions in the necessary concentrations, consists of:

- Medical oxygen cylinder (OZONE & LIFE®).
- Flow regulating valve with pressure gauge (OZONE & LIFE®).
- Ozone generating device, which is registered with ANVISA, under nº 8150910000, brand OZONE & LIFE®, model O & L1.5 RM, which generates concentrations between 1 and 72mg / L of ozone.
- Liquid ozonator tower (OZONE & LIFE®).
- Kit-7423 Vacu-vials®.
- Portable NDUV photometer (Anseros®-Ozone Analyzer GM-RTI).
- Spectrophotometer.

The use of the liquid ozonator tower has the purpose of producing microbubbles that increase the contact surface of O_3 with the aqueous solution. The bubble diffusers have a porous device to break the gas into small bubbles at the bottom of the water column, allowing the reduced bubbles to rise slowly to the top of the column and thus dissolving in the water[14]. A torre ozonizadora também permite manter a segurança no processo de ozonização devido ser um sistema fechado e vedado de borbulhamento, possuindo um catalisador acoplado para decomposição do O_3 excedente.

Ozone Generation

The O_3 was produced from pure O_2 through an OZONE & LIFE® generator, model O&L1.5 RM. Its production was caused by a low flow of medicinal O_2 (¼ L / min). And different ozonized solutions were obtained by bubbling them at a concentration of 56mg / L. In the experiment, the volume of the solutions was standardized in 500ml (milliliters), bubbling them continuously with a mixture of oxygen gas - ozone (O_2 / O_3), with a maximum exposure time of 30 minutes.

Before the beginning of the tests, the calibration of the ozone generator was examined using the portable NDUV photometer (Anseros®-Ozone Analyzer GM-RTI), which aims to monitor the O_3 in the gas phase in dry or humidified conditions, ensuring the gas concentration produced by the generator.

Preparation of aqueous solutions for ozone determination

No single method is consistently used by the scientific community or industry to assess O_3 concentrations in aqueous solutions, making comparisons and standardization of exposures difficult. Five methods for measuring O_3 are regularly cited in the literature, often using different units of measurement and with little or no explanation of the reasons that led to the choice of a particular one. Among them are:

- Neutral buffered iodometric method[15].
- Modified neutral buffered iodometric method[16].
- Iodometric titration method[17].
- DPD colorimetric method[18].
- Oxidation / reduction potential[19].

However, there are still significant disadvantages that limit its application[20].

In this study, the determination of ozone concentrations was based on the oxidative staining reaction of N,N'-diethyl-p-phenylamine (DPD) through fenton reactions in aqueous solutions, the results of which are expressed in parts per million (ppm) or milligrams / liter (mg / L) of O_3[21-22]. This is a candidate method for the standard analysis of aqueous solutions, due to the ease of execution and calibration for absolute determinations[23].

After ozonizing the solution and before analyzing the 25 ml sample of the solution in question, five drops of the chemical reagent (A-7400) were added. The activator solution A-7400 contains potassium iodide and when added to the sample before analysis, O_3 reacts with iodide and iodine is released. Therefore, the free iodine reacts with the reagent in the kit ampoule, generating a pinkish solution[24].

It is relevant to point out that the DPD method is more specific but requires the use of toxic materials and forms an unstable pink complex, whose color intensity is directly proportional to the O_3 concentration and, therefore, requires rapid determination. According to the manufacturer's instructions, in one minute, the reaction solution was read to measure the absorbance value at 415 nm (nanometer), using a dedicated photometer - SAMs (Single Analyte Meters).

The analysis of the O3 concentration took place in the following times of exposure of the solutions to the O_3: 05, 10, 15, 20, 25 and 30 minutes. The temperature of the environment of the experiment showed 11 ° C of variation during the analysis period, about seven hours, corresponding to the spectrum of values between 19 ° C and 30 ° C.

Results and Discussion

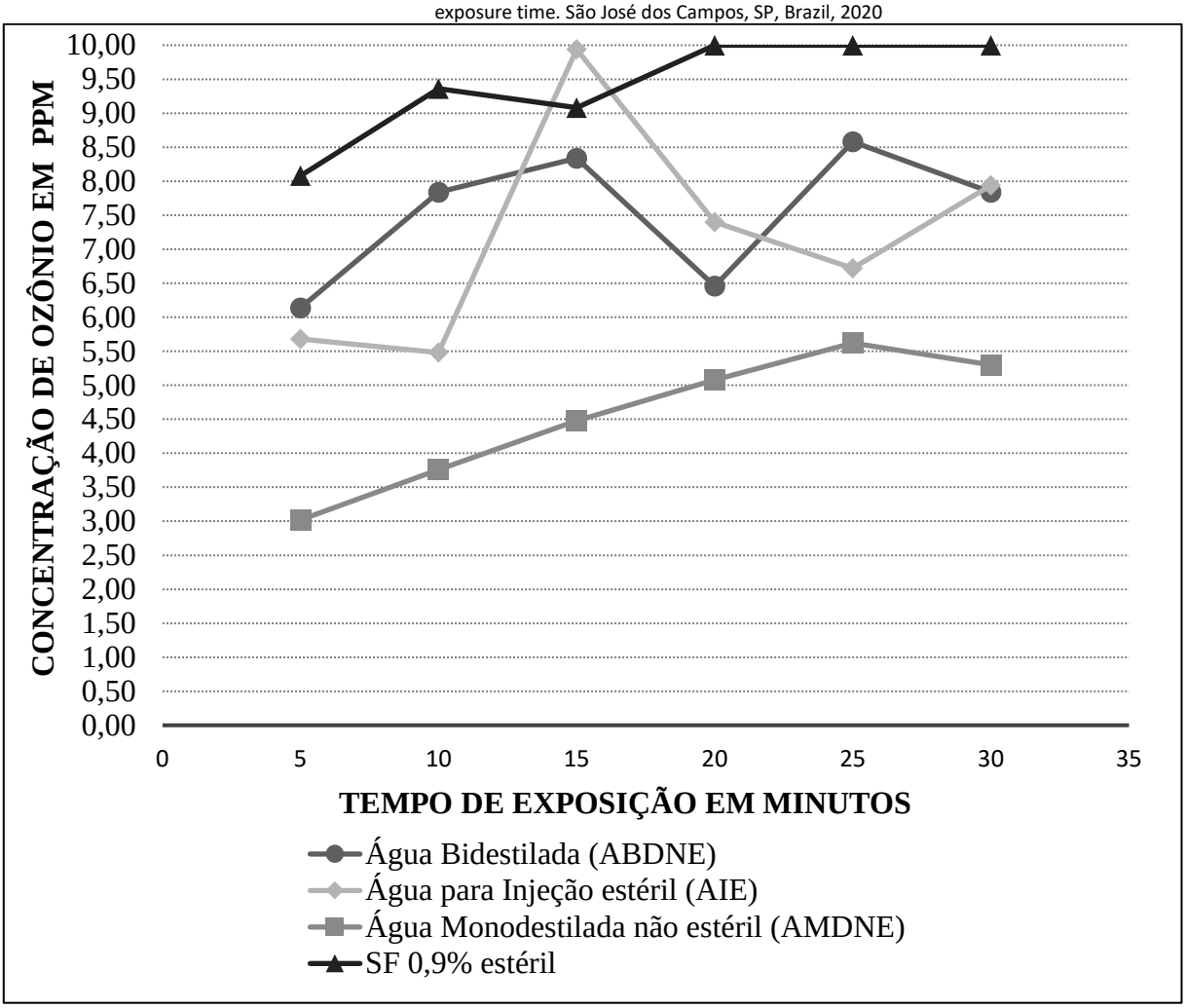
Next, the results and discussion of the experiments with the aqueous solutions used are presented and for a better explanation of the findings, the discussion was categorized in two parts: a) Saturation time (Tsat) and b) Saturation concentration (Csat).

Saturation time (Tsat)

Ozonation of all tested aqueous solutions was stopped within a maximum of 30 minutes.

Maximum csat of all solutions (except AMDNE which peaked at 25 minutes) happened at 15 minutes of ozonation, indicating that the maximum O_3 saturation happened at this time (Figure 1).

Figure 1. Saturation concentration (Csat) of ozone as a function of Saturation time (Tsat) to gas according to the different types of aqueous solution and exposure time. São José dos Campos, SP, Brazil, 2020



About 5 to 10 minutes of exposure to double distilled water is the time necessary to obtain a satisfactory O_3 saturation[25]. However, when using half the capacity of the ozonizing tower, we changed the pressure of the solution to the gas and minimized the contact time and, in this study, half the capacity of the ozonating tower (500ml) was used for the procedures.

This explains why the Tsat related to maximum Csat happened at 15 minutes, exceeding the time elapsed in the Madrid Declaration[25]. The final concentration of ozone in the water is a function of the concentration in the gas phase, the pressure of the gas, the temperature of the water and the technology of exchange between liquid and gas[26].

Ozonation with half the capacity of the column, at the same time and the same concentration has less phase transfer, with the volume of 1000 ml of water it is only necessary 05 minutes of exposure, with a generator calibrated at 60 mg / L, to obtain a final concentration of 10 to 15 mg / L[27].

In the present study, we showed that with 500 ml, and generator calibrated at 56 mg / L, with ¼ of O_2 flow, exposing the IEA (which had the highest Csat among the tested solutions) to 15 minutes of ozonation, we obtained the equivalent of concentration of 9.94 mg / L. The fact that we achieve a satisfactory Csat with half the capacity of the ozonator tower shows that ozonized water can be produced with smaller volumes and without wasting the solution.

From the twentieth minute of Tsat, an instability in the behavior of Csat is noticeable due to ozone degradation. A recent study showed that the O_3 degradation rate increased with increasing pH of the solution[28].

Saturation concentration (Csat)

In the tests carried out, ABDNE showed an increasing Csat in the 5, 10 and 15 minutes in the ozonation process, starting from 6.14ppm and reaching the peak of saturation with 8.34 ppm at 15 minutes of exposure. AMDNE presented a different pattern with more linear kinetics, however with much lower values of saturation when compared to ABDNE, and its peak was reached with 25 minutes of ozonation, with Csat of 5.62mg / L.

The solubility of O_3 in pure water is in line with Henry's law (1803), which states that the state of gas saturation in water is proportional to its concentration. There is evidence that the kinetics of O_3 in water corresponds to a reaction in relation to the concentration of the gas, which in turn is catalyzed by hydroxyl ions (OH^-) on the pH with an interval between 1-8[29].

At 15 min of ozonation, the difference in Csat between AMDNE and IEA waters was 5.46 ppm. The factor

that may have contributed to this result is the absence of ions and organic matter in the IEA. As it presents quantities of ionic species and, since it is not an extremely pure water, AMDNE reacts with ozone gas and forms by-products, increasing its degradation and decreasing its effectiveness[30].

In this regard, AMDNE contains ionic traces that function as substrates for the formation of radicals and potentially toxic substances, which are unusable for therapeutic purposes, as they do not have the capacity to disinfect[2]. Above all, there is the fact that it is not sterile, whose biological fragments can alter the pH and accelerate the consumption of O_3 in the solution.

The O_3 decomposition is considered a complex process of radical chain reactions[2]. The interaction of O_3 with hydroxyl ions, which are reactive species of O_2 metabolism, initiate the process of oxide-reduction reactions[31]. The hydroxide ions start the decomposition of O_3 and form the hydroperoxide radical (HO_2^-), which, in turn, produces hydroxyl radical (OH^-) and superoxide (O_2^-) which can lead to an additional decomposition of O_3, denominating it if radical chain reaction[32]. A study published in 1987, already reported that in the decomposition of O_3 at pH below 7, at any temperature, the radicals OH^- initiate the direct decomposition of O_3 and are the main cause of ozone decomposition in this condition[30].

Ozone degradation in double distilled water is reported in several studies and can be simplified with the scheme below[2,33]:

Initiation:	$O_3 + H_2O \rightarrow 2OH^- + O_2$
Propagation:	$O_3 + OH^- \rightarrow HO_2^- + O_2$ $O_3 + HO_2^- \rightarrow OH^- + 2O_2$
End of O_3 degradation:	$OH^- + OH\cdot \rightarrow H_2O_2$ $O_3 + H_2O_2 \rightarrow OH^- + HO_2^- + O_2$ $OH^- + HO_2^- \rightarrow H_2O + O_2$

At the end of the O_3 decomposition process in the aqueous medium, we invariably obtain water (H_2O) and oxygen (O_2); it is not recommended that water that has already undergone this process be ozonated again[27].

In this study, it was observed that the EIA showed the highest Csat peak of O_3 among all the aqueous solutions tested (9.94 ppm) (Figure 2). The Csat of O_3 in the IEA increased to the maximum in the exposure time of 15 minutes, corroborating with the literature that observes maximum amount of ozone between 8 and 15 minutes of exposure[2]. In the bubbling concentration at 56 mg / L, the IEA Csat ranged from 5.48 ppm to 9.94 ppm, and the highest O_3 content that was obtained among the tested solutions occurred with a sterile, clear, hypotonic, sterile injectable solution. and pyrogenic, whose pH ranges from 5.0 to 7.0[34].

The 0.9% ozonated SF solution reached the maximum of Csat after 10 minutes exposed to the O_2 / O_3 mixture. At 5, 10 and 15 minutes the concentrations were 8.08 mg / L, 9.36 mg / L and 9.08 mg / L, respectively.

From the twentieth minute of exposure, the spectrophotometer was unable to perform the reading, possibly due to the presence of oxidized compounds, they can significantly alter the analysis. In this sense, the manufacturer of the kit used presents as possible interfering compounds present in the sample as interfering compounds that can produce high results in the test, such as: chlorine (Cl); chlorine dioxide (ClO_2); ferric ion (Fe^{+3}); cupric ion (Cu^{2+}); among others[21-22]. This is an important fact, since substances oxidized by O_3 can directly interfere in the analysis and if there is no care in this judgment, it is possible to infer high values of O_3 concentration instead of reporting the formation of oxidized compounds possibly harmful to health.

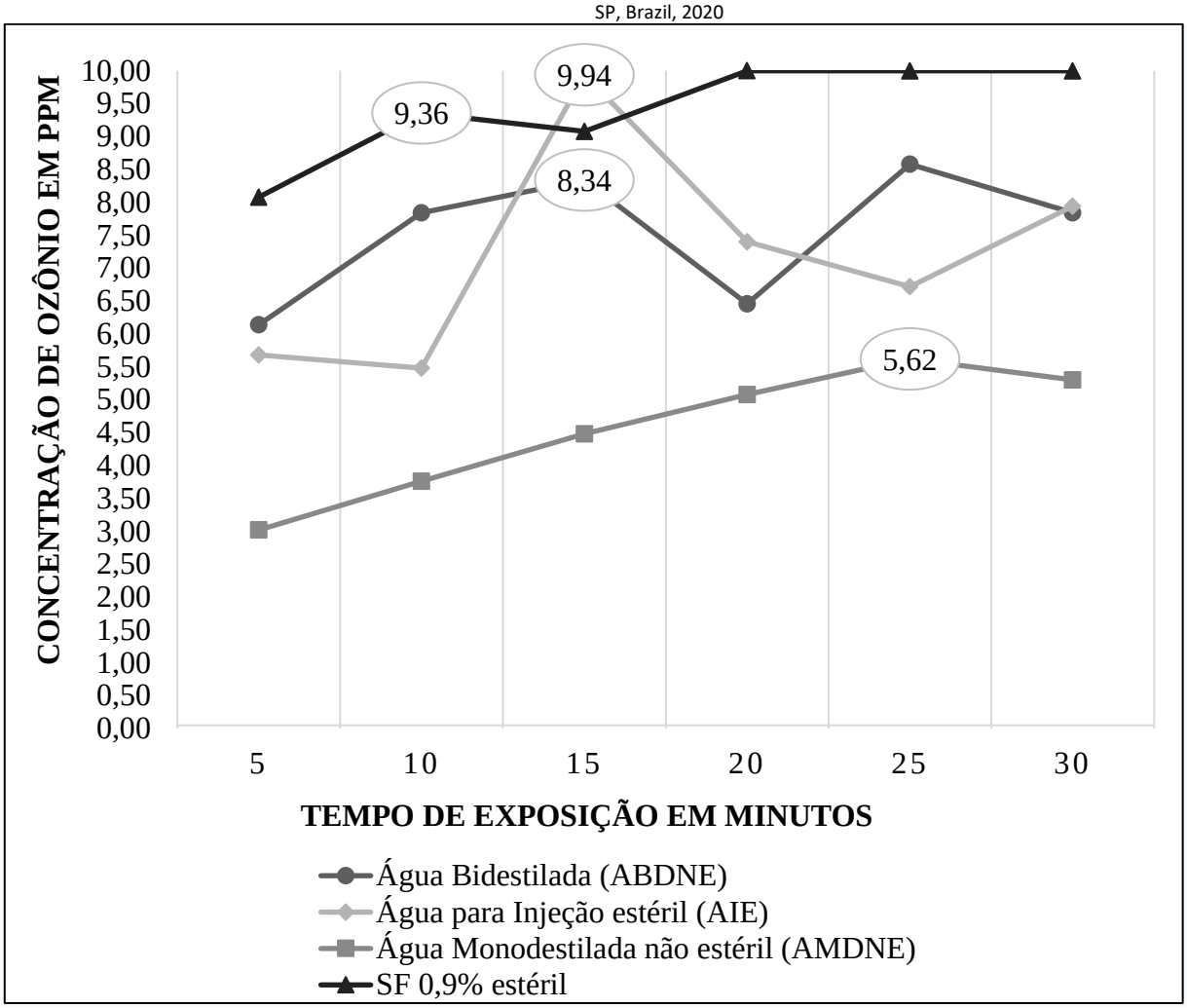
When evaluating the absorption speed of O_3 in saline solution, it was found that the absorption and degradation of O_3 in this solution is faster due to the interaction with NaCl molecules. Ozone can oxidize chloride ions in saline to form chlorate, and the chlorate content gradually increases over time. In addition, higher concentrations of O_3 result in increased levels of chlorate (ClO_3^-)[35]. It is important to note that O_3 can react with its container and, thus, increase the levels of toxic substances in the ozone solution[36].

Recently, a study[36] confirmed the production of ClO_3^- by ozonizing 0.9% SF with O_3 at concentrations of 20, 40 and 60 µg / ml. To simulate the 0.9% ozonation scenarios of SF 0.9% in clinical practice, the same study used common polypropylene containers and blood transfusion bags resistant to O_3 such as Polyvinyl Chloride (PVC) associated with plasticizers (additives that provide flexibility and suppleness) such as di- (2-ethylhexyl) adipate - DEHA, and di-(2-ethylhexyl) phthalate - DEHP. The results suggested that, under the same conditions, PVC associated with plasticizers produced lower ClO_3^- contents. However, over time (3, 6 and 15 days) the content of the compound increased significantly.

There are reports of ozonated saline for topical use. Study published in 2019[37], reports the use of 0.9% ozonated SF for the treatment of diabetic foot neuroinfection. Jets of SF0.9%, ozonized in high concentrations (80 ppm), were applied to guarantee the debridement of the loose necrotic tissue, as well as, of the purulent exudate.

However, the topical application of the ozonized saline solution should be further investigated to verify the decontamination and debridement of wounds, for example, versus the potential for alteration and deleterious effects at the cellular level, by the oxidized compounds of the solution during ozonation.

Figure 2. Peak saturation concentrations (Csat) of ozone according to the time of exposure to gas in different types of aqueous solution. São José dos Campos, SP, Brazil, 2020



It is pointed out that the materials of the ozonation container, as well as its packaging, can react with O_3 and release toxic substances in the solution. As an intravenous infusion therapy, 0.9% ozonated SF requires repeated administrations over a short period36. Further experimental and clinical studies are needed to determine whether recurrent exposure to ClO_3^- can cause damage to the individual's various organ systems.

Chloride (Cl^-), sodium hypochlorite (NaClO) can be generated, as well as their oxidized species such as ClO_3^-, substances that are very irritating and harmful, even at low concentrations, and imperceptible to analytical methods[38].

The intravenous infusion of ozonated saline is potentially toxic to some authors and, therefore, this practice is not recommended by them. Ozonated saline may contain toxic substances, including hydrogen peroxide, bromous acid and hypochlorous acid[38]. Reactive substances can be formed from ozonation of SF 0.9% and the toxicological evaluation of these products is a complex challenge that must be better studied and elucidated.

In contrast, 0.9% ozonated SF results from an alternative need to greater auto-hemotherapy, whose method requires the legally recognized handling of blood and be assisted, often, by hematologists and hemotherapists.

Researchers point out that chlorine gas is formed in acidic media with a pH below 3.0; hypochlorous acid (HClO) forms between a pH of 3.0-7.5; while ClO_3^- are formed from weak alkaline solutions with a pH greater than 7.5. In view of the pH of SF 0.9%, which is between 5.5-5.7 and blood, which is 7.4, the formation of HClO is imminent both in ozonated saline and in the blood[2]. Which, theoretically, allows the recommendation of 0.9% ozonated SF, as well as ozonation via major autohemotherapy, as in both cases there is the formation of HClO.

There is no consensus for the use of 0.9% ozonated SF in the world. In Russia, this practice is quite widespread, however, scientific articles published only in Russian, make it difficult to access this information and, consequently, the dissemination of this practice[2,39].

It is necessary to conduct more studies in vitro and in vivo to better clarify the possible deleterious effects versus the application of intravenous and topical infusion of 0.9% ozonized SF for various therapeutic purposes.

Final Considerations

With the present study, we determined the ozone concentrations in different types of aqueous solutions (ABDNE, AMDNE, IEA and SF 0.9%), using the DPD method. Among the aqueous solutions used, it was noticed that the IEA obtained a more satisfactory Csat than the other solutions.

We evidence that the ozonation time of 500 ml AIE (concentration of O_3 at 56 mg / L and ¼ of O_2 flow), with a temperature between 19 ° C and 30 ° C, is 15 minutes.

Ozonized aqueous solutions are neglected by researchers and health professionals in clinical practice, who choose to use systemic application routes (rectal insufflation and major auto-hemotherapy) and / or ozonized oil, whose concentrations of O_3 and / or its by-products are more accessible and clearer.

The fragility of O_3 in water was noteworthy, resulting from the vulnerability of using the ozonized aqueous solution for therapeutic purposes. The high degradation of O_3 in aqueous media makes it difficult to standardize doses and the specificity of the water to be used. Studies with ozonized solutions, whose methods of determining concentration and time of ozonation are not clear, produce dubious results and open to question, making it difficult to demystify and consolidate ozone therapy, as an integrative therapy in clinical practice, including in nursing and several other areas. In the meantime, nursing is essential for providing quality care throughout the organizational health structure[40], contributing to the production of knowledge about ozone therapy.

The effect of ozonization of SF 0.9% corroborates with the concept that other oxidized compounds can mask the results of Csat of O_3. Ozonized saline solutions should be better investigated both in vivo and in vitro to better determine their benefits from their use systemically and topically versus their possible deleterious effects at the cellular level and to various organ systems.

The determination of O_3 and its by-products in different aqueous solutions remains an important challenge. The development of tests with strict control of pH, temperature, and comparison between several simultaneous methods, will be opportune to outline a pattern of O_3 behavior in different aqueous solutions. In this perspective, more studies about the dosage and concentrations of O_3, as well as, of its by-products should be carried out, to support the best decision making about safe doses applied in ozone therapy.

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