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Original Article

Therapeutic Effect and Safety of Rectal Ozone Therapy in Mild and Moderate Symptomatic SARS CoV-2 Positive Patients

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Abstract

Background: COVID-19 an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Ozone therapy would be a therapeutic option for COVID- 19. Objective: To explore therapeutic effect and safety of rectal ozone therapy in mild and moderate symptomatic SARSCoV-2 positive patients, is the purpose of this study.

Methods: An exploratory, controlled, open and monocentric study was carried out in 32 patients, distributed at random in two groups of 16 patients each. The first group received rectal ozone therapy (ROT) with Standard treatment (ST) and the second one only ST. ROT were applied every 12 h for 10 days. Patients aged 19-80 years were included, after signing the informed consent, with positive SARS-CoV 2 symptomatic. RT-PCR and clinical signs evolution were primary efficacy variables. Ferritin, C-reactive protein, oxidative stress biomarkers, inflammatory cellular indicators, and biochemical and hematological variables.

Results: Patients (81%) had negative RT-PCR after ROT tenth application, with significant differences to ST group (43%). ROT significantly increases Glutation (GSH) levels compared to ST group, but not other REDOX markers as SOD, MDA, ON and AOPP. Catalase activity increased in both groups.

Conclusion: This study demonstrates the efficacy and safety of ROT in both mild and moderate symptomatic SARS-CoV 2 positive patients.

Keywords: *COVID 19, SARS CoV-2, Rectal Ozonetherapy, REDOX Balance, Superoxide dismutase, Glutation*

Introduction

COVID-19 is the third known zoonotic coronavirus disease after Acute Respiratory Response Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS-CoV), which also originates from the β cluster –coronavirus [1].

Among the complexities of the pathophysiological process of this viral infection, the so-called "storm of decompensated cytokines" stands out, which damage the vascular system, causing activation of the coagulation system, the consequent formation of thrombosis, which prevents perfusion into the tissues, causes multiple organ failure and death of patients [2].

The generation of free radicals is one of the pathogenic mechanisms of viruses, to cause inflammation and tissue damage. Oxidative stress is induced after the virus enters the host cell to facilitate its replication [3]. This suggests that the use of immunomodulatory and stimulatory therapeutic forms of the endogenous antioxidant response, such as ozone therapy, could counteract the pathophysiological development of COVID 19.

Several therapeutic effects justify the use of ozone in COVID-19 patients [4]. Ozone therapy stimulates Nrf2 [5,6], that would be an important physiological mechanism to blocked virus (SARS CoV 2) replication endogenously, preventing receptor contact to the virus, by reducing the expression of ACE2 and TMPRSS2, inactivating the virus replication ability [7]. The rebalancing of REDOX state achieved with ozone therapy is also important to cytokine synthesis induction by monocytes and lymphocytes, heme-oxygenase (HO-1) and shock proteins release, which are powerful activators of the immune system [8,9].

The immunomodulatory action of inflammatory response mediated by pro-inflammatory cytokines and increase in endogenous antioxidant activity, accompanied by the increase in nuclear transcription factor Nrf2 (5), by ozone therapy, was demonstrated in both preclinical [10-14] and clinical chronic pathological processes [15,16].

The rectal ozone therapy efficacy and safety was studied in 12 clinical trials, including chronic pathologies (angiopathy, chronic inflammation, immune imbalances, chronic rheumatological inflammation, among others), reaching improvement in both clinical and biochemical parameters, without adverse effects, only in one clinical trial mild irritation was evidenced in two patients [17].

Recently, the ozone therapy benefits applied by MAHT as adjuvant treatment in severe patients with COVID-19 have been demonstrated in different countries such as China [18], Italy [19,20] and Spain [21]. On the other hand, rectal ozone therapy insufflation also demonstrated its benefits in severe COVID 19 patients [22,23].

Due to all these antecedents of ozone therapy and the eminent need to provide a therapeutic solution to this COVID- 19 disease, the objective is to explore the therapeutic effect and safety of rectal ozone therapy in both mild and moderate symptomatic SARS-CoV 2 positive patients.

Materials and Methods

Study Design

The study was conducted following the ethical principles reflected in the 2013 Helsinki declarations and WHO recommendations. The exploratory study was an open-label and randomized trial. The study was conducted from May to August 2020 and the Hospital Health Care Ethics Committee authorized the study and ozone treatment. Positive confirmed COVID 19 patients, hospitalized in "Salvador Allende" Hospital, La Habana, Cuba were recruited for the study. Eligibility criteria for the study were the age and positively tested COVID 19 at least for 48 h later. The institutional review board of the hospital, Cuban Ministry of Public Health (MINSAP) and the Cuban Regulatory Agency (CECMED) approved this study and registered in the clinical trials public register with the number: RPCEC 0000320.

Inclusion Criteria

Adults of age 19 to 80 from both sex with positive reverse transcription-polymerase chain reaction (RT-PCR) from the nasopharyngeal swab test result, presenting mild to moderate clinical signs and willing were included in the study. All hospitalized patients included were informed of the procedures and potential risks and gave written informed consent.

Exclusion Criteria

1) Pregnancy or lactation; 2) G-6PD (glucose 6-phosphate dehydrogenase) deficiency (favism); 3) Patients with uncontrolled hyperthyroidism, 4) patients with abnormal coagulation, thrombocytopenic and active bleeding. 5) Allergic or intolerance to ozone. 6) Patients that use immunosuppressive medication. 7) Patients participating in another clinical trial. 8) Patients with psychiatric diseases. 9) Patients suffering from uncontrolled chronic disease.

Groups

We screened 32 patients positive SARS-CoV 2, confirmed by RT-PCR and hospitalized in the "Salvador Allende Hospital". Patients were randomly assigned to two groups of 16 patients each. Randomized treatment was open-label. Patients were assigned to a serial number by the study coordinator. Each serial number is linked to a computer-generated randomization list assigning the treatment regimens. The first group received rectal ozone therapy combined with standard treatment (Ozone + standard treatment (ST) and the second group with ST, where the patients were provided with conventional care as recommended in clinical management protocol for COVID 19 advocated by MINSAP.

Ozone Rectal insufflation

Medical Ozone obtained by Ozomed Plus®, (Ozone Generator) National Centre for Scientific Research, BioCubaFarma, Habana, Cuba. For rectal administration, the patients were placed in lateral decubitus position with lower limbs flexed and then lubricated rectal catheter was introduced rectally with patient's collaboration. A hemostatic clamp was placed on the catheter before the gas was insufflated. Ozone from the generator and immediately insufflated through the catheter, after removing the hemostat clamp. The insufflation time will be a few minutes, at an administration rate of 1 ml/s. The Ozone therapy rectal insuflation schedule was the following described in TS1.

Standard Treatment (ST) Approved by MINSAP Protocol for COVID-19, Version 1.4

- Kaletra ® (Capsules 200 mg lopinavir + 50 mg ritonavir) Medsol, Havana, Cuba.
- Chloroquine (Tablets 250 mg), one every 12 h, for 10 days.
- Heberferon® (Interferon α-2b human recombinant + interferon-gamma human recombinant 3,5 M UI Ampoule (lyophilized)) according to the National Formulary, IM, three times a week. Heber Biotec, S.A. Havana, Cuba
- Ceftriaxone one bulb every 12 h during 10 days, in the cases with pulmonary infection diagnostic.

Analysis of Primary Efficacy Parameters

The primary endpoint in this study was the patient's percentage having negative RT-PCR test for SARS-CoV-2 in nasopharyngeal swab samples on 5th and 10th treatments. A global response was considered too, where RT-PCR and clinical signs are classified into the complete response if RT-PCR test was negative and clinical signs disappear; partial response if RT-PCR was negative and clinical symptoms presented at inclusion time did not worsen or disappear at least two of them and non-response, if RT-PCR was still positive, regardless of clinical signs.

Secondary variables are C-reactive protein (CRP), neutrophil/lymphocytes ratio and redox parameters. The secondary variables

were determined at the initial and after the 5th day of treatment. A high percentage of patients have a negative PCR on the fifth day and were discharged, for that reason most of the evaluations were made on the fifth day and not on the tenth day.

All redox parameters were determined in serum by spectrophotometric methods using Zuzi Spectrophotometer (Japan). Serum reduced glutathiones (GSH) concentrations were measured by kinetics assay using the glutathione reductase reaction [24]. Malondialdehyde (MDA) concentrations were analyzed with the LPO-586 kit obtained from Calbiochem (La Jolla, C.A., USA) [25]. Superoxide dismutase (SOD) activities were assayed by a modified pyrogallol autoxidation method [26]. Catalase (CAT) activity was measured according to the method of Claiborne [27]. Serum advanced oxidation protein products (AOPP) was measured according to the methods of Witko-Sarsat *et al*, 1998 [28]. Nitrates and nitrites relation (NO) levels were measured according to Griess methods described by Granger et al 1996 [29].

Blood parameters such as hematocrit, hemoglobin, and erythrocyte sedimentation rate, were screened by Hematological counter MICROS 60. Others as triglycerides, creatinine, cholesterol and alanine aminotransferase activity were performed by standard procedures in HITACHI analyzer 912. As an efficacy response, the values of hemoglobin, hematocrit, erythrocytes, leukocytes, platelets and differential of leukocytes (lymphocytes, monocytes, basophils, neutrophils and eosinophils), erythrocyte sedimentation were considered to normalize. Also as an efficacy response, the values of albumin, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), gamma-glutamyl transferase (GGT), glucose, creatinine, bilirubin, uric acid (AU), cholesterol, triglyceride, and high-density lipoproteins (HDL) were considered to remain within their normal values. The safety and tolerability were evaluated through complementary variables (hemogram and blood chemistry). Analysis of adverse reactions occurrence was included too. In addition, a subjective assessment of tolerability was performed, according to following categories: Very good (no adverse events-AE), Good (mild and transient AE), Fair (moderate AE), Poor (severe AE) [30-53].

Statistical Analysis

For the evaluation of the response, the proportions of response by groups were estimated for the main variables (a negative RT-PCR test and the evolution of the clinical signs). The groups were compared using Fisher's exact test. This analysis was carried out for the variable "global response" according to the success criteria defined in the protocol. For the secondary variables (laboratory variables) were compared at times 5 and 10 days with respect to baseline using the paired t-test (before-after) or the Wilcoxon signed-rank test, as appropriate.

Results

With respect to baseline characteristics of the 32 patients included in the study there were no statistical differences, between the groups according to demographics, gender and age of patients (p>0.05), except for comorbidities risk which was high in the Ozone group (87%) in comparison to 68,8% for the control group (p=0.0021). Regarding clinical symptoms classification (mild and moderate), the ozone group had 50% for both mild and moderate symptoms, however, the control group had 69% mild symptoms patients and 31% with moderate symptoms, which showed significant differences between both groups (p=0.0236).

After the fifth day of ozone therapy treatment, 81% of patients had a negative RT-PCR, with significant differences (p=0.01) compared to the control group (43%). After 10 days of treatment, 93.8% of patients showed a negative RT-PCR in the ozone group, with significant differences (p=0.01) with regards to the control group (62.5%) (Table 1).

The severity of clinical symptoms and signs improved significantly after 5 days of treatment in ozone group, compared to control group (p<0.05), without differences at 10 days of treatment (Table 2). Regarding global response, there were significant differences for total, partial and non-response between groups. In the ozone group, the percentage of patients who had a total response (25%) increased significantly (p<0.05) compared to the control group (0%) after 5 days of treatment. Furthermore, 56. 3% of patients from control group had non-response on the fifth day, compared to ozone group (18.8%) (p<0.05). After 10 days, the results were similar, in ozone group increased significantly (p<0.05) the patient percentage (37.5%) with a total response with regards to control group (12.5%) (Table 2).

Ozone and control, showed a reduction in the levels of C reactive proteins after 5 days of treatment, but only was significant (p<0.05) for the control group. Regarding related indicators, such as neutrophil/lymphocyte ratio (N/L R), both groups experience a reduction of N/L R on the 5th day of treatment. However, only the ozone group

Table 1: RT-PCR SARS CoV 2 analysis in swab samples from each group.

Immunological response			
RT-PCR		**Ozone**	**Control**
5th day	Negative	13 (81.3%)	7 (43.8%)**
	Positive	3 (18.8%)	9 (56.3%)
10th day	Negative	15 (93.8%)	10 (62.5%)**
	Positive	1 (6.3%)	6 (37.5%)

**Pearson's chi-squared test.

Table 2: Disease evolution according to clinical symptoms and signs and global response to treatments.

	5th day		**10th day**	
	Ozone	**Control**	**Ozone**	**Control**
Disease evolution according to clinical symptoms and signs+, n patients (%)				
Improved severity of the disease	7 (43.8%)	1 (6.3%)*	7 (43.8%)	3 (18.8%)
Global Response to treatments, n patients (%)				
Total	4 (25.0%)	0 (0%)*	6 (37.5%)	2 (12.5%)*
Partial	9 (56.3%)	7 (43.8%)	10 (62.5%)	9 (56.3%)
Non-reponse	3 (18.8%)	9 (56.3%)*	0 (0.0%)	5 (31.3%)*

+Symptoms and signs: fever, headache, fatigue, sore throat and dry cough. *p<0.05 comparing both groups Fisher exact test.

achieved a statistically significant reduction ($p<0.05$) from 2.5 to 1.5 mg/L (Table 3).

The behavior of the Redox state, shown by the values of antioxidant indicators (levels of glutathione and activity of CAT and SOD) and pro-oxidants (AOPP, MDA and NO), are shown in Table 4, for each group, at the beginning (baseline) and at 5 days after starting the treatments. Both groups began the study with similar GSH values, without significant differences between them. The group of patients that received treatment with rectal ozone showed a significant increase ($p=0.025$) in GSH levels on the fifth day of treatment. However, the control group did not experience significant changes on the fifth day with regards to the initial value. On the other hand, both groups show significant differences ($p=0.009$) between them on the fifth day after starting the study. Regarding CAT activity, both groups show significant increase ($p=0.001$) on the fifth day of treatment in comparison to the baseline value, without significant differences between the groups. SOD activity did not reveal significant changes between groups. The pro-oxidant indicators (AOPP, NO and MDA) did not reach significant changes in any of the study groups.

Table 3: Inflammation variables.

Groups	Baseline	5th day
C reactive protein mg/L (reference < 6)		
Ozone	17.8 ± 22.7	9.0 ± 11.4
Control	10.7 ± 16.2	5.1 ± 6.6**
N/L R		
Ozone	2.5 ± 1.5	1.5 ± 0.9**
Control	2.3 ± 1.4	1.6 ± 1.0

**Comparison within groups (before and after) Wilcoxon signed-rank test.
N/L R: neutrophil /lymphocytes ratio.

Table 4: The behavior of the REDOX indicators for each of the groups.

Variables	Ozone		Control	
	Baseline	5th day	Baseline	5th day
GSH (mmol/mg Hb)	448.1 ± 105.2	511.7 ± 58.3*+	424.0 ± 72.2	398.6 ± 68.7
CAT (U/mg Hb min)	266.4 ± 47.6	302.2 ± 47.8*	223.4 ± 35.6	257.6 ± 39.3*
SOD ((U/mg Hb min)	3.01 ± 0.6	3.23 ± 0.4	2.27 ± 0.4	2.77 ± 0.4
AOPP (µM/cloramina T	20.6 ± 1.8	20.7 ± 2.7	21.66 ± 1.5	21.88 ± 2.7
NO ([NO_2] µM)	31.3 ± 4.8	31.7 ± 7.4	33.3 ± 9.4	40.7 ± 19.0
MDA (mmol/mg Hb)	3.3 ± 0.6	3.2 ± 0.6	3.0 ± 0.4	2.9 ± 0.5

SD: standard deviation, CAT: catalase, SOD: superoxide dismutase, MDA: malondialdehyde, GSH: glutathione, AOPP: advanced oxidation protein product. +Compare between groups and *$p<0.05$ differences between the baseline and the 5th day, by t student test or Wilcoxon test.

The hematological indicators evaluated did not show differences between groups at the evaluation times (baseline and the fifth day) except for the percentage of neutrophils and lymphocytes, where in both groups, neutrophils were significantly reduced at the fifth day of treatment (after the 10th application of ozone treatment) compared to baseline. Furthermore, the lymphocytes percentage increased in both groups, but only with a significant ($p<0.05$) difference in the control group. Both figures of neutrophils and lymphocytes (baseline and 5th day) were within the reference values.

The biochemical behavior in blood serum in terms of triglyceride values, there was a significant increase for both groups on the fifth day regarding the baseline values, being the value in the control group within the range of normal values. Cholesterol values were significantly reduced in the control group in comparison with the baseline value. In the group of patients treated with rectal ozone therapy on the fifth day, a significant reduction in ALT levels was observed in the ozone group in comparison with the baseline. During all the biochemical analyzes carried out on the blood, it was found that, despite observing some significant differences in some indicators at the fifth day of treatment in comparison with the baseline value, none of these are outside the values of references reported as normal.

With respect to adverse events, there are no significant differences among the study groups. In 12 patients presented AE for 75%, and only 4 patients did not present AE (25%). In the group of control patients, 9 of them (56.3%) who presented AE were registered, and 7 (43.8%) who did not present AE. There were no significant differences between the two groups. The intensity of the side effects was considered mild and moderate for both groups.

The adverse events associated with rectal ozone application were feeling of full intestines, tenesmus, colics and intestinal peristaltic movements. Adverse events were recorded daily.

No deaths or serious AE were reported during the study. These patients have their general condition compromised, which together with the adverse reactions generated by the conventional drugs (Heberferon, kaletra, chloroquine) that they are taking, mask the real response to the tolerability of ozone therapy.

Table 5: Physical Safety Indicators.

Parameter	Ozone (n=16)		Control (n=16)	
	Initial	5 d	Initial	5d
Weight (kg)	78.59 ± 17.9	77.83 ± 18.02*	79.88 ± 16.6	78.64 ± 16.6*
Temperature (°C)	36.4 ± 0.76	36,2 ± 0.54	36.3 ± 0.77	36.3 ± 0.56
Systolic blood pressure (SBP) (mmHg)	117.8 ± 6.04	115 ± 6.32	120 ± 9.7	114 ± 8.9
Diastolic blood pressure (DBP) (mmHg)	75.6 ± 7.3	73.5 ± 8.0	75.9 ± 6.6	74.1 ± 6.6
Heart rate (HR)	79.7 ± 6.2	77.8 ± 5.7	77.4 ± 6.4	77.4 ± 5.05
Respiratory rate (RR)	17.5 ± 0.9	17.2 ± 0.9	17.4 ± 0.9	16.7 ± 1.6*

*Comparison within groups (before and after) Wilcoxon signed-rank test.

The physical safety indicators evaluated showed a significant reduction in the bodyweight of the patients after the fifth day of treatment in both groups (Table 5). On the other hand, a significant reduction in respiratory rate was evidenced in the control group on the fifth day, but despite reaching statistical significance, is considered not relevant within the analysis of the general condition of the patient, since none of these worsened their clinical symptoms.

No deaths or serious AE were reported during the study.

Discussion

This exploratory clinical trial results showed negative RT-PCR in the 81% of patients treated with conventional treatment plus ozone rectal insufflation every 12 h after 10 applications of ozone therapy, with significant differences with regards to the control group (conventional treatment) where only 43% of the patients obtained negative RT-PCR in the same time. Regarding the percentage of negative RT-PCR on the 10th day (20 applications of ozone therapy), the significant differences in favor of ozone therapy are maintained. This result, is considered the first evidence on the effect of rectal ozone therapy on the PCR result in COVID 19 positive patients with mild and moderate symptoms. Similar results were reported in a clinical trial in COVID-19 positive patients with mild and moderate symptoms, who were treated with the combination of rectal ozone therapy and minor autohemotherapy (minor AHT). The scheme used was rectal ozone therapy twice daily (150 mL of ozone volume with a concentration of 40 mg/L) and minor AHT less than 25 mg/L of ozone concentration, once a day. The results confirm that 77% of the patients had a negative RT-PCR at day 5, compared to 43% in the control group. After the 10th day, 100% of the cases showed negative RT-PCR in the ozone therapy group, which was significantly higher compared to 70% in the control group [30].

On the other hand, it is important to point out that the negative RT-PCR in patients with rectal ozone therapy was accompanied by a significant improvement in clinical symptoms on the 5th day, compared to the control group. Regarding the evaluation of the treatment's global response, it was identified that rectal ozone therapy favored significantly the total response (negative RT-PCR and disappearance of clinical symptoms) compared to the control group, both in the analysis to 5 days. Similar results are reported in the clinical trial [30], where the cough and dyspnea, improved on the 5th and 10th day of treatment with rectal ozone therapy and minor AHT, compared to the control group.

In a recent study, the effectiveness of rectal ozone therapy was reported on the clinical symptoms of COVID-19. This study was carried out in four patients with severe pneumonia [23]. It describes that after 10 and 39 days with failed evolution under conventional treatment (retroviral drugs, IL6 and IL1 inhibitors, antibiotics and methylprednisolone), rectal ozone therapy was applied compassionately, five applications of 100 mL volume with a 35 mg/L ozone concentration, which resulted in a significant improvement dyspnea, respiratory rate, and oxygen saturation.

Other studies report the success of ozone therapy, applied by major autohemotherapy (M-AHT), in patients with COVID 19 in critical condition, hospitalized in intensive care units (ICU), in which the efficacy of the treatment was reported in terms of the improvement of the patient's health status, or condition in a much shorter time than in conventional treatment [21]. A percentage of 53% of SARS-CoV 2 positive patients, treated with ozone therapy via M-AHT, significantly improved clinical symptoms compared to the control group [31].

C-reactive protein (CRP) is synthesized in the liver and is an acute reactive phase protein that is increased in the blood in a wide range of inflammatory diseases. This protein is increased in 73-93% of patients infected with COVID-19, particularly in the severe phase of the disease [32]. All the COVID-19 patients included in this trial, characterized by mild and moderate symptoms, presented mean values of CRP higher than those reported as normal (<6 mg / L). Both, ozone therapy group and conventional treatment reduced CRP levels on the 5th day of evolution, with percentages of changes of 49.4% and 52.3%, respectively. This reduction was only statistically significant in the control group. Although the ozone group did not show statistical significance, the possibility that this result is influenced by the fact that the number of patients was low and there was a high standard deviation should be considered. As reported in other studies, rectal ozone therapy reduces CRP in patients with COVID-19 (mild and moderate) [32] and also in severe patients treated with ozone therapy via MAHT [24]. Other inflammatory and thromboembolic markers such as IL-6 and Dimer-D were reduced by MAHT ozone therapy in COVID-19 patients hospitalized in intensive care units. In addition, ozone improved the respiratory function indicators, such as oxygen saturation percentage (Sat O2) and the arterial pressure index of oxygen/fraction of inspired oxygen (PaO2 / FiO2) [19].

Regarding the analysis of the cellular indicators of the inflammatory response, a significant reduction in N/L ratio was observed in the group treated with ozone after 5 days of treatment, which corresponds to the increase in the lymphocyte count in this group. Although in the control group there were no statistically significant differences regarding this indicator, a tendency to decrease was observed after 5 days of treatment. The N/L ratio index is a predictive prognostic factor for the risk of death in hospitalized SARS CoV 2 positive patients undergoing endotracheal intubation with prognostic values of N/L > 4.94 reported by Tatum D. *et al.* (2020) [33]. In this study, the patients presented baseline N/L values of 2.5 and 2.3 in the ozone and control group, respectively, decreasing to 1.5 and 1.6 in each group after 5 days of treatment, which is consistent with the improvement of patients and favors the prognosis of the disease.

On the other hand, considering the oxidative stress indicators evaluated in the study is analyzed was verified that pro-oxidant indicators (MDA, PAOP and NO) do not suffer significant changes in the patient group treated with rectal ozone therapy. Some results support the association between oxidative stress, inflammation, and the pathogenesis of SARS-COV infection [34]. In the preclinical setting, it is evidenced that the overproduction of Reactive Oxygen Species (ROS) and a deprived system of antioxidants play a major role in the pathogenesis of SARS-CoV infection, as well as in the progression and severity of the respiratory disease. Experimental animal models of severe acute respiratory syndrome have shown increased ROS levels and impaired antioxidant defense during SARS-CoV infection [35]. Some authors suggest that the appearance of a

severe lung injury in patients infected by SARS-CoV depends on the activation of oxidative stress that is coupled with innate immunity and activates transcription factors, such as NF-kB, resulting in a response proinflammatory in the host in an exacerbated form [36].

In this study, it is highlighted that rectal ozone therapy significantly increased the GSH content on the fifth day of treatment, in comparison with the basal content and with the value of the control group on the fifth day [36]. These results correspond to those achieved in other trials carried out in chronic diseases (rheumatoid arthritis) [37], heart failure [38], multiple sclerosis [39] and coronary artery disease [40], among others, where demonstrates the stimulating action of ozone therapy on endogenous antioxidant systems. On the other hand, both treatments experienced an increase in CAT activity, without differences between them. The pro-oxidant indicators analyzed (MDA, PAOP) did not show significant changes on the fifth day of treatment.

Several studies indicate that higher glutathione levels can improve an individual's responsiveness to viral infections. It protects the host's immune cells through its antioxidant mechanism and is also responsible for the optimal functioning of a variety of cells that are part of the immune system. Glutathione inhibits the replication of various viruses at different stages of the viral life cycle, and this antiviral property of GSH appears to prevent the increase in viral load and the subsequent massive release of inflammatory cells in the lung ("cytokine storm"). Endogenous glutathione deficiency appears to be a crucial factor that increases the oxidative damage of the lung induced by SARS-CoV-2 and, as a result, leads to severe manifestations such as acute respiratory distress syndrome, multiple organ failure and death in patients with COVID-19 [41].

The nuclear transcription factor Nrf2 is the main regulator of the antioxidant response element (ARE) that directs the expression of cytoprotective proteins. Nrf2 confers protection against these pulmonary disorders [42], stimulates the innate immune system, eliminating numerous pathogenic bacteria and viruses [43]. Recently, a study in 40 patients showed that COVID-19 severity was directly related to the age and inflammatory response intensity was inversely associated with Nrf2 expression [44]. Patients with COVID-19 showed suppression of Nrf2 pathway, however, the pharmacological inducers of Nrf2 inhibited the replication of SARS-CoV2 and decreased the levels of inflammatory response [47]. Nrf2 agonists induce interferon (IFN)-independent antiviral program that is widely effective in limiting virus replication and suppressing pro-inflammatory responses of human pathogenic viruses, including SARS-CoV-2 [45].

It is well known that ozone therapy stimulates endogenous antioxidant systems through the expression of Nrf2 [5], which was confirmed in a clinical trial of multiple sclerosis, where rectal ozone therapy modulated the inflammatory response mediated by cytokines and increased antioxidant activity, accompanied by increased expression of Nrf2 [46]. There are several studies where have been well demonstrated that ozone therapy increases the level of Nrf2, with an improvement of the antioxidant defense system [47,48].

Although the physical examination of the patients showed a significant reduction in the weight of the patients 5 days after starting the treatment, for both groups, this indicator did not constitute a parameter of non-safety of the treatments, since it considers the influence of other aspects, such as the change of diet, the hospitalization of the patient and the general clinical symptoms that he presented, which prevented him from eating properly and therefore maintaining his body weight. Furthermore, no significant changes were observed in terms of hematological and blood chemistry indicators. The rest of the variables remained within the ranges of normal values.

Both treatments were tolerable. Within the tolerability classification, the highest percentage was regular for both treatments, both with high percentages, ozone therapy 60% and control 50%. The tolerability obtained for ozone therapy in this study is contradictory, as previous studies have shown that rectal ozone therapy has been very well tolerated [49]. This result could be subject to the fact that these patients, unlike those in other clinical trials, despite presenting mild and moderate symptoms of COVID-19, have implications for the general intake of their status, which together with adverse reactions generated by conventional drugs (Heberferon, Kaletra, chloroquine), mask the real response to the tolerability of ozone therapy. On the other hand, if we consider that ozone therapy significantly reduced the clinical symptoms of the patients compared to the control, we could confirm that this classification was influenced by the general deterioration of the patients as they were under so many adverse effects caused by the conventional medications.

AE occurred in 75% of the patients in the group that received treatment with rectal ozone therapy and in 56% of the patients in the control group. This difference between the groups with respect to this indicator was not statistically significant. These results were not as expected, since, according to experience and reports in the literature on clinical trials with rectal ozone therapy, this procedure, in general, does not cause AE in this sense, only very few and of mild intensity have been reported [50-52].

But, in this trial for the first time, rectal ozone therapy is being used in patients with COVID-19 in conjunction with conventional treatment (retroviral and others) who have adverse reactions. For example, in the study performed in COVID-19 convalescent patients treated with rectal ozone therapy, 80% (28/36) of the patients reported the feeling of fullness of the intestines, without other reports, that disappeared rapidly and in no case, treatment was required [53]. In addition, a high index of well-being was observed in the group of patients who received ozone and the safety in the use of this technique confirmed its therapeutic usefulness.

The compliance with the treatments by the ozone therapy group should be highlighted, in which no patient interrupted the treatment for any reason. However, in the control group 6 patients (37.5%) did not comply with the administration of Kaletra and chloroquine and 3 (18.8%) received incomplete treatment, which could be the reason why the analysis of the presentation of AE, had an increasing trend in the group that received rectal ozone therapy, although this was not statistically significant in comparison with the control group.

In conclusion, the results of this exploratory clinical trial show that rectal ozone therapy applied at the doses and therapeutic scheme described was effective and safe as an adjunctive treatment in positive patients for COVID-19. Ozone therapy significantly achieved a

negative RT-PCR in 81 % of patients and reduced clinical signs after five days of treatment. This primary efficacy result was accompanied by an increase in the glutathione content and the activity of CAT in the serum of the patients, improving the endogenous antioxidant response. This exploratory study demonstrates the efficacy and safety of rectal ozone therapy in both mild and moderate symptomatic SARS-CoV 2 positive patients. The combined treatment showed superior efficacy to conventional treatment by reducing the time in which patients improve clinical symptoms and obtain a negative RT-PCR. In both groups, oxidative stress indicators and cellular markers of inflammation improve, and the treatments are safe and well-tolerated. As it is an exploratory study, the number of patients included limits the scope of these conclusions, so future Phase III studies should confirm the results found.

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