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Original Article

Complementary application of the ozonized saline solution in mild and severe patients with pneumonia COVID-19: A non-randomized pilot study

[Aplicación de la solución salina ozonizada como terapia complementaria en pacientes COVID-19 con neumonía de estadía mediana a severa: Estudio piloto no aleatorizado]

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Abstract

Context: Currently, there is no effective antiviral therapy recommended for novel coronavirus pneumonia 2019 (COVID-19).

Aims: To assess the safety of ozonized saline solution (O_3SS) used as a complementary therapy in adult COVID-19 patients.

Methods: Twenty-five adult patients hospitalized with mild to severe symptoms of COVID-19, who met the inclusion criteria and were treated from April 18 to April 26, 2020, at Virgen De La Paloma Hospital, Madrid, Spain were included in this study. Patients were assigned to receive standard care consisting ceftriaxone (250 mg – 2 g twice daily for 7 days) plus azithromycin (500 mg once daily for 5 days), of 200 - 400 mg hydroxychloroquine twice daily for 5-7 days plus tocilizumab 400 mg twice daily for 5 days, low molecular weight heparin and 40 to 60 mg metil-prednisone plus O_3SS, 200 mL, 3-5 µg/mL per day for 10 days. No control group was included, the data was compared to clinical trials in this subject. Secondary endpoints assessed included the clinical status of participants, laboratory examinations, and duration of viral shedding.

Results: Patients with COVID-19 with mild to severe symptoms who received intravenous O_3SS as an adjunct treatment experienced no side effects. The main results of O_3SS treatment were a tendency to improve clinical symptoms without side effects. None of the patients treated died.

Conclusions: Early evidence of efficacy shown improvements in symptoms such as dyspnea, weakness, and reduction in body temperature were observed and corresponded to improvements in laboratory results including D-dimer, fibrinogen, lactate dehydrogenase, and C-reactive protein. These preliminary data will serve as the basis for a future study of the effectiveness of this therapy.

Keywords: COVID-19; ozone therapy; ozonized saline solution; pneumonia; SARS-CoV-2.

Resumen

Contexto: Actualmente, no existe una terapia antiviral eficaz recomendada para la neumonía por el nuevo coronavirus 2019 (COVID-19).

Objetivos: Evaluar la seguridad de la solución salina ozonizada (O_3SS) como terapia complementaria en pacientes adultos con COVID-19.

Métodos: Se incluyeron veinticinco pacientes adultos con síntomas leves a severos de COVID-19, que cumplieron con los criterios de inclusión. Estos fueron tratados del 18 al 26 de abril de 2020, en el Hospital Virgen de La Paloma, Madrid, España. Los pacientes fueron asignados para recibir atención estándar [ceftriaxona (250 mg - 2 g cada 12 h por 7 días); azitromicina (500 mg cada 24 h por 5 días); 200-400 mg de hidroxicloroquina cada 12 h por 5-7 días; tocilizumab 400 mg cada 12 h por 5 días; heparina y 40 a 60 mg de metil-prednisona] más O_3SS, 200 mL, 3-5 µg/mL por día durante 10 días. No se incluyó ningún grupo de control, los datos se compararon con ensayos clínicos similares. Los criterios de valoración secundarios incluyeron el estado clínico de los participantes, los exámenes de laboratorio y la duración de la diseminación viral.

Resultados: Los pacientes con COVID-19 tratados que recibieron O_3SS intravenoso como tratamiento complementario no experimentaron efectos secundarios. El tratamiento con O_3SS tendió a mejorar los síntomas clínicos. Ninguno de los pacientes tratados falleció.

Conclusiones: Se observaron evidencias tempranas de mejoras en síntomas como disnea, debilidad y reducción de la temperatura corporal, que correspondieron con una mejora de variables como: el dímero D, fibrinógeno, lactato deshidrogenasa y proteína C reactiva. Estos datos preliminares servirán de base para un futuro estudio de la eficacia de esta terapia.

Palabras Clave: COVID-19; ozonoterapia; solución salina ozonizada, neumonía; SARS-CoV-2.

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INTRODUCTION

A coronavirus called Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) has been isolated as a causative agent of severe pneumonia. This infection has been named coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO), which officially declared COVID-19 a pandemic on March 11, 2020 (Park, 2020). COVID-19 has rapidly spread to at least 215 countries and territories, and killed more than 1 million people as of October 29, 2020. There are no specific therapies available to treat Covid-19 infection.

Hydroxychloroquine (HCQ) and chloroquine (CQ) have gained unprecedented attention as potential therapeutic agents against COVID-19. In addition to their antimalarial use, they have also shown *in vitro* activity against COVID -19 (Pawar, 2020). However, there is a growing body of scientific evidence on their side effects, particularly in HCQ prolongation and cardiac arrhythmias (Pastick et al., 2020). Moreover, current data are insufficient to judge the efficacy of remdesivir for COVID-19, and the results of additional randomized studies are awaited (Pardo et al., 2020).

There are multiple physiological pathway dysregulations that appear to be disrupted by SARS-CoV-2 and related viruses. The angiotensin-converting enzyme (ACE2) has recently been identified as the receptor for SARS-CoV-2. The ACE2 system is an essential protection route against inflammatory injuries due to excessive oxidative stress. Unregulated ACE2 dysfunction worsens COVID-19 and could trigger multi-organ failure. The imbalance in the action of the peptides derived from ACE1 and ACE2 [angiotensin II (Ang II) and angiotensin 1-7 (Ang 1-7), respectively] may explain most of the pathological consequences of SARS-CoV-2 infections (Sriram and Insel, 2020).

Health risk factors that predispose patients with COVID-19 to disease progression to a more advanced stage include pro-inflammatory conditions such as hypertension, diabetes, and cardiovascular disease (Cheng et al., 2020). Previous studies of SARS-CoV virus infections in primates suggest that severe lung injury was due to an exacerbated inflammatory response mediated by activation of the innate immune system and upregulation of the NF-κB pathway (Smits et al., 2011). A recent human study has shown that in critically ill patients, the SARS-CoV-2 virus causes an exacerbated inflammatory response that is not self-limiting but produces an inflammatory cytokine storm in an uncontrolled manner. This happens because theoretically, the cytotoxic NK and T cells (CD8 and $CD4^{+}$) should inhibit the activity of macrophages, but on downregulation, this inhibition does not occur because infected cells lack the major histocompatibility complex, which should inhibit the action of the macrophage. This results in a suppression of functional lymphocytes (lymphopenia) leading to decreased immune function and increased susceptibility to infections (Yao et al., 2020). Additional animal studies have demonstrated that pulmonary fibrosis, which is a hallmark of the Severe Acute Respiratory Syndrome (SARS) disease process is mediated by induction of TGF-beta1 through upregulation of the ROS/p38-MAPK/STAT3/Egr-1 pathway both *in vitro* and *in vivo* (Li et al., 2016). Host genetics may also play a role in pathogenesis since studies in knockout mice have shown that a genetic deficiency of ACE2 receptors results in a reduction of Ang 1.7 thus increasing oxidative stress and susceptibility to advanced disease progression (Leon et al., 1998).

Low dose of ozone (O_2/O_3) is produced by a mixture of oxygen (carrier) and ozone (active component) in a carrier of 99.9% pure oxygen. Medical ozone (O_{3X}) therapies are effective in treating a range of human pathologies that have a physiological basis for inflammatory deregulation (oxidative stress). Correctly dosed and timed treatments can induce endogenous oxidative preconditioning (Leon et al., 1998). Potentially, O_{3X} may improve symptoms of COVID-19 acting as an inducer of bone adaptation, a modulator of pro-inflammatory cytokines, and improving tissue oxygenation (Martínez-Sánchez et al., 2020). Preliminary case reports showed the benefit of ozone treatment of 204 COVID-19 patients in China, Cu-

ba, Spain, Italy, Iran and U.S.A (Brownstein et al., 2020; Fernandez-Cuadros et al., 2020; Franzini et al., 2020; Hernández et al., 2020; Peña-Lora et al., 2020; Razzaq et al., 2020; Schwartz and Narros, 2020; Wu et al., 2020b; Zheng et al., 2020) also in 18 patients treated in Ibiza (Spain) as part of a prospective single-center cohort study (Hernández et al., 2020). These results allowed us to assess the safety and effectiveness of ozonized saline solution (O_3SS) in patients with mild to severe COVID-19 as an adjunct therapy. Here, we report data from 25 patients who received standard care (SC) plus O_3SS. The results suggest that O_3SS as a complementary therapy shows no side effects, early evidences of efficacy suggest favorable recovery of patients, stabilizes their biochemical index, reduces the need for oxygen support.

MATERIAL AND METHODS

The complementary application of O_3SS was carried out following the principles of the Declaration of Helsinki (World Medical, 2013) and the good clinical practice of the International Conference on Harmonization (Battershill and Fielder, 1998). All patients and/or legal representatives have been informed of the objectives and risks of participation. They had time to carefully read and sign the informed consent form. Random online clinical monitoring and quality control were performed. A virtual independent data safety and monitoring board (DSMB), made up of O_{3X} experts, clinicians and infectious disease experts from AEPROMO (Spanish Association of Health Professionals in Ozone Therapy) and ISCO3 (International Scientific Committee for Therapeutic Ozone), was selected to review the protocol and hold daily meetings to monitor the daily results of O_3SS application. The trial was reported following the Consolidated Standards of Reporting Trials (CONSORT) guideline (Cuschieri, 2019). The pilot study protocol was approved by the management of the site hospital (fast track) on April 17 and the full randomized clinical trial by the Madrid Regional Ethics Committee (number 05/20) on the 18 May. This manuscript is a partial report (pilot study) of the full study involving the comparison between two parallel groups SC alone and SC plus O_3SS. The pilot study will not be publicly registered as the data acquisition will be used to conduct the full randomized clinical trial [EudraCT number, 2020-002425-28 and AEMPS number, 20-0381], which will be publicly registered and performed after administrative approval, using the pilot study experience. The results will be reported in a future manuscript.

Design and site

Site

The complementary application of O_3SS was done following the criteria of a pilot, open-label clinical trial, from April 18 to April 26, 2020, aimed at initially treating hospitalized patients with the mild respiratory syndrome to severe secondary to SARS-CoV-2 infection COVID-19; and as a second objective, to assess the safety and efficacy of O_3SS. These patients were hospitalized at the Viamed Virgen de la Paloma Hospital, Madrid (declared a COVID-19 center during the epidemic). The hospital has all source documents recorded in an electronic medical registration system. Routine clinical analyses, laboratory examinations, and chest X-rays can be obtained locally.

Participants

Hospitalized patients with clinical suspicion of COVID-19 (i.e., history of fever and any respiratory symptoms, e.g., cough or rhinorrhea); male or female aged 18 to 98 at the time of registration; within a week of the onset; who have not participated in other clinical studies within the past three months; willing and able to sign informed consent for participation in the O_3SS application; Chest X-rays confirmed lung damage (for moderate cases); were included. Patients were recruited before laboratory confirmation of COVID-19 by reverse transcription-polymerase chain reaction (RT-PCR COVID-19), considering that this procedure could delay inclusion. The flowchart (Fig. 1) shows the suspected clinical-epidemiological cases as well as the cases already confirmed by RT-PCR COVID-19.

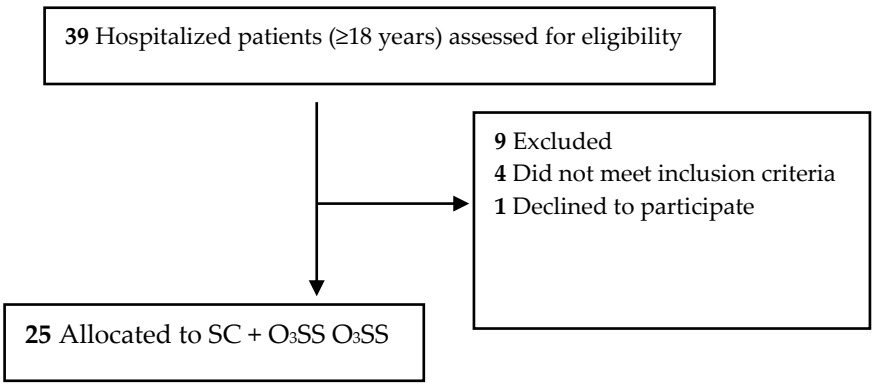

Figure 1. Study flow chart.

Eligible participants were allocated to receive standard care (SC), basically: 40 - 60 mg methylprednisone daily for 5 - 7 days plus tocilizumab 400 mg twice daily for 5 days, low molecular weight heparin (LMWH) and hydrocortisone plus O_3SS, 200 mL, 3 - 5 µg/mL daily for 10 days plus ozonized saline solution (O_3SS), 3 - 5 µg/mL daily for 10 days.

Exclusion criteria included: Participants who were pregnant, lactating or planning to become pregnant during the trial. Patients with significant renal or hepatic impairment or with scheduled elective surgery or other procedures requiring general anesthesia while applying O_3SS. Participants who had participated in a clinical trial involving an investigational product in the past 12 weeks before the study. Patients with G-6PD (Favism) defect. Patients who have used an immunosuppressant continuously or who have received an organ transplant within the past 6 months. Patients with a history of uncontrolled hyperthyroidism, an unstable period of severe cardiovascular disease, intravenous copper or iron supplementation, or any condition that did not allow patient safety during the study. The patient had to be transferred to a non-participating hospital within 72 h. Patients receiving copper or iron supplementation i.v.

Participants were assigned at the time of inclusion and subsequently identified only by their assigned number (always assigned in chronological order) throughout the O_3SS application. This is O_3SS's open-label application.

This is a preliminary report, focused on assay the safety of the application of O_3SS. Therefore, a control group was not included. To analyze the early efficacy criteria, the results were compared with those reported for groups of patients treated in similar circumstances in Spain or the rest of the world, in the same period of time. These preliminary results will serve to carry out a comparative clinical study to determine the efficacy.

Sample size calculation

According to the recommendations of Whitehead et al. (2016), the sample size calculation of this pilot complementary application of O_3SS is estimated. A medium sample (25 subjects) was selected for the main future experiment, which was designed to have a power of 90% and a two-tailed 5% significance.

Procedures

According to the hospital protocol, all patients who met the same study criteria (i.e. Acute Respiratory Distress Syndrome) received intravenous ceftriaxone (250 mg – 2 g twice daily for 7 days) plus azithromycin (500 mg once daily for 5 days), Enoxaparin (Clexane®) 40 - 60mg daily, HCQ 200 mg, methylprednisone 40 mg or prednisone 5 mg systematically, from day 0. Tocilizumab (Actemra®), 0.4 mg twice daily for 5 days, has also been prescribed if influenza infection is suspected. O_3SS consists of bubbling and saturating 200 mL of sterile physiological solution (0.9%) with an O_2/O_3

mixture for 10 min, at concentrations ranging from 3 to 5 µg/NmL. Continuous bubbling using the infusion set, the solution was administered i.v. (the basilic brachial or cephalic veins) for 15 to 30 min. Ozonation (bubbling) was stopped when about 50 mL of liquid remained in the bottle (ISCO3, 2020). Patients received SC plus O_3SS. In the first 5 days, the bubbling concentration used was 5 µg/NmL (total dose per day 250 µg O_3), administered daily. During the next 5 sessions, the bubbling concentration was lowered to 3 µg/NmL (total dose per day 150 µg O_3) and administered daily. Patients received a total of 10 O_3SS sessions. The ozone concentration was measured by a spectrophotometer integrated into the ozone generator (254 nm). The concentration of ozone in saline during the continuous bubbling flow has been calculated as ¼ of the bubbling concentration (Yoldi et al., 2019). Under these ozonization conditions, it was demonstrated that no H_2O_2 or HOCl appeared at the appropriate concentration (H_2O_2 not exceeding 0.0004% (Maslennikov et al., 2008) HOCL concentrations are less than 0.001 g/mL (Peretiagyn et al., 2006). The decomposition processes of ozone in aqueous solutions of 0.9% NaCl are not accompanied by the formation of products other than oxygen (Razumovskii et al., 2010).

The ozone was generated by a class IIb CE medical device (Ozonobaric P®, SEDECAL®, Spain). The container that administered the solution was disposable, made of medical-grade materials, free of phthalates, and fully ozone compatible. It has been classified by Bexozone® (Bexen Medical®, Spain) as a class IIb medical device. Physiological saline solution (0.9% NaCl) from (Lab. ERN, Spain) was used.

Clinical parameters were measured daily by routine clinical staff from day 0 to discharge or death, then on day 28 for discharged patients, to assess efficacy (days 7 and 14) and safety outcomes. Laboratory parameters and electrocardiograms were performed at the discretion of the clinician. Data were recorded on the case report form and transferred to an electronic database (Excel®, Microsoft®), which was then validated by external trial monitoring staff.

Dyspnea was assessed as follows: Grade 0, no dyspnea; grade 1, slight dyspnea; grade 2, moderate dyspnea; grade 3, severe dyspnea; grade 4 very severe dyspnea (Fletcher, 1952). Weakness was assessed as follows: 0, paralysis; 1, severe weakness; 2, slight weakness; 3, normal strength (Vanhoutte et al., 2012).

Outcomes

Safety criteria included adverse events occurring during treatment, serious adverse events, and premature or temporary discontinuation of treatment. Adverse events were classified according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NIH, 2010). The null hypothesis was that the complementary application of O_3SS in the experimental group would have a mortality rate of 50% lower than the mortality reported only for SC on day 14. Thus, the primary endpoint was mortality on day 14. Secondary endpoints included the participant's clinical status, laboratory examinations, chest X-rays on days 7 and 14, daily clinical status during hospitalization, duration of mechanical ventilation (if needed) and supplemental oxygen (if needed) and the time (in days) between the start of treatment and death. Here we present analyzes up to day 14, with lethality as the primary endpoint. Virological measurements included the detection of viral RNA was performed daily until 2 consecutive negative values were obtained.

To assess the efficacy result, the seven-category order scale was used, which consisted of the following categories: 1, not hospitalized with the resumption of normal activities; 2, not hospitalized, but unable to resume normal activities; 3, hospitalized, not requiring supplemental oxygen; 4, hospitalized, requiring supplemental oxygen; 5, hospitalized, requiring high-flow nasal oxygen therapy, non-invasive mechanical ventilation, or both; 6, hospitalized, requiring extracorporeal membrane oxygenation, invasive mechanical ventilation, or both; and 7, death (Wang et al., 2020b). Illness severity was defined as mild, no evidence of pneumonia on imaging; Moderate, fever and respiratory symptoms with radiologic signs of pneumonia;

Severe dyspnea, respiratory rate ≥30/min, blood oxygen saturation ≤93%, PaO2/FiO2 ratio <300 and/or pulmonary infiltrates >50% in 24 to 48 h; critical respiratory failure, septic shock, and/or multiple organ dysfunction/failure (Shang et al., 2020).

Laboratory analysis

Hematology and biochemistry analysis were performed in automated machines. Samples (2 nasopharyngeal swabs or 1 oropharyngeal swab) were subjected to the Novel Coronavirus (2019-nCoV) real-time RT-PCR assay, using a kit from Biopath-Unilabs (France) de Cobas z480 qPCR (Roche), with the use of LightMix Modular SARS-CoV-2 (COVID-19). Sampling did not stop when a swab at some point was negative. Baseline throat swabs were tested for the detection of the E gene, RdRp gene, and N gene. The samples on subsequent visits were qualitatively detected for the E gene.

Statistical analysis

Descriptive statistics were used for demographic, laboratory, and clinical data. To assess the safety of SC + O_3SS compared with SC alone, the proportion (and 95% CI) of death in the SC + O_3SS group was compared to the historical proportion (and 95% CI) of death in patients who did not use O_3SS in Spain and Europe during the same period (Borobia et al., 2020; Casa Rojo et al., 2020; Lechien et al., 2020). For the qualitative variables, χ^2 tests and Fisher's exact tests were performed. We used the t-test or the Mann-Whitney test to compare the means and the medians. Wilcoxon's signed-rank test and Hodges-Lehmann estimate were used to compare inter-quantile ranges (IQR). Statistical analyzes were performed in Statistical Version 17 of IBM SPSS, and a two-tailed $p<0.05$ was considered significant.

RESULTS

Demographic and clinical characteristics

A total of 25 patients who were assigned to the SC + O_3SS group completed the study (Fig. 1). Some patients (4 out of 25 [16%]) had COVID-19 confirmed retrospectively by a reverse transcription-polymerase chain reaction test. Patients with an unconfirmed initial disease and whose clinical and epidemiological presentation was compatible with COVID-19 were analyzed together. The Overall baseline characteristics are presented in Table 1.

Baseline characteristics show an overall median age (min-max) of 44 (30 - 95) years and a predominance of females (14 [65%]). The most common comorbidities were hypertension (4 out of 25 [16%]), asthma (3 out of 25 [12%]), hypothyroidism (3 out of 25 [12%]), smoking (2 out of 25 [8%]) and obesity (2 in 25 [8%]). Hypertension was more common in men than in women (4 of 11 [36%] *vs.* 0). On admission, oxygen supply was required in 14 of 25 patients (56%), which was more common in men than in women (10 of 11 [90%] *vs.* 4 of 14 [28%]). Baseline body temperature was above 37.5°C in 13 of 25 patients (52%); with a higher frequency in men than in women (8 out of 11 [72%] *vs.* 5 out of 14 [35%] respectively). The main clinical presenting symptoms were weakness (21 of 25 [84%]), dyspnea (19 of 25 [76%]), dry cough (14 of 25 [56%]), and anosmia (12 of 25 [48%]). Polymyalgia and headache were more common in women (both 43%) than in men (27% and 18%, respectively).

Laboratory results (Table 2) show borderline low hemoglobin levels in male patients. Increased levels of serum ferritin, fibrinogen, D-dimer, LDH, CPR, ALT, and AST were observed in all patients. Serum ferritin values were significantly ($p<0.05$) higher in women than in men and CRP was significantly ($p<0.05$) higher in men than in women. All patients were positive for qualitative SARS-CoV-2 PCR at baseline.

The most common X-ray result on a chest X-ray was the infiltration of ground-glass opacity (Table 2), in 40% of patients and pleural effusion in 20%. Pulmonary auscultation revealed rales, rales/rhonchi, and wheezing in 56% of patients. Most patients met the severe disease status (76%) and 6 (24%) met the criterion of mild disease.

Table 1. Demographic and clinical findings of patients at baseline.[a]

Variable	Total	Men	Women
n	25	11	14
Age, median (min-max) years	55 (30-95)	55(30-90)	55(45-95)
Current smoker n (%)[b]	2 (8)	2 (18)	0
History of drug abuse n (%)	1 (4)	1 (9)	0
Comorbidities n (%)			
Hypertension	4 (16)	4 (36)*	0
Asthma	3 (12)	2 (18)	1 (7)
Hypothyroidism	3 (12)	1 (9)	2 (14)
Obesity	2 (8)	2 (18)	0
Alcohol use disorder	1 (4)	1 (9)	0
COPD	1 (4)	1 (9)	0
Rheumatic diseases	1 (4)	1 (9)	0
Raynaud's syndrome	1 (4)	0	1 (7)
Tuberculosis	1 (4)	1 (9)	0
Chronic kidney disease	1 (4)	1 (9)	0
Diabetes	1 (4)	0	1 (7)
Heart disease	1 (4)	1 (9)	0
Peripheral arterial disease	1 (4)	0	1 (7)
Oxygen therapy on admission	14 (56)	10 (90)*	4 (28)
Body temperature, °C			
<37.5	12 (48)	3 (27)*	9 (64)
37.5-38.0	1 (4)	1 (9)	0
38.1-39.0	12 (48)	7 (63)*	5 (35)
Blood pressure (mm Hg)			
Systolic, mean (min-max)	120 (110-151)	120 (110-151)	120 (110-125)
Diastolic, mean (min-max)	80 (70-90)	80 (70-90)	80 (70-80)
O_2 saturation, median (min-max) %	93 (80-98)	90 (80-93)	93 (83-98)
Clinical symptoms[c]			
Weakness n (%)	21 (84)	9 (82)	12 (86)
Dyspnea n (%)	19 (76)	10 (91)	9 (64)
Dry cough n (%)	14 (56)	5 (45)	9 (64)
Anosmia n (%)	12 (48)	5 (45)	7 (50)
Polymyalgia n (%)	9 (36)	3 (27)*	6 (43)
Headache n (%)	8 (32)	2 (18)*	6 (43)
Diarrhea n (%)	6 (24)	3 (27)	3 (21)

[a]In all cases the race was white; [b]No former smoker was found; [c]Symptoms with frequency lower than 20 % n (%): cough with phlegm 4(16); central chest pain 4(16); pharyngodynia 3(12); abdominal distension 3 (12); abdominal colic 3 (12); flatulence 2(12); lateral chest pain 3(12); lower limb edema 1(4) and oliguria 1(4). COPD, chronic obstructive pulmonary disease. *Significant difference ($p<0.05$), $\chi 2$ tests for proportion between gender.

Table 2. Laboratory and radiographic findings of patients at baseline.

Variable	NR	Total	Men	Women
n		25	11	14
Leucocytes count, mean ± SD	(4.5 – 11) × 10^9 cells/L	7.00 ± 3.68	5.97 ± 1.86	8.30 ± 4.97
Lymphocytes count, mean ± SD	(1.0 – 4.8) × 10^9 cells/L)	1.49 ± 1.64	1.35 ± 0.52	1.67 ± 2.46
Platelets count, mean ± SD	(150 - 450) × 10^9 cells/L	287 ± 100	270 ± 99	309 ± 101
Eosinophils, mean ± SD	(0 - 0.4) × 10^9 cells/L	0.04 ± 0.05	0.03 ± 0.04	0.06 ± 0.06
Hemoglobin, mean ± SD	Male 138 - 172 g/L Female 120 - 156 g/L	135 ± 16	131 ± 15 ↓	140 ± 16
Serum ferritin, mean ± SD	Male 18 - 350 µg/L Female 18 - 204 µg/L	561 ± 567 ↑	335 ± 256 ↑*	829 ± 718 ↑
Fibrinogen, mean ± SD	2 – 4 g/L	7.6 ± 3.2 ↑	6.7 ± 3.2 ↑	8.7 ± 3.0 ↑
D-Dimer, mean ± SD	<250 µg/L	905 ± 769 ↑	807 ± 695 ↑	1030 ± 872 ↑
LDH, mean ± SD	<270 U/L	423 ± 182 ↑	333 ± 111 ↑	538 ± 194 ↑
ALT, mean ± SD	<48 U/L	68 ± 58 ↑	50 ± 21 ↑	91 ± 80 ↑
AST, mean ± SD	<42 U/L	49 ± 22 ↑	39 ± 16	61 ± 24 ↑
CRP, mean ± SD	<10 mg/L	33.7 ± 71.0 ↑	46.9 ± 86.1 ↑*	9.2 ± 9.5
Radiologic findings				
GGOI	Unilateral n (%)	4 (16)	1 (9)	3 (21)
	Bilateral n (%)	6 (24)	3 (27)	3 (21)
Pleural effusion	n (%)	5 (20)	4 (16)	1 (7)
Pulmonary auscultation				
Rales	Unilateral n (%)	2 (8)	1 (9)	1 (7)
	Bilateral n (%)	8 (32)	5 (45)	3 (21)
Rales/rhonchi	Bilateral n (%)	3 (12)	2 (18)	1 (7)
Wheezing	Unilateral n (%)	1 (4)	1 (9)	0
Disease severity				
Mild disease (4)[a]	n (%)	6 (24)	1 (9)	5 (36)
Severe disease (5)[a]	n (%)	19 (76)	10 (91)	9 (64)

[a]Value according to seven-category ordinal scale; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGOI: Ground-glass opacity infiltration; LDH: Lactate dehydrogenase; NR: Normal Range; CRP: C-reactive protein; ↑ Above the reference range; ↓ Below the reference range; disease severity was done according to the criteria of Chinese Center for Disease Control and Prevention (Shang et al., 2020). No significant difference ($p>0.05$), $\chi 2$ tests were found between data expressed as proportion; *Significant difference ($p>0.05$) between gender within the same series.

Clinical outcomes

The overall mortality rate in patients in our group was zero. Safety results were evaluated at 7 and 14 days. Hematologic and laboratory results were not significantly altered with the application of O_3SS therapy. No decrease in hemoglobin or an increase in LDH, ALT, or AST from baseline was observed. No side effects related to the study drug (O_3SS) were detected. The occurrence of epistaxis was detected in 3 patients between days 3 - 4 of treatment with a heparin suspension reversing symptoms. A gradual reduction in body temperature was observed in the patient with >37.5°C at baseline (Fig. 2A). From day 3 a significant reduction ($p<0.05$) was found. On day 8 all patients returned to lower body temperature values below 37.5°C.

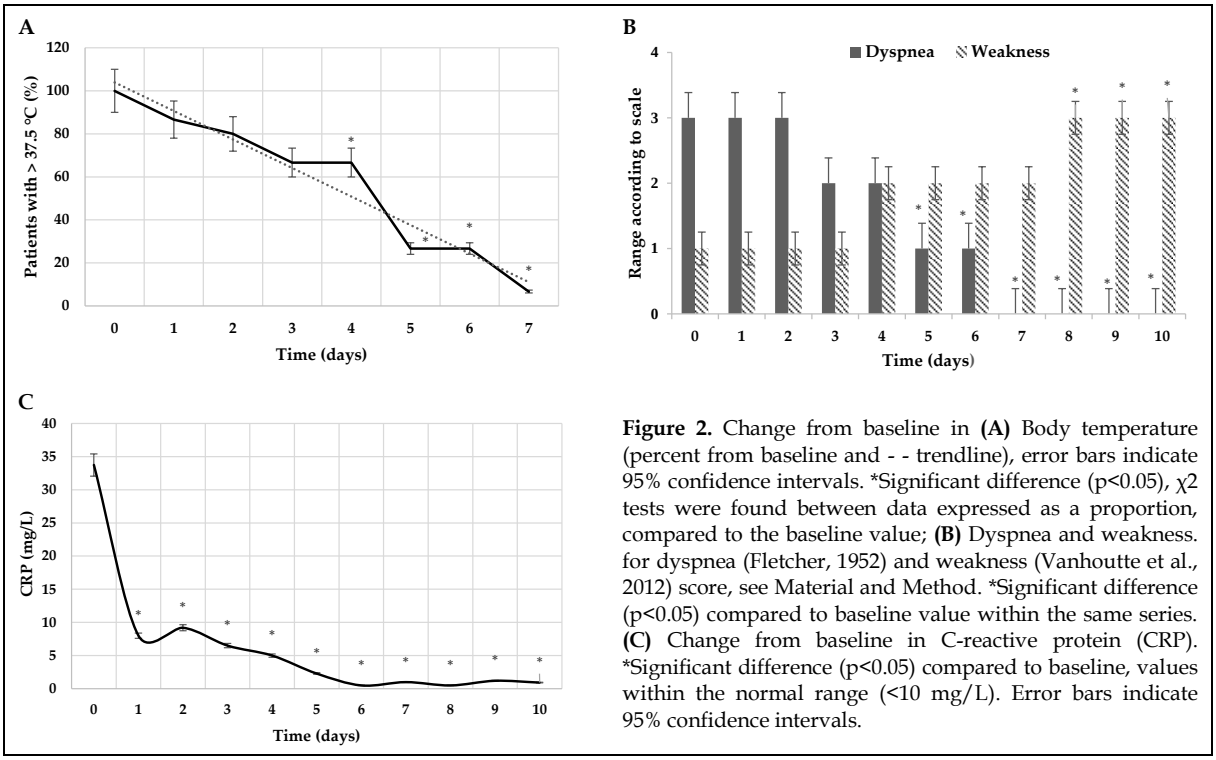

Figure 2. Change from baseline in **(A)** Body temperature (percent from baseline and - - trendline), error bars indicate 95% confidence intervals. *Significant difference ($p<0.05$), $\chi 2$ tests were found between data expressed as a proportion, compared to the baseline value; **(B)** Dyspnea and weakness. for dyspnea (Fletcher, 1952) and weakness (Vanhoutte et al., 2012) score, see Material and Method. *Significant difference ($p<0.05$) compared to baseline value within the same series. **(C)** Change from baseline in C-reactive protein (CRP). *Significant difference ($p<0.05$) compared to baseline, values within the normal range (<10 mg/L). Error bars indicate 95% confidence intervals.

Dyspnea and weakness were gradually reduced (Fig. 2B). From the 7th day, the prevalence of dyspnea was reduced by 40% (mild dyspnea) on day 14, only 1 patient (4%) remained with this symptom. Weakness improved on day 7 when 86% of patients went from severe weakness to mild weakness. By day 14, 91% of patients had gone from severe weakness to mild weakness. CRP values (Fig. 2C) entered the normal range within 24 h of the first application of O_3SS.

Serum ferritin, fibrinogen, D-dimer, and LDH gradually decreased during treatment (Fig. 3A). On day 10, fibrinogen and LDH values entered normal ranges in all patients. ALT and AST were also reduced during this time and on Day 10 remained above normal in 7 of 25 patients (28%) and 9 of 25 patients (36%), respectively. The rate of decline in ALT and AST activity on day 10 was 82 ± 117 U/L and 71 ± 65 U/L, respectively.

The mean duration of viral shedding was 8 days (IQR 6.0 - 11.5). Throughout the application of O_3SS, none of these 25 patients withdrew. The mean duration of hospitalization from inclusion to discharge was 14 days (IQR 9.5-15) (Table 3). Efficacy results based on the seven-category ordinal scale show improvement on day 7 in 19 out of 25 patients (76%). Of these 19 patients, 17 patients (68%) went from 5 to 3 and 2 patients (8%) went from 4 to 2 on the ordinal scale.

At the end of O_3SS treatment (day 14), most patients (18 of 25 [72%]) were in score 2 (discharge), (6 of 25 [24%]) in score 3 (hospitalized, not requiring supplemental oxygen) and (1 in 25 [4%]) were admitted to an intensive care unit (ICU).

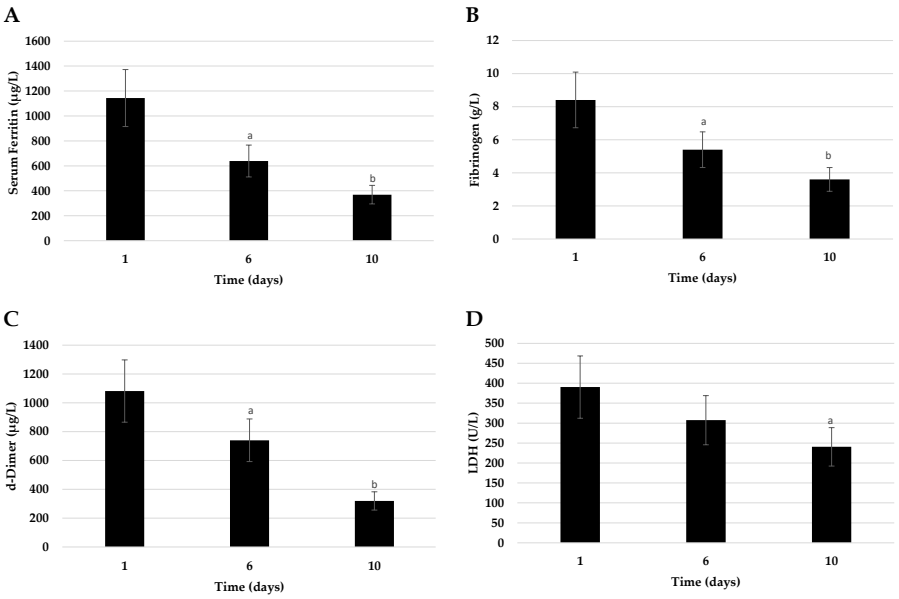

Figure 3. Follow-up of biochemical findings.

(A) Serum ferritin; **(B)** Fibrinogen; **(C)** D-Dimer; **(D)** LDH. Values represented a mean ± S.E.M. The baseline and follow-up values correspond to patients with biochemical index out of normal range that complete the three-point test: for serum ferritin, n = 18; fibrinogen, n = 10; D-dimer, n = 10; LDH, n = 20. [a]Significant differences ($p<0.05$) compared to day 1; [b]Significant difference ($p<0.05$) compared to days 1 and 6.

Table 3. Efficacy outcomes.

Characteristic	**SC + O_3SS (n = 25)**
Hospital stay — median (IQR) No. of days	14 (11-18)
Time from inclusion to average discharge (IQR) No. of days	14 (9.5-15)
Oxygen support — days (IQR)	9 (6 - 14.5)
Score on the seven-category scale at day 7 — No. of patients (%)	
2. Not hospitalized, but unable to resume normal activities	2 (8)
3. Hospitalization, not requiring supplemental oxygen	17 (68)
4. Hospitalization, requiring supplemental oxygen	4 (16)
5. Hospitalization, requiring HFNC or noninvasive mechanical ventilation	1 (4)
6. Hospitalization, requiring ECMO, invasive mechanical ventilation, or both	1 (4)
7. Death	0
Score on the seven-category scale at day 14 — no. of patients (%)	
2. Not hospitalized, but unable to resume normal activities	18 (72)
3. Hospitalization, not requiring supplemental oxygen	6 (24)
4. Hospitalization, requiring supplemental oxygen	0
5. Hospitalization, requiring HFNC or noninvasive mechanical ventilation	0
6. Hospitalization, requiring ECMO, invasive mechanical ventilation, or both	1 (4)
7. Death	0

ECMO, extracorporeal membrane oxygenation; HFNC, a high-flow nasal cannula for oxygen therapy; IQR, interquartile range; SC + O_3SS, standard care (SC) plus ozonized saline solution (O_3SS).

DISCUSSION

The COVID-19 pandemic represents a global public health crisis. Given the severity with which this disease has developed, empirical treatment recommendations for COVID-19 are made based on unsupported studies. Due to the mortality and morbidity associated with the disease, untested drugs with a questionable safety profile at higher doses are prescribed on a compassionate basis (Borba et al., 2020). To deal with this pandemic, the reuse of existing therapeutic agents is proving to be the only pragmatic approach as an urgent response, most of these drugs having already been tested for their safety (Tu et al., 2020). These agents can be classified into two categories: 1) agents that directly target the virus replication cycle, and 2) agents based on immunotherapy approaches. "The development of a vaccine represents a longer-term strategy to prevent COVID-19 outbreaks in the future" (Tu et al., 2020). O_{3X} has been used to treat various pathologies including viral diseases (ISCO3, 2020). Various well-known mechanisms presuppose the utility of O_{3X} in COVID-19 infection (Martínez-Sánchez et al., 2020). In this indication, O_{3X} can be classified as an immunomodulator.

Developing a vaccine represents a longer-term strategy to prevent future COVID-19 outbreaks (Tu et al., 2020). O_{3X} has been used to treat various pathologies including viral diseases (ISCO3, 2020). Various well-known mechanisms presuppose the utility of O_{3X} in COVID-19 infection (Martínez-Sánchez et al., 2020). In this indication, O_{3X} can be classified as an immunomodulator (Chang et al., 2005), either stimulating innate antiviral immune responses (Paulesu et al., 1991) or attenuating the damage induced by deregulated inflammatory responses (Caliskan et al., 2011).

The population distribution by age in this complementary application of the O_3SS (30-50 years [28%], 50-70 years [52%] and >70 years [20%]) was following the international epidemiological data reported for this infection (Epidemiology Working Group for Ncip Epidemic Response and Prevention, 2020) and the infected Spanish population (Borobia et al., 2020; Casa Rojo et al., 2020). This finding highlights that people of any age can get an infection with COVID-19. However, middle-aged and older adults are most commonly affected. The mean age in Spain was 18 to 102 years (Casa Rojo et al., 2020). Similar to the mean age of our 55-year-old patients (30 to 95 years). In other studies of patients hospitalized with confirmed COVID-19, the mean ranged from 49 to 56 years (Chen et al., 2020a; Huang et al., 2020). Co-morbidities have been associated with serious illness and mortality, but results from the O_3SS application indicate that only (9 of 25 [36%]) patients did not experience co-morbidities. The most frequent comorbidities were hypertension, asthma, hypothyroidism, smoking, and obesity, following the data available from infected patients in Spain (Borobia et al., 2020; Casa Rojo et al., 2020). Except for hypothyroidism, all other conditions are considered to be risk factors for infection with SARS-CoV-2 (Zou et al., 2020). All of the patients were white, so an analysis of race differences was not performed. In general, more men were affected by the disease as reported in cohort studies in China, Italy, and the United States (Chen et al., 2020b; Onder et al., 2020; Richardson et al., 2020). However, we recruited more women (14) than men (11), but an analysis of the incidence ratio by sex, in this case, is not valid, due to the small number of subjects. Also, in a Madrid study of 2226 cases, it was found that the proportion of affected female patients was relatively high (51.8%) (Borobia et al., 2020).

Fever (defined as an axillary temperature above 37.5°C) is not a universal finding on the presentation of COVID-19. In our sample (13 out of 25 [52%]) had a fever at baseline (Table 1). In a study of more than 5,000 patients hospitalized for COVID-19 in New York City, only 31% had a temperature >38°C at presentation (Richardson et al., 2020). In another study involving 1,099 patients from Wuhan and other parts of China, fever was present in only 44% of cases on admission but was ultimately noted in 89% during hospitalization (Guan et al., 2020). In an epidemiological study carried out in 18 European hospitals with 1420

patients, fever appeared in 45.5% of subjects (Lechien et al., 2020). However, the febrility of our patients treated with O_3SS (Fig. 2A) gradually decreased, which is consistent with their good recovery.

All clinical manifestations found in patients (Table 1) were similar to clinical features of disease onset (Wang et al., 2020a). The main clinical manifestations of the disease (dyspnea and weakness) had a favourable course of resolution in patients treated with O_3SS. On days 7 and 8 respectively (Fig. 2B), these symptoms were found to be significantly improved ($p<0.05$). Acute respiratory distress syndrome (ARDS) is the main complication of patients with severe illness. ARDS was shown in 20%, on average 8 days after symptom onset in a study involving 138 COVID-19 patients; in this study, mechanical ventilation was implemented in 12.3% (Wang et al., 2020a). Also, some patients who initially have no severe symptoms may progress over a while (within a week). In another study, the median time to dyspnea was 8 days (Huang et al., 2020). However, without exception, our 25 patients after treatment with O_3SS had resolution of dyspnea. Elevation of inflammatory markers (e.g., ferritin, D-dimer, CPR) has been observed in our COVID-19 patients, consistent with the results of other recent reports (Terpos et al., 2020) and the Madrid epidemiological report during the same period (Casa Rojo et al., 2020). Elevated ferritin has also proven to be a poor prognostic factor.

In a study involving 6424 subjects in Madrid, increased serum ferritin levels were associated with the development of ARDS (Wu and McGoogan, 2020). Also, 72.4% of patients had increased ferritin levels. In a descriptive study, among 99 COVID-19 cases in Wuhan, China, a higher D-dimer was detected in 36% of patients (Chen et al., 2020a), but the reported value in Madrid during the same period was lower (61.5%) (Casa Rojo et al., 2020). Higher D-dimer levels are significantly associated with an increased risk of ARDS (Wu et al., 2020a). The increase in disease severity and the development of ARDS are related to the increase in CRP (Terpos et al., 2020). According to different studies from China and Singapore, the average CPR of patients who do not need supplemental oxygen is 11.1 (IQR: 0.9 - 19.1 mg/L). The number of patients requiring oxygen was 65.6 (IQR: 47.5 - 97.5 mg/L) (Young et al., 2020), while the mortality group was 109.25 (IQR 35.00 - 170.28 mg/L) (Deng et al., 2020). In our patients, based on the improvement of their clinical conditions, the average baseline value of CPR after the first 24 - 48 h was 12.5 (IQR: 2.5-19.3 mg/L) (Fig. 2C).

Fibrinogen is also higher in our sample, confirming that hypercoagulation in SARS-CoV-2 patients is an important complication. In clinical studies, higher levels of D-dimer and fibrinogen were found in COVID-19 patients compared with healthy controls ($p<0.001$) (Han et al., 2020). Also, high LDH levels are significantly associated with severe COVID-19 at the time of admission (Li et al., 2020). In our findings, the administration of O_3SS reduced the levels of inflammatory markers (such as ferritin, D-dimer, CPR, LDH) and fibrinogen as a marker of coagulation function (Fig. 3).

Radiographic findings and auscultation found that 60% and 56% of patients had signs of pneumonia (Table 2), which were similar to the results reported by COVID-19 patients in Madrid during the same period, and 52.4% of patients had rales (Casa Rojo et al., 2020). Chest radiographs of early or mild disease may be normal. In a retrospective study of 64 patients with COVID-19 records in Hong Kong, 20% of the patients showed no abnormalities on chest radiographs at any time during the illness (Wong et al., 2019). The main abnormalities of imaging examination are consolidation and ground-glass opacity, bilateral, peripheral, and lower lung zone distribution (Vancheri et al., 2020). According to imaging results or auscultation, in 25 patients treated with O_3SS, bilateral pneumonia only accounted for 24-32% (Table 2). Generally, the degree of lung involvement increases with the course of the disease, and the severity peaks 10 to 12 days after the onset of symptoms (Pan et al., 2020). However, in our patients, after the third to fifth O_3SS treatment sessions, chest X-rays and auscultation changed dramatically after the third to fifth sessions of O_3SS, both showing improvement in their status.

Compared with other reports of patients who received only SC treatment in Madrid during the same period (only 10 days, range 1- 62 days), there was no significant difference in the admission time to discharge between patients treated with O_3SS and SC (>0.05) (Casa Rojo et al., 2020). However, including O_3SS as an adjuvant therapy can speed up the improvement of patients in clinical symptoms (Table 3) and laboratory biomarkers (Fig. 2). This improvement prevents the patient from transitioning to a critical state. Also, compared with other reports of 20.0 days (IQR 17.0-24.0) and 37 days, the time to reach the median virus shedding duration [8 days (IQR 6.0-11.5)] and the longest virus shedding duration (22 days) Reduced, respectively (Zhou et al., 2020). No deaths were recorded on the 7th or 14th day of the study period. However, the mortality rate of COVID-19 patients hospitalized in Madrid during the same period was 20.7 - 21.1% (Borobia et al., 2020; Casa Rojo et al., 2020).

The most likely mechanism related to low-dose ozone is the use of the physiological saline solution as a carrier and as a complementary therapy in COVID-19 patients, which is thought to regulate the "cytokine storm" through the balanced regulation of the Nrf2/NF-κB pathway (Martínez-Sánchez et al., 2020). The potential benefits of ozone in these clinical conditions deserve further study. Clinical studies with this rationale have been proposed (Marini et al., 2020).

CONCLUSIONS

The results of this pilot study show that by using O_3SS treatment as a complementary therapy to standard treatment, patients with mild to severe symptoms due to COVID-19 disease can be safe. No side effects were observed during O_3SS treatment. The use of O_{3X} as an adjuvant treatment of SARS-CoV-2 patients' infection has molecular and preclinical scientific evidence and clinical evidence in terms of cryoprotection and control of inflammation. Based on the results of this clinical trial, it is reasonable to use this therapy to conduct further clinical studies on other viral diseases with similar clinical and pathophysiological characteristics. Without a sufficiently powerful randomized controlled trial, it is not recommended to apply O_3SS to COVID-19 or other viral infections.

SUPPLEMENTARY DATA

The article contains clinical data to support the results of the study. Individual patient data can be contacted with the corresponding author via email. Preprinted articles can be found at https://www.preprints.org/manuscript/202006.0233/v3.

CONFLICT OF INTEREST

The funders have no role in the design of the protocol applicable to the patient; in the collection, analysis, or interpretation of the data; in the writing of the manuscript, or in the decision to publish the results. Gregorio Martínez-Sánchez is Chairman of ISCO3 (International Scientific Committee of Ozone Therapy), Adriana Schwartz is Secretary of ISCO3 and Chairman of AEPROMO (Spanish Association of Medical Professionals in Ozone Therapy). This research was partially funded by AEPROMO.

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AUTHOR CONTRIBUTION:

Contribution	Schwartz A	Martínez-Sánchez G	de Lucía AM	Viana SM	Constanta AM
Concepts or ideas	x	x	x	x	x
Design	x	x			
Definition of intellectual content	x	x	x	x	x
Literature search	x	x	x	x	x
Clinical studies	x		x	x	x
Data acquisition	x	x	x	x	x
Data analysis	x	x	x	x	x
Statistical analysis	x	x			
Manuscript preparation	x	x			
Manuscript editing	x	x	x	x	x
Manuscript review	x	x	x	x	x

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