Article

Complementary Application of the Ozonized Saline Solution in Moderate and Severe Patients with Pneumonia Covid-19: Efficacy and Tolerability.

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Abstract: Currently, there is no effective antiviral therapy recommended for the new coronavirus disease 2019 pneumonia (COVID-19). The purpose of this study was to evaluate the safety and efficacy of Ozonized Saline Solution (O_3SS) used as a complementary therapy in adult patients with mild to severe COVID-19. Twenty-five adult patients who were hospitalized with mild to severe COVID-19 symptoms, who met the inclusion criteria and were being treated from April 3rd to April 26th, 2020, at the Viamed Virgen De La Paloma Hospital, Madrid, Spain were included in this study. Patients were allocated to receive standard care (SC) that included 200-400 mg hydroxychloroquine twice daily for 5-7 days plus Tocilizumab 400 mg twice daily for 5 days, low molecular weight heparin (LMWH) and 40 mg-60 mg metil-prednisone plus O_3SS, 200 mL, 3-5 μg/mL daily for 10 days. Primary outcomes of treatment with O_3SS were an improvement of clinical symptoms and a reduction in mortality. Secondary end points evaluated included participant clinical status, laboratory examinations, and duration of viral shedding. None of the patients treated with SC + O_3SS died. Improvements in symptoms such as dyspnea, weakness, and reduction in body temperature were observed and corresponded with an improvement of laboratory finding including D-dimer, fibrinogen, LDH, and CRP. No side effects from the O_3SS treatment were observed. Conclusions: COVID-19 patients with mild to severe symptoms who received intravenous O_3SS as a complementary therapy demonstrated improved clinical symptoms, improved laboratory values and a reduction in mortality.

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

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1. Introduction

A recently identified coronavirus named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) was isolated from lower respiratory tract samples as the causative agent of severe pneumonia. The current outbreak of infections with SARS-CoV-2 has been termed Coronavirus Disease 2019 (COVID-19) by the World Health Organization (WHO). COVID-19 rapidly spread into at least 213 countries and killed more than 420,000 people by June 13, 2020. WHO officially declared COVID-19 a pandemic on March 11, 2020 [1].There are not specific therapies available to treat the Covid-19 infection.

Hydroxychloroquine (HCQ) and chloroquine (CQ) have garnered unprecedented attention as potential therapeutic agents against COVID-19. Apart from their antimalarial use, they have also shown an in vitro activity against COVID -19 [2]. However, there is a growing body of scientific data of their side effects particularly in QTc prolongation and cardiac arrhythmias [3].

There are multiple physiological pathway dysregulations that appear to be disrupted by the SARS-CoV-2 and related viruses. Angiotensin-converting enzyme (ACE2) has recently been identified as the SARS-CoV-2 receptor. The ACE2 system is a critical protective pathway from inflammatory injuries due to excess oxidative stress. An unregulated ACE2 dysfunction worsens COVID-19 and could initiate multi-organ failure. The imbalance in the action of ACE1 and ACE2-derived peptides [Angiotensin II (Ang II) and Angiotensin 1-7 (Ang 1-7), respectively] may explain most of the pathological consequences of SARS-CoV-2 infections [4].

Health risk factors that predispose COVID-19 patients for a progression of the disease to more advanced stage include proinflammatory conditions such as hypertension, diabetes and cardiovascular disease [5]. Earlier studies of the related SARS-CoV virus infections of primates suggest that the severe lung injury was due to an exacerbated inflammatory response mediated through an activation of the innate immune system and an upregulation of the NF-κB pathway [6]. A recent human study demonstrated that in critically ill patients, the SARS-CoV-2 virus cause an exacerbated inflammatory response which is not self-limiting, it is uncontrolled generating an inflammatory cytosine storm. This is happening because, in theory the lymphocytes NK and T cytotoxic (CD8 and CD4+) should inhibit the macrophage activity, but by down regulation this inhibition does not occur because the infected cells lack Major Histocompatibility Complex, who should inhibit the action of the macrophage. This is caused a suppression of functional lymphocytes (lymphopenia) resulting in decreased immune function and increased susceptibility to infection [7]. Additional animal studies have demonstrated that the pulmonary fibrosis that is a hallmark of the SARS disease process is mediated through an induction of TGF-beta1 by way of an upregulation of the ROS/ p38 MAPK/ STAT3/Egr-1 pathway both in vitro and in vivo [8]. Host genetics may also play a role in pathogenesis since studies on knock-out mice have demonstrated that a genetic deficiency of ACE2 receptors resulted in a reduction of Ang 1,7 thus increasing oxidative stress and susceptibility to advanced disease progression [9].

Medical ozone (O_2/O_3) at a low dose which is produced by a mixture of oxygen (carrier) and ozone (active component) in a carrier of 99.9% pure oxygen. Medical ozone therapies (O_{3X}) have been demonstrated to be effective in treating a range of human pathologies that have a physiological basis of inflammatory dysregulation (oxidative stress). Properly dosed and timed treatments have the ability to induce endogenous oxidative preconditioning [10]. Potentially, O_{3X} may improve the symptoms of COVID-19 acting as an inductor of adaptation to OS, a modulator of pro-inflammatory cytokines and improving tissues oxygenation [11]. These findings allowed us to evaluate the safety and efficacy of ozonized saline solution (O_3SS) in patients with mild to severe COVID-19 as a complementary 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 accelerates the recovery of the

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patients, stabilizes their biochemical index, reduces the need for oxygen support and shows no side effects.

2. Experimental Section

The complementary application of O_3SS was done in accordance with the principles of the Declaration of Helsinki [12] and the Good Clinical Practice Guidelines of the International Conference on Harmonization [13]. All patients and/or legal representatives were informed about the objectives and risks of participation. They were given 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), composed of O_{3x} experts, clinicians, and experts in infectious diseases from AEPROMO (Spanish Association of Medical Professionals in Ozone Therapy) and ISCO3 (International Scientific Committee of Ozone Therapy), was selected to review the protocol and hold daily meetings to follow the daily results of the application of O_3SS. The trial was reported according to the Consolidated Standards of Reporting Trials (CONSORT) reporting guideline [14]. The protocol was approved by the Regional Etic Committee of Madrid (Number 05/20). This manuscript is a partial report of the study.

2.1. Design and Site

2.1.1. Site

The complementary application of O_3SS was done following the criteria of a pilot, open label, phase III clinical trial, between April 3th to April 26th 2020, aiming to first treat hospitalized patients with mild to severe respiratory syndrome secondary to SARS-CoV-2 infection COVID-19; and as a second aim, to assess the safety and efficacy of O_3SS. These patients were hospitalized at the Viamed Virgen de la Paloma Hospital, Madrid (declared COVID-19 center during the epidemic). The hospital has all source documents registered in an electronic medical recording system. Clinical analyses, laboratory examinations, and routine Chest radiographs are also available locally.

2.1.2. Participants

Hospitalized patients with clinical suspicion of COVID-19 (i.e., history of fever and any respiratory symptom, e.g., cough or rhinorrhea); male or female aged 18-98 years old at the time of inclusion; within 1 week of onset; who did not participate in other clinical studies within the last three months; willing and able to sign the informed consent for participation in the application of O_3SS; Chest radiographs confirmed pulmonary lesions (for moderate cases); were included. Patients were enrolled before laboratory confirmation of COVID-19 by reverse transcription–polymerase chain reaction (RT-PCR Covid-19), considering that this procedure could delay inclusion. The flow chart (Fig. 1) presents clinical-epidemiologic suspected cases as well as cases already confirmed by RT-PCR Covid-19.

The exclusion criteria included: Female participants who were pregnant, lactating or planning pregnancy during the course of the trial. Patients with significant renal or hepatic impairment or with scheduled elective surgery or other procedures requiring general anesthesia during the application of O_3SS. Participants who had participated in any clinical trial involving an investigational product within the past 12 weeks prior to the study. Patients with G-6PD defect

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(Favism). Patients who continually used immunosuppressant, or were organ transplant recipients within the last 6 months. Patients with history of not controlled hyperthyroidism, unstable period of severe cardiovascular disease, copper or iron supplementation via IV or any situation that did not allow the patients 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.

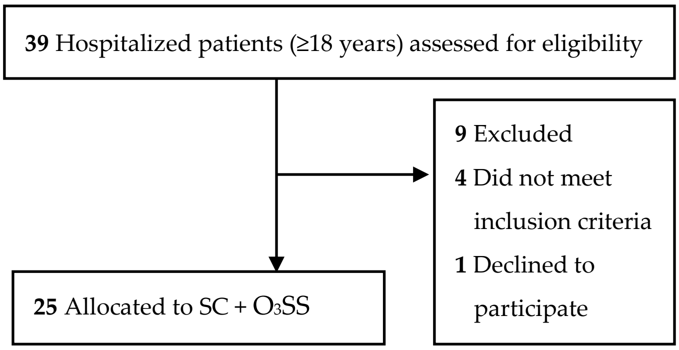

Figure 1. Study flow chart. Eligible participants were allocated to receive standard care (SC), basically: (40 mg-60 mg methyl prednisone daily for 5-7 days plus Tocilizumab 400 mg twice daily for 5 days, low molecular weight heparin (LMWH) and hydrocortisone) plus O_3SS, 200 mL, 3-5 μg/mL daily for 10 days) plus ozonized saline solution (O_3SS), 3-5 μg/mL daily for 10 days.

Participants were allocated at the time of inclusion and were subsequently identified throughout the application of O_3SS only by their allocated number, always assigned in chronological order. This was an open label application of O_3SS.

2.3. Sample Size Calculation

The sample size calculation for this complementary application of O_3SS was estimated according to the suggestion of Whitehead A.L. et al [15]. The sample for the primary outcome (i.e., reduction in lethality rate) was calculated assuming a 20% lethality incidence in critically ill patients [16,17], and that O_{3x} (SC + O_3SS) would reduce lethality by at least 50% compared with the SC group. Thus, considering 90% power and 5% α, 25 participants were needed. Sample calculation was performed in the R version 3.6.1 (R Project for Statistical Computing), with the functions implemented in the *TrialSize* and *gsDesign* packages.

2.4. Procedures

According to hospital protocol, all patients meeting the same criteria of the study (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), Enoxaparina (Clexane®) 40 – 60 mg daily, HCQ 200 mg, methyl prednisone 40 mg or prednisone 5 mg systematically, starting on day 0. Tocilizumab (Actemra®), 0,4 mg twice daily for 5 days, was also prescribed when influenza infection was suspected.

O_3SS consists of bubbling and saturating a physiological solution (0.9%) with O_2/O_3 mixture during 10 min, at concentrations ranging 3-5 μg/NmL. Its administration takes about 20-30 min. Patients in O_{3x} group, received SC plus O_3SS. The first 5 days the concentration used was 5 μg/NmL, administered daily. In the following 5 sessions, the concentration was lowered to 3 μg/NmL and administered daily. Patients received 10 sessions of O_3SS in total.

Ozone was generated by a medical class IIb CE device (Ozonobaric P®, SEDECAL®, Spain). The container that administered the solution was disposable, made of medical-grade materials, free of phthalates and fully compatible with ozone. It had a classification as medical devise class IIb obtained from Bexozone® (Bexen medical®, Spain). Physiological Saline Solution (NaCl 0.9 %) from (Lab. ERN, Spain) was used.

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

Dyspnea was scaled as: Grade 0, no dyspnea; grade 1, slight dyspnea; grade 2, moderate dyspnea; grade 3, severe dyspnea; grade 4 very severe dyspnea [18]. Weakness was scaled as: 0, paralysis; 1, severe weakness; 2, slight weakness; 3, normal strength [19].

2.5. Outcomes

Safety outcomes included adverse events that occurred 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. The null hypothesis was that the complementary application of O_3SS in the experimental group would have a mortality rate that was 50% lower than the mortality reported for only SC by day 14. Thus, the primary end point was mortality by day 14. Secondary end points included participant clinical status, laboratory examinations, chest radiographs on days 7 and 14, daily clinical status during hospitalization, duration of mechanical ventilation (if applicable) and supplementary oxygen (if applicable), and the time (in days) from treatment initiation to death. Here we present analyses until day 14, with lethality as the primary outcome. Virologic measures included viral RNA detection was performed daily until 2 consecutive negative values was obtained.

To evaluate the efficacy outcome the seven-category ordinal scale was used, and consisted of the following categories: 1, not hospitalized with 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 nasal high-flow oxygen therapy, noninvasive mechanical ventilation, or both; 6, hospitalized, requiring extracorporeal membrane oxygenation, invasive mechanical ventilation, or both; and 7, death [20].

2.6. Laboratory Analysis

Hematology and biochemistry analyses were performed in automatized machines. Samples (2 nasopharyngeal or 1 oropharyngeal swabs) were submitted to Novel Coronavirus (2019-nCoV) Real Time RT-PCR test, using a kit from Biopath-Unilabs (France) by Cobas z480 qPCR (Roche), with the use of LightMix Modular SARS-CoV-2 (COVID19). Sampling did not stop when a swab at a given time point was negative. Baseline throat swabs were tested for detection of E gene, RdRp gene, and N gene, and samples on the subsequent visits were qualitatively detected for E gene.

2.7. Statistical Analysis

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Descriptive statistics were used for demographic, laboratory, and clinical data. To assess the safety of the SC + O_3SS compared to SC, the proportion (and 95% CI) of deaths in SC + O_3SS group was compared with the historical proportion (and 95%CI) of deaths in patients who did not use O_3SS in other countries.[16,17,21,22] For qualitative variables, $\chi 2$ tests and Fisher exact tests were performed. We used the *t* test or Mann-Whitney test to compare means and medians. The Wilcoxon signed-rank test and Hodges–Lehmann estimate was used to compare inter quantile ranges (IQR). Statistical analyses were performed in IBM SPSS statistic version 17, and a 2-tailed $P < 0.05$ was considered significant.

3. Results

5.1. Demografic and clinical characteristics

A total of 25 patients that were allocated to the SC + O3SS group completed the study. (Fig. 1). Some patients (4 of 25 [16%]) had COVID-19 confirmed a posteriori by reverse transcription–polymerase chain reaction testing. The patients with initial unconfirmed disease who had clinical and epidemiological presentation compatible with COVID-19 were analyzed together. Overall baseline characteristics are presented in Tab. 1.

Baseline characteristics show an overall median (min -max) age of 44 (30 -95) years and a predominance of women (14 [65%]). The most frequent comorbidities were hypertension (4 of 25 [16%]), asthma (3 of 25 [12%]), hypothyroidism (3 of 25 [12%]), smoking (2 of 25 [8%]), and obesity (2 of 25 [8%]). Hypertension was more frequent in male vs female (4 of 11 [36%] vs 0). On admission, oxygen support was required in 14 of the 25 patients (56%), which was more frequent requirement in males vs females (10 of 11 [90%] vs 4 of 14 [28%]). Baseline body temperature was greater than 37.5 °C in 13 of 25 patients (52%); with a greater frequency in males vs females (8 of 11 [72%] vs 5 of 14 [35%] respectively). Main presenting clinical 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 present more frequently in females (both 43%) than in males (27% and 18 % respectively).

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

The most common radiographic finding on a chest radiograph was ground-glass opacity infiltration (Tab. 2), in 40 % of patients and pleural effusion in 20 %. Pulmonary auscultation found rales, rales/rhonchi and wheezing sound in 56 % of the patients. A majority of patients fit the severe disease status (76%) and 6 (24%) meet the criterium of mild disease.

5.2. Clinical outcomes

Overall mortality rate in our group patients was zero. Safety outcomes were evaluated at 7 and 14 days. Haematological and laboratory findings did not undergo notable modification with the application of O3SS therapy. Non decrease in haemoglobin levels, or increased in LDH, ALT or AST compared to base line were found. No side effects associated with the investigational drug (O3SS) was detected. Occurrence of epistaxis was detected in 3 patients between days 3-4 of treatment with a suspension of heparin reversing the symptoms.

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

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 (%)[2]	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[3]			
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)

Legend: 1. In all cases the race was white; 2. No former smoker was found; 3. Symptoms with frequency lower that 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 ($p<0.05$), $\chi2$ tests for proportion between gender.

Progressive reduction of body temperature was observed in patient with >37.5 °C at baseline (Fig. 2a). From the day 3 a significant ($p<0.05$) reduction was found, at day 8, all patients return to values of body temperature lover than 37.5 °C.

Dyspnea and weakness was gradually reduced (Fig. 2b). From day 7 dyspnea prevalence was reduced by 40 % (slight dyspnea) on day 14, only 1 patient (4 %) remained with this symptom. Weakness was improved on day 7 when 86 % of the patients passed from severe weakness to slight weakness. On day 14, 91 % of patients shifted from severe weakness to slight weakness. The CRP values (Fig. 2c) entered into the normal range within 24 h of the first application of the O_3SS.

Serum ferritin, fibrinogen, D-dimer and LDH were progressively decreasing during the treatment (Fig. 3a). By day 10, fibrinogen and LDH values entered the normal ranges in all patients.

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ALT and AST were also decreased during this time and by the day 10 remained above the normal range in 7 of 25 patients (28%) and 9 of 25 patients (36%), respectively. The rate of decline in activity of ALT and AST by day 10 were 82±117 U/L and 71±65 U/L, respectively.

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) x 10^9 cells /L	7.00±3.68	5.97±1.86	8.30±4.97
Lymphocytes count, mean ±SD	(1.0 – 4.8) x 10^9 cells /L)	1.49±1.64	1.35±0.52	1.67±2.46
Platelets count, mean ±SD	(150-450) x 10^9 cells /L	287±100	270±99	309±101
Eosinophils, mean ±SD	(0-0.4) x 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)[2]	n (%)	6 (24)	1 (9)	5 (36)
Severe disease (5)[2]	n (%)	19 (76)	10 (91)	9 (64)

Legend: 1. Viral RNA SARS-CoV-2 load in throat swabs sample; 2. 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 the criteria of Chinese Center for Disease Control and Prevention [23]. No significant difference (p>0.05), $\chi 2$ tests was found between data expressed as proportion; *, significant difference (p>0.05) between gender within the same series.

Average duration of viral shedding was 8 days (IQR 6.0-11.5). None of the 25 patients withdrew throughout the application of O_3SS. The average duration of hospitalization from inclusion to discharge was 14 days (IQR 9.5-15) (Tab. 3). Efficacy outcome based on the seven-category ordinal scale shows at day 7 an improvement in 19 out of 25 patients (76%). Out of these 19 patients, 17 patients (68%) shifted from 5 to 3 and 2 patients (8%) shifted from 4 to 2 on the ordinal scale.

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

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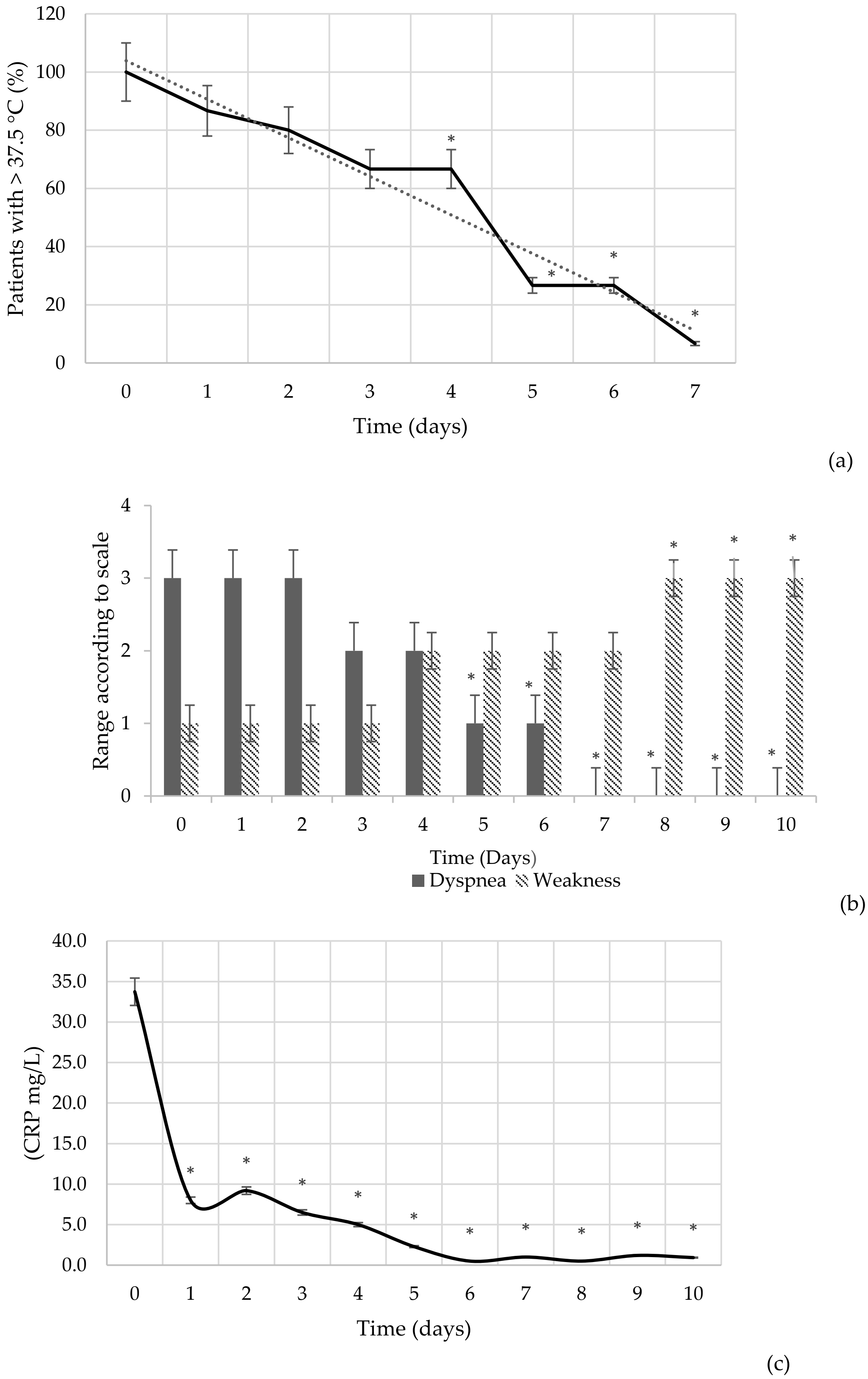

Figure 2. Change from baseline in: (**a**) body temperature (percent from baseline and - - trendline), error bars indicate 95% confidence intervals. * significant difference ($p<0.05$), $\chi 2$ tests was found between data expressed as proportion, compared to baseline value; (**b**) Dyspnea and weakness. For Dyspnea [18] and Weakness [19] 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.

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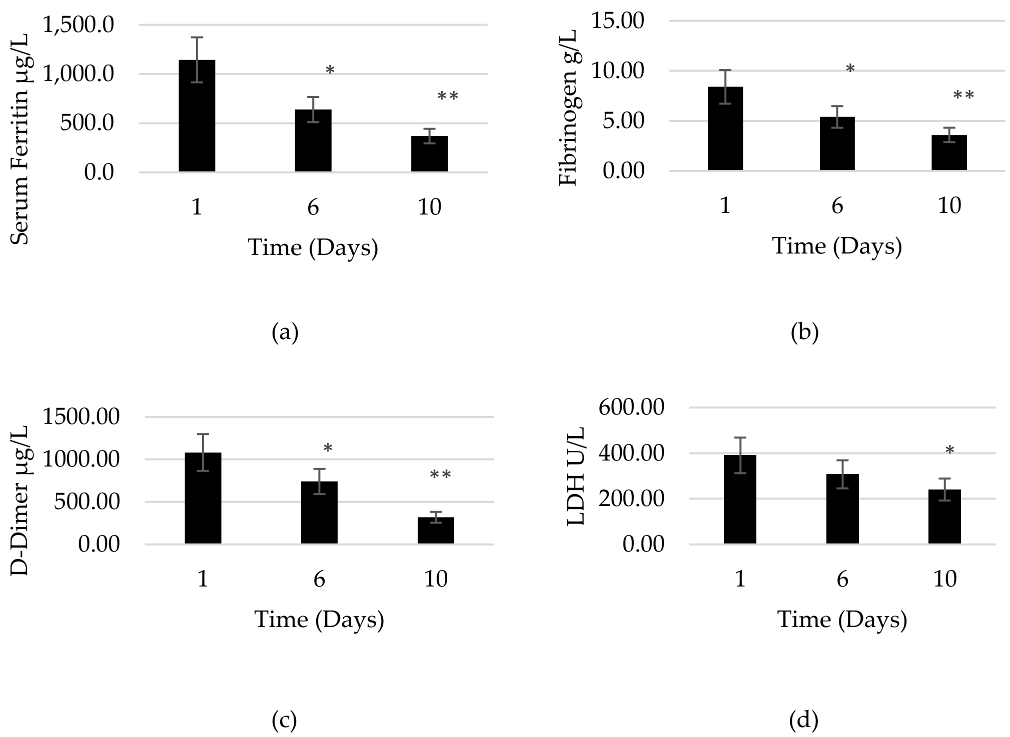

Figure 3. Follow-up of biochemical findings. (**a**) Serum ferritin; (**b**) Fibrinogen; (**c**) D-Dimer; (**c**) LDH. Values represented a mean ± S.E.M. The baseline and follow-up values correspond to patients with biochemical index out of normal range that complete the three-point test: for serum ferritin, n=18; fibrinogen, n=10; D-dimer, n=10; LDH, n=20. *, significant differences ($p < 0.05$) compared to day 1; **, significant difference ($p< 0.05$) compared to day 1 and 6.

4. Discussion

The COVID-19 pandemic represents the greatest global public health crisis since the pandemic influenza outbreak of 1918. Given the severity with which this disease has unfolded, empirical treatment recommendations for COVID-19 are being made based on unpowered studies. Because of the mortality and morbidity associated with the disease untested drugs with a questionable safety profile at higher doses are being prescribed on a compassionate basis [26]. On the other hand, the repurposing of the existing therapeutic agents previously designed for other viral infections and pathologies happens to be the only practical approach as a rapid response measure to the emergent pandemic, as most of these agents have already been tested for their safety. These agents can be divided into two broad categories: 1) those that can directly target the virus replication cycle, and 2) those based on immunotherapy approaches either aimed to boost innate antiviral immune responses or alleviate damage induced by dysregulated inflammatory responses. The development of vaccine represents a more long-term strategy to prevent COVID-19 outbreaks in the future [27]. O_{3x} has been used to treat different pathologies including viral diseases [28]. There are well known different mechanisms that presuppose the utility of O_{3x} in COVID-19 infection [11]. In this indication, O_{3x} can be classified as immunomodulator, either boosting innate antiviral immune responses or alleviating damage induced by dysregulated inflammatory responses.

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Table 3. Efficacy outcomes.

Characteristic	SC + O_3SS n=25	Standard Care[1] n=100
Hospital stay — median (IQR) no. of days	14 (11-18)	16 (13-18)**
Time from inclusion to average discharge (IQR) no. of days	14 (9.5-15)	14 (11-16)**
Oxygen support — days (IQR)	9 (6 - 14.5)	13 (6 - 16)**
Score on seven-category scale at day 7 — no. of patients (%)		
2. Not hospitalized, but unable to resume normal activities	2 (8)	0
3. Hospitalization, not requiring supplemental oxygen	17 (68)*	17 (17)
4. Hospitalization, requiring supplemental oxygen	4 (16)*	51 (51)
5. Hospitalization, requiring HFNC or noninvasive mechanical ventilation	1 (4)*	21 (21)
6. Hospitalization, requiring ECMO, invasive mechanical ventilation, or both	1 (4)	4 (4)
7. Death	0*	7 (7)
Score on seven-category scale at day 14 — no. of patients (%)		
2. Not hospitalized, but unable to resume normal activities	18 (72)*	28 (28)
3. Hospitalization, not requiring supplemental oxygen	6 (24)	24 (24)
4. Hospitalization, requiring supplemental oxygen	0*	20 (20)
5. Hospitalization, requiring HFNC or noninvasive mechanical ventilation	0*	6 (6)
6. Hospitalization, requiring ECMO, invasive mechanical ventilation, or both	1 (4)	5 (5)
7. Death	0*	17 (17)

Legend: 1. According to Cao *et al*. (2020) [24] n=100. ECMO, extracorporeal membrane oxygenation; HFNC, high-flow nasal cannula for oxygen therapy; IQR, interquartile range; SC + O_3SS, standard care (SC) plus ozonized saline solution (O_3SS); *, significant difference ($p<0.05$), $\chi 2$ tests was found between data standard care plus O_3SS treated patient and SC. **. No significant difference ($p>0.05$) was found between IQR data SC plus O_3SS treated patient and SC (Hodges–Lehmann estimate) and 95% confidence intervals.

The population distribution by age in this complementary application of O_3SS (30-50 years [28%], 50-70 years [52%] and >70 years [20%]) was in line with the international epidemiologic data reported for this infection [29]. This finding emphasises that individuals of any age can acquire COVID-19 infection, although adults of middle age and older are most commonly affected. In several cohorts of hospitalized patients with confirmed COVID-19, the average age ranged from 49 to 56 years [30,31] similar with the average age of our patients 55 (30-95). Comorbidities have been associated with severe illnesses and mortality, however the results of application of O_3SS indicates that only (9 of 25 [36%]) patients did not show comorbidities. The most frequently comorbidities were hypertension, asthma, hypothyroidism, smoking and obesity. Except hypothyroidism, all other conditions are considered risk factors for SARS-CoV-2 infection [32]. All patients were white, therefore an analysis of differences between races was not performed. In general, more males were

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affected by the disease and males have comprised a disproportionately high number of deaths in cohorts from China, Italy, and the United States [17,33,34]. However, we enrolled more women (14), than men (11), but an analysis of the ratio of gender incidence in this case is not valid, because the small number of subjects.

Fever (defined as an axillary temperature over 37.5°C) is not a universal finding on presentation of COVID-19. In our sample (13 of 25 [52%]) had fever at baseline (Tab. 1). In a study of over 5000 patients who were hospitalized with COVID-19 in New York, only 31% had a temperature >38°C at presentation [33]. In another study of 1099 patients from Wuhan and other areas in China, fever was present in only 44% on admission but was ultimately noted in 89% during the hospitalization [35]. Nevertheless, our patients treated with O_3SS (Fig. 2a) had their febrility reduced gradually in line with their favorable recovery. Nevertheless, our patients treated with O_3SS (Fig. 2a) fever reduced gradually in line with their favorable recovery.

All clinical manifestations found in patients (Tab. 1) were similar to the clinical features of the disease onset [36]. The main clinical manifestations of disease (dyspnea and weakness) had a favorable course of resolution in O_3SS treated patients. On days 7 and 8 respectively (Fig. 2b) these symptoms were found to be significantly ($p<0.05$) improved. Acute respiratory distress syndrome (ARDS) is the major complication of patients with severe disease and it can manifest shortly after the onset of dyspnea. In a study of 138 COVID-19 patients, ARDS developed in 20%, on average of 8 days after the onset of symptoms; mechanical ventilation was implemented in 12.3% [36]. Some patients with initially no severe symptoms may progress over the course of a week. In another study, the median time to dyspnea was 8 days [31]. However, without exceptions, our 25 patients after the treatment with O_3SS, had a resolution of the dyspnea. Elevation of inflammatory markers (e.g., ferritin, D-dimer, CPR), were observed in our COVID-19 patients, in line with the results of other recent reports [37]. Elevated ferritin has also emerged as poor prognostic factors, Wu et al. showed that higher serum ferritin was associated with ARDS development [23]. Elevated D-dimer was detected in 36% of patients in a descriptive study of 99 COVID-19 cases in Wuhan, China [30]; in which increased levels of D-dimer were significantly associated with increased risk of ARDS [38]. Increased disease severity and ARDS development were associated with elevated CRP [37]. According to different studies from China and Singapore, mean values of CPR in patients that did not require supplemental O_2 were 11.1 (IQR: 0.9-19.1 mg/L); in patients that required O_2, 65.6 (IQR: 47.5-97.5 mg/L) [39] and in the mortality group, 109.25, (IQR 35.00-170.28 mg/L) [40]. In our patients, in correspondence with their clinic improvement, the average baseline values of CPR was 12.5 (IQR: 2.5-19.3 mg/L) after the first 24-48 h (Fig. 2c).

Fibrinogen was also higher in our sample, confirming that the coagulation function in patients with SARS-CoV-2 is significantly deranged compared with healthy people. In a prospective study, evaluating the coagulation profile of patients with COVID-19, D-dimer, and fibrinogen levels were markedly higher among patients as compared with healthy controls ($p<0.001$) [41]. In addition, high LDH level were significantly associated with severe COVID-19 on admission [42]. In our findings administration of O_3SS reduced the levels of inflammatory markers (e.g., ferritin, D-dimer, CPR, LDH) and fibrinogen as marker of coagulation function (Fig. 3).

Radiographic findings and auscultation revealed signs of pneumonia in 60 and 56 % of patient respectively (Tab. 2). Chest radiographs may be normal in early or mild disease. In a retrospective study of 64 patients in Hong Kong with documented COVID-19, 20 % did not have any abnormalities on chest radiograph at any point during the illness [43]. Common abnormal radiograph findings were consolidation and ground glass opacities, with bilateral, peripheral, and lower lung zone distributions. The 25 patients treated with O_3SS, bilateral signs of pneumonia was present only in 24-32% of them, according to the radiographic findings or auscultation, respectively (Tab. 2). Normally lung involvement increased over the course of illness, with a peak in severity at 10 to 12 days after symptom onset [44]. However, in our patients, chest radiographs and auscultation drastically changed after the third to fifth session of O_3SS, with both showing an improvement of their status.

Time from inclusion to discharge in patients treated with O_3SS and SC was not significantly different (>0.05) as compared to other report of patients treated only with SC [24]. However, the inclusion of O_3SS as a complementary treatment, accelerated the improvement of patients in terms of clinical symptoms (Tab. 3) and laboratory biomarkers (Fig. 2). This improvement avoided the patient transit to critical status. In addition, the time to median duration of viral shedding [8 days (IQR 6.0-11.5)] and longest duration of viral shedding (22 days) were reduced compared to other reports of 20.0 days (IQR 17.0-24.0) and 37 days, respectively [22].

Low doses of ozone, using physiological saline solution as a carrier and applied as a complementary therapy in COVID-19 patients, shows beneficial clinical effects and outcomes. The most probable mechanism is associated to the modulation of the “cytokines storm” through the balanced regulation of the Nrf2/NF-κB pathway[11]. The potential benefit of ozone in these clinical conditions merits further research.

5. Conclusions

Results of this pilot study suggest that patients with mild to severe symptoms due to COVID-19 disease can experience improved clinical symptoms, improved laboratory biomarkers and decreased time of viral shedding by the inclusion of O_3SS treatment as a complementary therapy to standard care.

No side effects were observed during the O_3SS treatment. The use of O_{3x} as adjuvant treatment for the management of the infection by SARS-CoV-2 patients has molecular and preclinical scientific evidence and clinical justification in term of cryoprotection and control of the inflammatory response. Based on the results of this clinical trial, it would be reasonable to conduct further clinical studies with this therapy on other viral diseases with a similar clinical and pathophysiological profile.

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