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ORIGINAL

# Multicentric study on the effect of rectal Ozone on COVID-19: the Spanish and Slovakian experience

*Estudio multicéntrico sobre el efecto del Ozono rectal en COVID-19: la experiencia española y eslovaca*

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## Abstract

***Objective:*** To evaluate the effect of rectal Ozone ($O_3$) in severe COVID-19 pneumonia in two different cohorts differing in location (Madrid vs Zilina), ethnicity (Slovakian vs Spanish cohorts) and age.

***Material and Methods:*** In a multicenter-study, 32 severe bilateral-COVID-19-pneumonia patients and (+) RT-PCR (reverse transcriptase polymerase chain reaction) SARS-CoV-2 were evaluated (16 from each cohort). Primary outcomes: a) clinical ($O_2$-saturation); b) biochemical (Lymphocyte-count, Fibrinogen, D-Dimer, Urea, Ferritin, LDH [lactate dehydrigenase], IL-6 and CRP [c-reactive protein]); and c) radiological improvement. Secondary outcomes: a) days-of-hospitalization, b) mortality-rate before discharge.The Ozone-protocol consisted of 10 sessions of intra-rectal Ozone, total dose 5.25 mg (150 mL volume, 35 µg/ml concentration). The Standard-of-care protocol included $O_2$ supply, antivirals (Remdesivir / Isoprinosine), corticosteroids (Dexamethasone / Metilprednisolone), monoclonal antibodies (Anakinra / Tocilizumab), antibiotics (Azytromicine), anticoagulants (Enoxaparine / Fraxiparine).

***Results:*** Patients in Slovakian cohort were younger (53.38 vs 84.69 years). Grade of severity was worse in Spanish-cohort (4.78 vs 3.30 points). Length of stay was superior in Spanish-cohort (27.38 vs 10.07 days). Both cohorts improved $O_2$-saturation and Lymphocyte-count. Inflammation biomarkers (Fibrinogen, D-Dimer, Urea, Ferritin, LDH, CRP and IL-6) decreased in both cohorts. In Spanish-cohort, Urea and Ferritin improvement was not significant (p>0.05), while in Slovakian-cohort, Urea, Fibrinogen and LDH were not significant (p>0.05) Radiological signs of bilateral pneumonitis decreased on both cohorts. Mortality was similar between both cohorts (12.5%) but inferior if compared to an external control group (23%).

***Conclusion:*** After Standard of care protocol, Rectal Ozone improved $O_2$-saturation, decreased inflammation biomarkers and improved Taylor's radiological scale in both cohorts. Although age, grade of severity and days of hospitalization were inferior in Slovakian cohort, mortality was similar in both cohorts, but inferior if compared to an external control cohort.

*Keywords:* Ozone, Ozone therapy, Pneumonia, COVID-19, SARS-CoV-2, Rectal insufflation.

## *Resumen*

***Objetivo.*** Evaluar el efecto del Ozono ($O_3$) rectal en la neumonía grave por COVID-19 en dos cohortes diferentes en cuanto a localización (Madrid vs Zilina), etnia (cohortes eslovaca vs española) y edad.

***Material y métodos:*** En un estudio multicéntrico, se evaluaron 32 pacientes con neumonía bilateral grave por COVID-19 y (+) RT-PCR (reacción en cadena de la polimerasa con transcriptasa inversa) SARS-CoV-2 (16 de cada cohorte). Resultados primarios: a) clínicos (saturación de $O_2$); b) bioquímicos (recuento de linfocitos, fibrinógeno, dímero D, urea, ferritina, LDH [deshidrogenasa láctica], IL-6 y PCR [proteína c reactiva]); y c) mejoría radiológica. Resultados secundarios: a) días de hospitalización, b) tasa de mortalidad antes del alta. El protocolo de ozono consistió en 10 sesiones de ozono intra-rectal, dosis total de 5,25 mg (volumen de 150 ml, concentración de 35 µg/ml). El protocolo de cuidados estándar incluyó suministro de $O_2$, antivirales (Remdesivir / Isoprinosina), corticosteroides (Dexametasona / Metilprednisolona), anticuerpos monoclonales (Anakinra / Tocilizumab), antibióticos (Azitromicina), anticoagulantes (Enoxaparina / Fraxiparina).

***Resultados:*** Los pacientes de la cohorte eslovaca eran más jóvenes (53,38 vs 84,69 años). El grado de gravedad fue peor en la cohorte española (4,78 frente a 3,30 puntos). La duración de la estancia fue superior en la cohorte española (27,38 frente a 10,07 días). Ambas cohortes mejoraron la saturación de $O_2$ y el recuento de linfocitos. Los biomarcadores de inflamación (fibrinógeno, dímero D, urea, ferritina, LDH, PCR e IL-6) disminuyeron en ambas cohortes. En la cohorte española, la mejoría de la Urea y la Ferritina no fue significativa (p>0,05), mientras que en la cohorte eslovaca, la Urea, el Fibrinógeno y la LDH no fueron significativos (p>0,05) Los signos radiológicos de neumonitis bilateral disminuyeron en ambas cohortes. La mortalidad fue similar en ambas cohortes (12,5%), pero inferior si se compara con un grupo de control externo (23%).

***Conclusiones:*** Tras el protocolo de cuidados estándar, el ozono rectal mejoró la saturación de $O_2$, disminuyó los biomarcadores de inflamación y mejoró la escala radiológica de Taylor en ambas cohortes. Aunque la edad, el grado de gravedad y los días de hospitalización fueron inferiores en la cohorte eslovaca, la mortalidad fue similar en ambas cohortes, pero inferior si se compara con una cohorte de control externa.

*Palabras clave:* Ozono, Ozonoterapia, Neumonía, COVID-19, SARS-CoV-2, Insuflación rectal.

## Introduction

Since the discovery of a new coronavirus on Wuhan, province of China, by December 2019, this new pandemic due to the new SARS-CoV-2 or COVID-19 virus has hit the economies and Sanitary Health Systems all over the world to an extent that the World Health Organization (WHO) has declared an exceptional situation of pandemic by the 3rd of March 2020[1].

To date, there is no effective treatment for the management of SARS-CoV-2 infection or COVID-19 disease. There are several clinical trials trying to find the most accurate treatment to fight against COVID-19 infection. Unfortunately, there is no evidence from controlled trials to recommend a specific treatment for suspected/confirmed COVID-19 patients[2].

In Spain, the pandemic situation in its first and second waves has saturated the Health System extremely that Material and Human Resources have been reorganized because of the shortage of them[3]; therefore, all medical activity has been focused and directed multidisciplinary to fight against this new pandemic situation. In that scenario, Ozone has been proposed as a complementary treatment to Internal Medicine Staff of our Hospital.

Currently, there are 9 clinical trials (CT) that postulate the potential use of Ozonized Autohemotherapy on the management of COVID-19 disease (1 CT from Brazil, 1 from Turkey, 2 CT from Italy, 2 CT from Spain and 3 CT from China); only 2 CT considers ozonized saline solution (1 CT from Spain and 1 CT from India); and 3 CT (2 CT from Cuba and 1 from India) consider rectal Ozone as an alternative for the management of COVID-19 infection. The studies from Cuba are still in phase of recruiting, and the study from India (Shah et al) has been recently published[4-7].

The Standard-of-care for COVID-19 is only supportive and acute respiratory distress syndrome (ARDS) will cause respiratory failure and finally death in COVID-19 patients. Although most patients will develop mild symptoms (fever, cough, myalgia and dyspnea); a small percentage of patients (15%) will develop a hyper inflammation or "cytokine storm" syndrome[8], and ARDS will constitute the leading cause of mortality, which occurs in 4.9% of patients[2]. Treatment of hyper inflammation will decrease the mortality rate[9]; therefore, we hypothesize that anti-inflammatory drug such as corticosteroids, monoclonal antibodies and even ozone could be recommended at this Stage.

Ozone ($O_3$) is capable of modulating pain and inflammation and recognized bactericidal, fungicidal, virucidal and anti-parasitic properties are attributed to ozone; a fact that is supported by clinical studies that come from countries where the practice of ozone therapies is well-regulated (Cuba, Italy, Germany, Russia, Turkey, India and Spain)[10,11]. Many water purification plants worldwide use ozone because of its germicidal effect[10]. Because of ozone biological properties, Fernández-Cuadros et al have postulated ozone as an alternative therapy for the management of the present SARS-CoV-2 pandemic[12]. Virucidal, immunomodulatory and vasodilator properties that favor $O_2$ transport to hypoxemic tissues postulate Ozone as a promising alternative in COVID-19 management[12].

Despite the preventive effect of vaccines on COVID-19 infection, there is no drug with anti-viral, anti-inflammatory and oxigenatory properties all in one as ozone does. Our study group postulated and further demonstrated ozone as a treatment once COVID-19 disease has started and viral spread, inflammation and hypoxia are hallmarks present in COVID-19 patients. In that sense, our Research Group has presented the preliminary results of rectal ozone for the management of mild-severe COVID-19, with very promising results[13,14]. Although our patients treated were older (mean age 83 years), there is no report of a similar study considering the treatment of younger patients, using the same protocol. There is a need to perform a multicenter study to evaluate our protocol in different groups and with different ages, that means in different cohorts, considering location, ethnicity and age, in order to recommend and to confirm the results observed previously in our country.

The objective of this article is to show the updated results of the effectiveness of a rectal ozone ($O_3$) protocol in a series of COVID-19 patients with severe bilateral pneumonia (Spanish cohort), and to compare them with another cohort of patients treated in another country (Slovakian cohort), in terms of clinical, biochemical and radiological variables. Mortality and Hospitalization time (length of stay) were also compared between cohorts. Cohorts differed on location (Madrid vs Zilina), ethnicity (Slovakian vs Spanish cohorts) and age.

## Material and methods

A prospective, before-and-after, multicentric study was performed in 2 Hospitals, one in Hospital Universitario Santa Cristina, Madrid-Spain (16 patients), and another in FNsP Hospital (Faculty Hospital and Poliklinic), Zilina-Slovakia (16 patients). The study included a total of 32 severe COVID-19 patients, with clinical symptoms and RT-PCR (reverse transcriptase polymerase change reaction) positive for SARS-CoV-2. The study run from August 2020 to February 2021. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki (1975), and the protocol was approved by the Ethics Committee of Santa Cristina University Hospital (15/4/20), by

Ethics Committee for Medical Investigation at Hospital Universitario la Princesa (25-06-20, acta CEIm 14/20, Registry number 4146), and by Zilina's Ethic Comission (17/1/2021). Three Committees authorized the study for ozone treatment for compassionate use in severe COVID-19 pneumonia (**Figure 1**).

***Inclusion criteria:*** 1) patients 18 years and older; 2) with positive result on RT-PCR SARS-CoV-2; 3) with clinical signs of moderate-severe pneumonia (SpO2 <93% or PaO2/FiO2<300 mmHg, or moderate/severe respiratory symptoms); 4) with radiological signs of COVID-19 pneumonia [bilateral "ground glass image" (compatible with lung lesions) on chest X-ray (according to Taylor's scale)] [15]; 5) who required hospitalization due to moderate or severe respiratory symptoms; 6) who required O2-supply with non-mechanical ventilation; 7) whose patient/legal representative gave informed consent to participate in the trial.

***Exclusion criteria:*** 1) Pregnancy or breast feeding; 2) Glucose 6-phosphate dehydrogenase (G6PD) deficiency (favism), rare in Spain; 3) Patients enrolled in other clinical studies. Comorbidities did not constitute a reason for exclusion in this study.

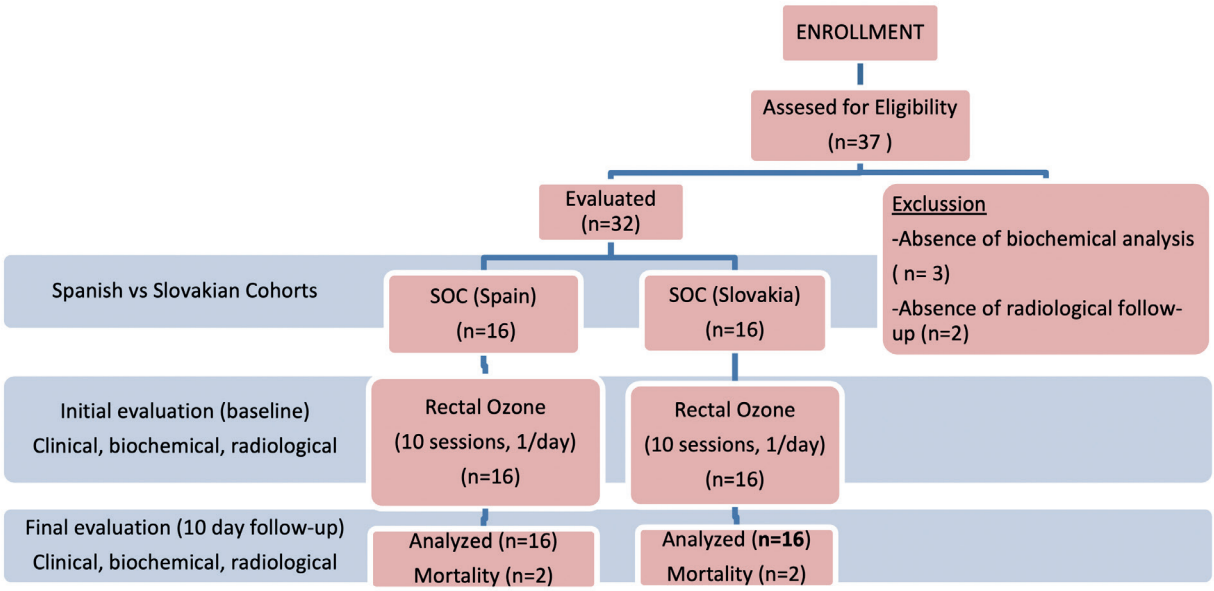
Once Standard-of-care (SOC) was completed (depending of the best available treatment on each country), no further clinical improvement was observed after Standard-of-care, and at this point, physicians considered that Ozone could be prescribed as compassionate use in COVID-19 treatment, the initial biochemical evaluation (leucocyte and lymphocyte count [Mindray BC-6000 kit], Ferritin [Abbott Alinity I kit], D-Dimer [IL ACL TOP 300 CTS kit], Fibrinogen [IL ACL TOP 300 CTS kit], CRP [C-reactive protein, Abbott Alinity c kit] and IL-6 [Snibe Maglumi 1000 kit]) and the initial radiography of the chest were performed. The procedure, indications and contraindications were explained to the patient and/or legal representative, and informed consent was signed prior to patient evaluation and treatment.

The Standard-of-care protocol in Spanish cohort included $O_2$-supply, antivirals (Remdesivir [200 mg/1day, the first day and 100 mg/day for 4 days]), corticosteroids [Dexamethasone 6 mg/day por 7 days] or Metilprednisolone [40 mg/day for 7 days]), antibiotics (Azytromicine [500 mg/day per 5 days]) and anticoagulants (Enoxaparine [40 mg SC [subcutaneous]/day, all hospitalization period], anti-IL-6 (Tocilizumab 8 mg/kg IV [intravenous] twice with an interval of 12 h, and up to a maximum of 800 mg per dose]) or anti-IL-1 (Anakinra 100 mg, single dose) and hyper immune serum (1 dose) if necessary.

The Standard-of-care protocol In Slovakian cohort included $O_2$-supply, antivirals (Remdesivir [200 mg IV/1 day, the first day and 100 mg/day for 4 days]; Isoprinosine 500 mg, 2 tablets/3 time/day in the case of lymphocytopenia), corticosteroids [Dexamethasone 8-24 mg IV/ 10-24 days], antibiotics [Azytromicine 500 mg/day for 6 days], anticoagulants [Fraxiparine, 1 dose per day if D Dimer > 1.0, 2 doses per day if D Dimer>2.0], and anti-IL-1 (Anakinra 100 mg, single dose in some cases).

The ozone protocol consisted in administering intra rectally a dose of 5,25 mg of ozone (rectal insufflation of 150 mL of Ozone at a 35 µg/mL concentration for 5 to 10 days), according to the severity of the patients. The supplies needed for ozone insufflation were: a) Ozonosan α-Plus® [Ozone Generator for Spanish cohort], or Ozofutura® [Ozone Generator for Slovakian cohort] ; b) Rectal probe;

**Figure 1:** Study protocol, enrollment, initial/final evaluation and follow-up in COVID-19 patients including both Spanish and Slovakian cohorts. SOC, Standard of care..



c) three silicone syringes of 50 mL capacity; and d) gel for lubrication of probe.

Prior to ozone administration, the patient was placed in the supine position (sedated patients) or lateral decubitus position (collaborative patients) with the lower limbs flexed. Three 50-mL silicone syringes of ozone were loaded with the corresponding concentration (35 µg/mL), and were slowly injected rectally through a 14 French rectal probe. The probe was previously lubricated with medical gel-type solution. The insufflation rate was of 1 mL / second.

The final evaluation was performed after of ozone protocol was completed. Clinical, biochemical analysis and chest radiographies were evaluated, and any adverse effects were recorded. Mortality and Hospitalization time were compared between both cohorts.

Chest Radiography was used to confirm diagnosis and to grade severity. Taylor Scale for Severe Acute Respiratory Infection grades severity (SARS), ranging from 1 to 5 degrees. Grade 1 is normal. Grade 2 shows patchy atelectasis, hyper inflammation or bronchial wall thickening. Grade 3 denotes focal alveolar consolidation but with no more than one segment or lobe involved. Grade 4 depicts multifocal consolidation and grade 5 shows diffuse alveolar consolidation[15].

For determination of sample size, the a priori power calculation was based on Taylor Scale, with an expected effect size of 0.2 between Groups at 10-days follow-up, a 2-time point of measurement (pretest-posttest evaluation) and a level of significance of 0.05 and a desired power of 0.80; all of which gave us a total sample size estimation of 30 patients. With an expected dropout of 10% during the study, a total of 32 patients were finally calculated (16 patients in each group).

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS®), Illinois, USA version 20.0. Frequencies and percentages were used to evaluate qualitative variables; while for the evaluation of quantitative variables, means/median and standard deviation were used. For evaluation of Taylor's Scale, median and Interquartile range (IQR) were used. The Wilcoxon test was the statistical tool used to evaluate a change before-and-after treatment in quantitative variables. The level of significance was 95% (p <0.05).

## Results

This is a multicenter study where two cohorts of 16 patients each who were treated using homogeneous ozone protocol (Total dose 5.25 mg, 150 ml at a 35 µg/ml concentration, every day for up to 10 days.

### Demographic Variables

In **table I**, we observe that the Slovakian cohort was younger than the Spanish cohort (53.38 vs 84.69 years, p=0.0001). Regarding COVID-19 severity, using Taylor scale as a radiological grading system, the Spanish cohort was more severe than the Slovakian cohort (4.78 vs 3.30, p=0.0001). No significant differences regarding sex were observed in both cohorts. The Length of Stay (LOS) was superior in the Spanish cohort (27.38 vs 10.07 days, p=0.0038). Number of Ozone sessions was similar in both cohorts (7.5 vs 6.43). No adverse events were observed in both cohorts. Mortality was similar in both cohorts (12.5%, p=0.7642). **Table I**.

### Primary Outcomes (clinical, biochemical and radiological variables)

In **table II**, in the Spanish cohort, all variables improved on an overall view. The improvement was significant for Saturation of $O_2$, Lymphocytes, Fibrinogen, D-Dimer, LDH, CRP, IL-6 and Taylor Scale (p<0.05). On the contrary, the improvement was not significant for Leucocytes, Urea and Ferritin (p>0.05). **Table II**.

In **table III**, in the Slovakian cohort, all variables improved on an overall view. The improvement was significant for Saturation of $O_2$, Lymphocytes, D-Dimer, Ferritin, CRP, IL-6 and Taylor Scale (p<0.05). On the contrary, the improvement was not significant for Leucocytes, Fibrinogen, Urea and LDH (p>0.05). **Table III**.

In **table IV**, when both the Spanish and the Slovakian cohort were evaluated together, all variables improved on an overall view. The improvement was significant for Saturation of O2, Lymphocytes, Fibrinogen, D-Dimer, CRP, IL-6 and Taylor Scale (p<0.05). On the contrary, the improvement was not significant for Leucocytes, Urea and Ferritin (p>0.05). **Table IV**. **Figures 2** and **3**.

**Table I:** Demographic and clinical Variables in patients treated in Spain and Slovakia, after rectal Ozone insufflation (n=32).

| Variable | Spanish Cohort | Slovakian Cohort | p |
|---|---|---|---|
| Taylor, median [IQR] | 4.78 [0.3] | 3.30 [0.82] | 0,0001** |
| Age (years) mean ± SD | 84,69 ± 12.23 | 53,38 ± 11.88 | 0,0001** |
| Male Sex n (%) | 12(75%) | 10(62.5%) | 0.3342 |
| Female Sex n (%) | 4(25%) | 6(37.5%) | 0.2982 |
| Length of Stay (days) mean ± SD | 27,38 ± 15.96 | 10,07 ± 3.81 | 0,0038** |
| Number of Ozone sessions mean ± SD | 7.5 ± 2.80 | 6.43 ± 1.71 | 0.1187 |
| Adverse events | 0 | 0 | 1 |
| Mortality (%) | 12.5 | 12.5 | 0.7642 |

*, p<0.05. %, percentage. **, p<0.01. IQR, Interquartile range. SD, standard deviation.

**Table II:** Main Variables in patients treated in Spain, after rectal Ozone insufflation (n=16).

| VARIABLES | PRE-O3 | POST-O3 | P |
|---|---|---|---|
| **CLINICAL** | | | |
| Sat $O_2$ mean ± SD | 89 ± 4.1 | 94.5 ± 3.4 | 0.0002** |
| **BIOCHEMICAL** | | | |
| Leucocytes cells/mL mean ± SD | 8602 ± 3676 | 7823 ± 2568 | 0.4165 |
| Lymphocytes cells/mL mean ± SD | 985 ± 484 | 1278 ± 583 | 0.0403* |
| Fibrinogen mg/dL mean ± SD | 713 ± 112 | 572 ± 163 | 0.0107* |
| D-Dimer ng/mL mean ± SD | 3240 ± 2484 | 1343 v 1320 | 0.0110* |
| Urea mg/dL mean ± SD | 67 ± 41 | 55 ± 24 | 0.1089 |
| Ferritin ng/mL mean ± SD | 989 ± 799 | 840 ± 1060 | 0.6043 |
| LDH U/L mean ± SD | 329 ± 111 | 241 ± 89 | 0.0209* |
| CRP mg/mL mean ± SD | 8.9 ± 6.1 | 2.46 ± 3.7 | 0.0040** |
| IL6 pg/mL mean ± SD | 85.07 ± 50.53 | 30.48 ± 38.10 | 0.0048** |
| **RADIOLOGICAL** | | | |
| Taylor median [IQR] | 4.78[0.3] | 3.0[0.0] | 0.0001** |

*, p<0.05. **, p<0.01. Sat $O_2$, Saturation of Oxygen. LDH, lactate dehydrogenase. CRP, C-reactive protein. IL-6, interleukin 6. . IQR, Interquartile range. SD, standard deviation.

**Table III:** Main Variables in patients treated in Slovakia, after rectal Ozone insufflation (n=16).

| VARIABLES | PRE-O3 | POST-O3 | P |
|---|---|---|---|
| **CLINICAL** | | | |
| Sat $O_2$ mean ± SD | 86 ± 6.99 | 94 ± 2.47 | 0.0001** |
| **BIOCHEMICAL** | | | |
| Leucocytes cells/mL mean ± SD | 9200 ± 4239 | 8826 ± 1713 | 0.7763 |
| Lymphocytes cells/mL mean ± SD | 1300 ± 564 | 2473 ± 897 | 0.0001** |
| Fibrinogen mg/dL mean ± SD | 289 ± 142 | 271 ± 127 | 0.4334 |
| D-Dimer ng/mL mean ± SD | 1389 ± 1106 | 622 ± 428 | 0.0172* |
| Urea mg/dL mean ± SD | 31 ± 12 | 32± 9 | 0.6655 |
| Ferritin ng/mL mean ± SD | 730 ± 531 | 415 ± 284 | 0.0173* |
| LDH U/L mean ± SD | 231 ± 139 | 202 ± 121 | 0.0963 |
| CRP mg/mL mean ± SD | 19.4 ± 19.4 | 2.4 ± 3.2 | 0.0040** |
| IL-6 pg/mL mean ± SD | 51.8 ± 42.8 | 5.8 ± 6.5 | 0.0026** |
| **RADIOLOGICAL** | | | |
| Taylor median [IQR] | 3.3 [0.82] | 1.9[0.0] | 0.0001** |

*, p<0.05. **, p<0.01. Sat $O_2$, Saturation of Oxygen. LDH, lactate dehydrogenase. CRP, C-reactive protein. IL-6, interleukin 6. . IQR, Interquartile range. SD, standard deviation.

**Table IV:** Main Variables in patients treated in Spain and Slovakia, after rectal Ozone insufflation (n=32).

| VARIABLES | PRE-O3 | POST-O3 | P |
|---|---|---|---|
| **CLINICAL** | | | |
| Saturation $O_2$ mean ± SD | 88.89 ± 4.39 | 93.92 ± 2.21 | 0.0001** |
| **BIOCHEMICAL** | | | |
| Leucocytes cells/mL mean ± SD | 88904 ± 3890 | 83067 ± 2210 | 0.4551 |
| Lymphocytes cells/mL mean ± SD | 11370 ± 538 | 18537 ± 955 | 0.0001** |
| Fibrinogen mg/dL mean ± SD | 509 ± 250 | 427 ± 210 | 0.0089** |
| D-Dimer ng/mL mean ± SD | 2349 ± 2130 | 996 ± 1044 | 0.0011** |
| Urea mg/dL mean ± SD | 50 ± 35 | 44 ± 22 | 0.1463 |
| Ferritin ng/mL mean ± SD | 864 ± 683 | 635 ± 804 | 0.1478 |
| LDH U/L mean ± SD | 282,0356 | 222,3537 | 0.0052** |
| CRP mg/mL mean ± SD | 14.01 ± 14.88 | 2.43 ± 3.46 | 0.0002** |
| IL-6 pg/mL mean ± SD | 69.04 ± 49.09 | 18.58 ± 29.57 | 0.0001** |
| **RADIOLOGICAL** | | | |
| Taylor median [IQR] | 4.07[2.0] | 2.48[1.0] | 0.0001** |

*, p<0.05. **, p<0.01. Sat $O_2$, Saturation of Oxygen. LDH, lactate dehydrogenase. CRP, C-reactive protein. IL-6, interleukin 6. . IQR, Interquartile range. SD, standard deviation.

**Figure 2:** Biochemical variables in both Spanish and Slovakian Cohort (n=32). *, p<0.05.

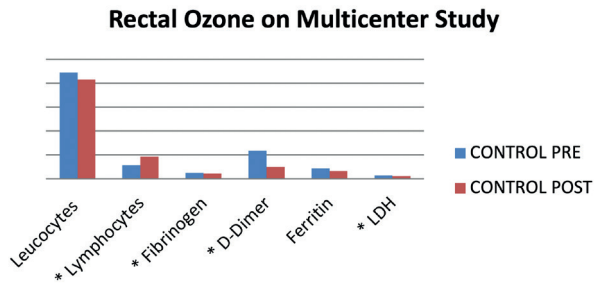
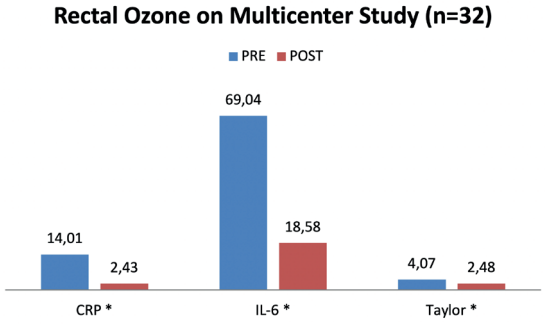
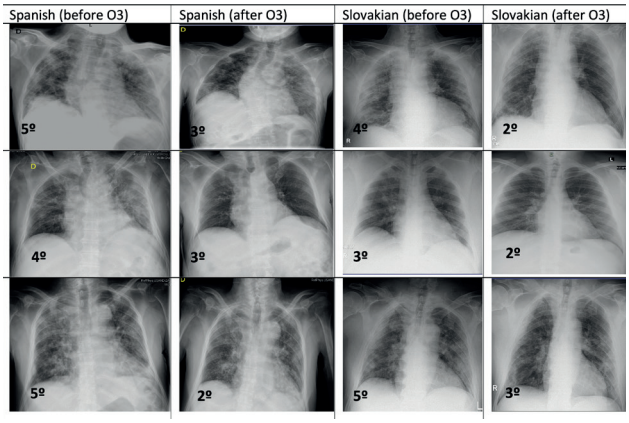



**Figure 3:** CRP, IL-6 and Radiological Taylor Scale in Spanish and Slovakian cohort. *, p<0.05 (n=32).



We present six cases of radiological improvement based on Taylor Scale, three patients from the Spanish cohort and three patients from the Slovakian cohort (**Figure 4**).

**Figure 4:** Three cases from Spanish and three cases from Slovakian cohorts are presented before/after Ozone ($O_3$).




## Discussion

To the best of our knowledge, this is the first multicenter study on the effectiveness of Rectal Ozone insufflation protocol in severe COVID-19 pneumonic patients in the light of SARS-CoV-2 pandemic. After Standard of care protocol was prescribed and no further improvement was observed, Rectal ozone improved clinical, biochemical and radiological variables in a significant manner ($p<0.05$) in both Spanish and Slovakian cohorts.

Since no further improvement was observed after standard of care was administered, ozone was prescribed as complementary treatment in both cohorts. Slovakian cohort was younger and radiological pneumonia was not as severe as Spanish cohort, as evaluated by Taylor Scale. This would explain the shorter length of stay in Slovakian cohort.

To date, almost 1661 clinical trials are in course to determine the best treatment for COVID-19 infection, but most of the pharmacological therapies investigated have not demonstrated effectivity in the management of SARS-CoV-2 pandemic[4,16]. From the clinical trials evaluating ozone therapy, only 2 of them have published their results[7,13]. This study presents the use of a homogeneous rectal ozone protocol in two different cohorts of COVID-19 patients regarding location, ethnicity and age, and the effects from one population were reproducible in the other one.

Our study group identified previously four properties that could cope with the complications derived from this COVID-19 infection (anti-viral, anti-oxidant, anti-inflammatory and $O_2$ delivery enhancer)[12]. In the mentioned study, it is expected that those properties could cope with the complications derived from this COVID-19 infection[12]. Our results confirm that those properties are responsible for the clinical, biochemical and radiological improvement, as stated by some others authors[17-19]. Improvement of $O_2$ delivery to tissues included the lungs, will be the reason why $O_2$ saturation improved in such patients. This comes in line with what Menendez-Cepero observed; she mentioned that Ozone is capable of modulating interferons and cytokines, decreasing inflammation biomarkers[20].

Ayanian et al. have identified that high level of at least 5 variables such as D-dimer, CRP, IL-6, Ferritin and LDH which produce negative outcomes in COVID-19 patients (ICU [Intensive Care Unit] admission, intubation and death. Reversely, lower levels of such biomarkers are related with positive outcomes (survival)[21]. Moreover, Lorenz et al. have suggested that severe COVID-19 patients have some characteristics features: neutrophilia, lymphopenia, hypercoagulability (D Dimer elevation) and hyper ferritinemic syndrome[22]. Copaescu has stated that clinical biomarkers (Saturation of Oxygen and respiratory rate) and inflammatory biomarkers (IL-6 and CRP [CRP as a surrogate of IL-6]) may predict severity in COVID-19, and such biomarkers may evaluate response to therapy[23]. Therefore, we intended to demonstrate that ozone was capable of reducing such outcome biomarkers whether clinical (Saturation of Oxygen) or biochemical (D-dimer, CRP, IL-6, Ferritin and LDH).

In a recent review, Cattel has stated that ozone is antiviral and might inactivate the virus and inhibit its viral replication. Moreover, ozone could reduce inflammation and lung damage and ozone might favor immunity and oxygenation, and increase Oxygen saturation and decrease $O_2$ support. As a consequence, an increase in lymphocyte count, a decrease of inflammation biomarkers (CRP, IL-6, Ferritin, D-Dimer and LDH), an improvement in $O_2$ saturation and a decrease in $O_2$ supply; and finally a negativization of RT-PCR SARS-Cov-2 Test is expected after ozone treatment[24]. We have evaluated similar clinical and biochemical variables in both cohorts, the Spanish and the Slovakian, and we did observe such positive effects in both cohorts.

Chirumbolo et al. have stated that ozone produces improvement in COVID-19 patients whatever the method of ozone delivery was used. Major autohemotherapy, ozonized saline solution and even rectal ozone has reduced inflammation in COVID-19 patients[25]. In our study, ozone by rectal insufflation was chosen because the technique is very simple, economic and safe[12].

To date, there are 21 manuscripts that postulate the use of ozone against COVID-19[26]. There are also 21 articles that state the benefit of ozone in treating 682 patients with COVID-19 (patients recruited from China, Spain, Cuba, Italy, Iran, India and USA). Ozone reduces

inflammation; time of ventilatory support and time to normalize (negativize) RT-PCR for SARS-CoV-2[26].

In our study, an improvement in Saturation of Oxygen was observed (Oxygen Saturation from 88.89 to 93.92%). Similarly, in another study, Franzini observed an improvement of 85 to 95% in Oxygen saturation ($p<0.001$)[27]. Araimo observed that ozone reduced the need for ventilatory support in COVID-19 disease[5]. Ozone improves saturation of Oxygen, as observed by Franzini, Araimo and our study[5,27].

In our multicentric study, an improvement in leucocyte count, in biomarkers of inflammation and in biomarkers of coagulation was observed (Fibrinogen, D-Dimer, LDH, CRP and IL-6). Similar results have been observed by Araimo[5], Franzini[27], Tascini[28], Schwartz[29] and Hernández[30], in different cohorts, in Italy and in Spain.

Finally, and improvement in radiological severity based on Taylor scale was observed in our multicentric study, (change in Taylor scale from 4.07 to 2.48 points). This comes in line Schwartz et al., who observed an improvement in lung affection from 60% to 24%; and the improvement, occurred at 3-5 days from ozone treatment[29].

Mortality of an external control cohort regarding similar age and sex in Spain using Standard of care was 21%[14]. The overall mortality in first and second wave in the Community of Madrid was 23% in hospitalized COVID-19 patients[29]. However, when mortality was compared to hospitalized patients treated by ozone, mortality decreased to 12.5% in both Spanish and Slovakian cohorts. These observations would suggest that ozone could decrease mortality in severe COVID-19 pneumonic patients.

Shah et al. and Hendawy et al. have published a case-control study and a case report, respectively; using rectal ozone insufflation. Shah et al. used a protocol of rectal ozone insufflation, 150 ml at a 40 µg/ml concentration, 2 times/day, for up to 10 days[7]. They observed clinical (oxygen saturation and clinical NEWS Score) and biochemical improvement (LDH, CRP, Ferritin), although the improvement was not statistically significant. Moreover, on 10$^{th}$ day of treatment, 100% of patients showed RT-PCR negativity vs 70% in control group ($p=0.01$)[7]. Ozone was superior to negativize SARS-Cov-2 infection[7].

Hendawy et al. reported the use of rectal ozone, one single insufflation, and a total dose of 25.2 mg (2000 ml at a 12.6 µg/ml concentration) in two cases, and an immediate improvement of oxygen saturation from 84% to 94-97% was observed[31]. The observations form both authors (Shah and Hendawy) are similar to ours[7,31]. Rectal ozone insufflation improved oxygen saturation, and decreased both biomarkers of inflammation and coagulation.

Peña-Lora and Fernandez-Cuadros have published another case in which, after 5 session of rectal ozone (100ml, once a day at a 35 µg/ml), oxygen saturation improved from 90% to 94%, biochemical variables ameliorated (IL-6, PCR, D dimer, Fibrinogen and ferritin) and radiological improvement was observed (from 5 to 3 on Taylor Scale)[32].

In the present study, we have used a dose of 35 µg/mL. The dose is similar to the one used in a Cuban protocol directed by Leon-Fernandez et al, which has been lately published[33].

A limitation of this multicentric study is the small sample size analyzed. Moreover, with such a small sample size (32 patients, 16 in each cohort), we cannot speculate why mortality was similar in both cohorts (12.5%). We expected to see some differences since Slovakian cohort was younger than the Spanish cohort. Maybe in a greater sample, these differences would be statistical, and could favor the younger cohort, because there is a linear correlation between age and mortality in COVID-19 patients[12]. We did not take into account comorbidities in these cohorts, and comorbidities might be related to age of population. In any case, the mortality was lower if compared to an external control group (23%). However, despite the number of patients evaluated, the fact that ozone improved all variables analyzed once standard of care was already finished; a larger sample in a new study is needed in order to confirm the effect of rectal ozone insufflation in the management of COVID-19 patients, as it was observed in this manuscript.

## Conclusions

In patients with severe COVID-19 pneumonia, after Standard of care protocol was prescribed and no further improvement was observed, rectal Ozone improved $O_2$ saturation, decreased inflammation biomarkers and improved Taylor's radiological scale with statistical significant difference in both Spanish and Slovakian cohorts. Although age, grade of severity and days of hospitalization were inferior in the Slovakian cohort, mortality was similar between both cohorts, but inferior if compared to an external control group. It is necessary to develop a Randomized Control Trial to confirm these promising observations.

### Author contributions

Conceptualization; drafting; investigation, project administration, supervision, writing, review, editing and translation: MEFC. Biochemical Analysis, data-curation and validation: JRDC and JV. Patients' treatment: MEFC, OSPM, MJAF, JV. All authors have read and agreed to the published version of the manuscript.

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## Informed Consent Statement
All patients signed informed consent for treatment and publication.

## Ethics approval
This study was approved by Ethics Committee of Hospital Universitario Santa Cristina (15-04-2020), by Hospital Universitario la Princesa (25-06-20, acta CElm 14/20, Registry number 4146), Madrid-Spain; and by Zilina´s Ethic Comission (17/1/2021).

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## Conflicts of interest
The authors declare no conflicts of interest.

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