Safety and efficacy of a COVID-19 treatment with nebulized and/or intravenous neutral electrolyzed saline combined with usual medical care vs. usual medical care alone: A randomized, open-label, controlled trial

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Abstract. Coronavirus disease 2019 (COVID-19) is currently the major public health problem worldwide. Neutral electrolyzed saline solution that contains reactive chlorine and oxygen species may be an effective therapeutic. In the present study, the treatment efficacy of intravenous and/or nebulized neutral electrolyzed saline combined with usual medical care vs. usual medical care alone was evaluated in ambulatory patients with COVID-19. A prospective, 2-arm, parallel-group, randomized, open-label, multi-center, phase I-II clinical trial including 214 patients was performed. The following two outcomes were evaluated during the 20-day follow-up: i) The number of patients with disease progression; and ii) the patient acceptable symptom state. Serial severe acute respiratory syndrome coronavirus 2 naso/oro-pharyngeal detection by reverse transcription-quantitative (RT-q) PCR was performed in certain patients of the experimental group. Biochemical and hematologic parameters, as well as adverse effects, were also evaluated in the experimental group.

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Abbreviations: ROS, reactive oxygen species; PASS, patient acceptable symptom state; RR, relative risk; CI, confidence interval; RPCEC, Cuban public registry of clinical trials; NSAIDs, nonsteroidal anti-inflammatory drugs; ARDS, acute respiratory distress syndrome; AST, aspartate aminotransferase; ALT, alanine aminotransferase; VAS, visual analog scale; CRP, C-reactive protein; ALP, alkaline phosphatase; NK, natural killer

Key words: COVID-19, neutral electrolyzed saline, SARS-CoV-2, reactive oxygen species, reactive chlorine species, immune system, inflammation, treatment
The experimental treatment decreased the risk of hospitalization by 89% [adjusted relative risk (RR)=0.11, 95% confidence interval (CI): 0.03-0.37, P<0.001] and the risk of death by 96% (adjusted RR=0.04, 95% CI: 0.01-0.42, P=0.007) and also resulted in an 18-fold higher probability of achieving an acceptable symptom state on day 5 (adjusted RR=18.14, 95% CI: 7.29-45.09, P<0.001), compared with usual medical care alone. Overall, neutral electrolyzed saline solution was better than usual medical care alone. Of the patients analyzed, >50% were negative for the virus as detected by RT-qPCR in nasso/oro-pharyngeal samples on day 4, with only a small number of positive patients on day 6. Clinical improvement correlated with a decrease in C-reactive protein, aberrant monocytes and increased lymphocytes and platelets. Cortisol and testosterone levels were also evaluated and a decrease in cortisol levels and an increase in the testosterone-cortisol ratio were observed on days 2 and 4. The experimental treatment produced no serious adverse effects. In conclusion, neutral electrolyzed saline solution markedly reduced the symptomatology and risk of progression in ambulatory patients with COVID-19. The present clinical trial was registered in the Cuban public registry of clinical trials (RPCEC) database (May 5, 2020; no. TX-COVID19: RPCEC00000309).

Introduction

Coronavirus disease (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is currently the major public health problem worldwide (1,2). Previous studies reported that the most common initial symptoms are systemic, upper respiratory symptoms and cough. Lower respiratory and gastrointestinal symptoms are less frequent and generally appear at the late stage of the disease (3). The symptoms, if present, with the longest duration are cough, loss of sense of smell or taste, sinus congestion, shortness of breath upon exertion, body aches and headache (3). A study on the time that COVID-19 patients require to achieve a usual state of health reported that 65 percent have returned to their usual state of health 7 days from the date of diagnosis, whereas 35% of patients had not returned to their usual state of health at 12-14 days after receiving a positive test result (4). Although most infections are self-limited, an estimated 15% of infected adults develop severe pneumonia that requires treatment with supplemental oxygen and hospitalization (5). However, the number of infected patients identified as having severe infection and requiring hospitalization varies among regions and countries, whether due to inherent conditions in the population (6) or to the strategy used in identifying individuals that are positive for the virus (7). In Mexico, 40.3% of confirmed cases are estimated to require hospitalization (8).

There are numerous experimental approaches for treating COVID-19. Initially, chloroquine appeared to be a promising treatment, but its lack of efficacy has since been demonstrated (9). Despite the numerous drugs that are currently recommended, such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticoids, antivirals, antibiotics and proinflammatory cytokine (interleukin) modulators, no specific drug therapy has been proven to be effective against SARS-CoV-2, yet (10). Treatment is symptomatic and oxygen therapy is the first step in addressing respiratory impairment (1). Noninvasive and invasive mechanical ventilation may be necessary in cases of respiratory failure that is refractory to oxygen therapy (1).

COVID-19 symptomatology and manifestations depend on the degree of immune dysregulation caused by the virus, characterized by systemic inflammation and remote organ injury (11,12). Viral infection is capable of producing an excessive immune reaction in the host. In severe cases, a reaction known as ‘cytokine storm’ occurs (1). A rapid and robust type I IFN-orchestrated response may lead to virus clearance, given that antiviral lymphocytes, such as natural killer (NK) cells, are activated and expanded. Conversely, late activation of innate immunity is usually associated with severe pathology that may lead to pneumonia, acute respiratory distress syndrome (ARDS), septic shock, multi-organ failure and, eventually, death (13). Different immune system alterations come together to produce severe disease. A key factor in the cytokine storm in COVID-19 is the elevation of monocytes, a circulating innate immune cell type producing IL-6 (14), combined with lymphocyte reduction that limits the systemic antiviral response (15,16). Inefficient SARS-CoV-2 clearance by alveolar macrophages may promote excessive viral replication, leading to severe pathology that is accompanied by increased viral shedding and, in turn, viral transmissibility (13). In the present study, it was postulated that administration of intravenous and/or nebulized electrolyzed saline may aid in modulating the body’s immune response to SARS-CoV-2, reducing symptomatology and preventing disease progression.

Electrolyzed saline is produced from a saline solution of sodium chloride, activated by a controlled process of electrolysis, producing reactive species of chlorine and reactive oxygen species (ROS). Significant examples of said reactive species are oxidant chlorine species, such as hypochlorous acid and oxidant ROS, such as hydrogen peroxide. Molecular hydrogen (H₂) is also produced (17). ROS are normally produced in the organism and have different physiological functions (18). Their most well-known activity is to control bacteria, parasites and viruses through the activity of cells of the innate immune response, macrophages and neutrophils that release ROS to structurally damage the invading pathogens, thus protecting the host from infection (19).

A series of studies have indicated that, in addition to the primordial innate immune response, ROS are secondary messengers in processes of exacerbated inflammation control and tissue repair in a process known as redox signaling. Redox signaling is ROS-dependent and the immune response varies, according to ROS concentrations and exposure time (19-23). Different studies have indicated that ROS are able to activate and repair phenotypes, such as M2 macrophages and regulatory T cells, acting as potentiators of the humoral immune response (24,25). ROS have been indicated to mediate the communication between the different cells of the immune system, such as polymorphonuclear cells, neutrophils, macrophages, antigen-presenting cells, B cells and T cells (23-26). Specifically, hypochlorous acid may act as a coadjuvant and adaptive immune response stimulator by modifying antigen proteins and increasing their recognition, processing and presentation by antigen-presenting dendritic cells (27). In addition, ROS have an important role in later stages of B-cell activation by promoting the sustained signaling of B-cell antigen receptors, thus favoring antibody production (28).
Numerous studies have also suggested that H₃ has beneficial effects in diverse animal models and human diseases (29). Its oral administration in an animal model limited the increase of IL-6 and tumor necrosis factor-alpha, producing a potent antioxidant and anti-inflammatory effect (30).

Therefore, the present study was designed to randomly select patients with COVID-19 receiving usual medical care and compare the safety and efficacy of two treatments: Usual medical care combined with electrolyzed saline [administered intravenously and/or through inhalation of the aerosol (nebulization), with dose escalation] and usual medical care alone (control).

Materials and methods

Study design. A prospective, randomized, single-blind, 2-arm, parallel-group, open-label, phase I-II clinical trial was performed between May and December 2020 and carried out according to the consolidated standards of reporting trials (CONSORT) statement guidelines for randomized controlled trials (31). The study aimed to evaluate the safety and efficacy of electrolyzed saline for preventing disease progression and it was approved by the ethics committee of the School of Medicine of the Universidad de Colima (Colima, México; April 8, 2020), and written informed consent was obtained from all of the participants. The trial was performed in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization-Good Clinical Practice guidelines. The present clinical trial was registered in the Cuban public registry of clinical trials (RPCEC) database (May 5, 2020; no. TX-COVID19: RPCEC00000309).

Study subjects. The inclusion criteria were as follows: Males and non-pregnant females aged ≥18 years, presenting with COVID-19 and a positive diagnosis of SARS-CoV-2 by reverse transcription-quantitative (RT-q) PCR, who had a medical consultation due to their illness and were indicated for at-home ambulatory treatment. Women of reproductive age, without permanent contraceptive methods and sexually active agreed to utilize effective non-hormonal contraceptive measures during the study period and for at least 15 days after the final drug administration of the study. Exclusion criteria were pregnant or breastfeeding females and patients presenting with any of the following conditions prior to the diagnosis of COVID-19: Cancer, ischemic heart disease, chronic decompensated systemic disease, creatinine 1.25 times higher than the normal value or creatinine clearance <50 milliliters/min (Cockcroft-Gault method), blood hemoglobin <10 g/dl, drug addiction (illegal drugs) or known liver disease with a doubling of liver function test values [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) or bilirubin]. In addition, the following elimination criteria were applied: Patients who decided to drop out of the study, patients who at any point of the study, presented with severe toxicity (grade 3 or higher, according to the Common Terminology Criteria for Adverse Events v5.0, US Department of Health and Human Services) (32), that was attributable to the administration of the experimental drug.

The physicians participating in the project identified candidates from primary and secondary healthcare centers (public or private) in the Mexican states of Colima, Chiapas and Morelos (in Colima: Regional University Hospital from the Health Ministry of the State of Colima, Colima Hospital, General Hospital of Zone 1 of the IMSS Colima, Medical Center Union Clinic, San Francisco Clinic; in Chiapas: Poliforum COVID-19 Respiratory Care Clinic; in Morelos: Private practice medical office Xochitepec). The physicians asked the patients for their permission, once they were at home, for the researchers to call them by telephone, requesting their participation in the study. Prior to said phone call, the candidates were randomly allocated to the experimental group (electrolyzed saline + usual medical care) or the control group (usual medical care alone). Randomization was performed using computer-generated random allocation cards. In that manner, the patients were directly asked to participate in one of the non-blinded groups.

The inclusion process was performed by researchers who did not participate in the evaluation of the results. Prior to entering the study, all of the patients were receiving usual treatment under the care of their family physician or specialist. When asked to participate in the study, the patients selected for the electrolyzed saline group were told they would receive an experimental treatment in addition to their usual medical care, as well as have sign and symptom follow-up and undergo certain laboratory tests. The patients receiving usual medical care alone (control group) were asked to participate in the study, with follow-up of signs and symptoms performed by telephone. All of the patients were advised that they would continue to be under the supervision of their regular physician or healthcare institution and that the research team would in no way modify or limit any intervention that their physician, or they themselves, considered pertinent, such as going to the emergency service if there were any alarming symptoms.

Neutral electrolyzed saline. The experimental treatment consisted of an aqueous saline solution of sodium chloride, activated by a controlled process of electrolysis (patent no. MX330845B), and thus resembled activated saline, electrolyzed saline or electrolyzed water. It had a neutral pH (6.0-7.5) and its active ingredient was 0.002% of active species of chlorine and oxygen. The good manufacturing practices for intravenous electrolyzed saline (HOMEOSTECH®) also met the required processes for sterile injectable products (33). As an intravenous (IV) electrolyzed saline, its formulation was 17.12 mEq/l of sodium chloride and 0.38 mM of active species of chlorine and oxygen. The vials utilized were 5-ml ampules, and the name and composition were indelibly printed on each one. The electrolyzed saline was provided by Esteriorpharma S.A. de C.V as an experimental (not commercial) product.

When the randomized patient was in the electrolyzed saline group, he or she was included in a dose escalation with overdose control design, as has previously been reported (34). Dose level 1 consisted of nebulizations (inhalation of the mist, produced by a nebulizer provided with a mask for inhalation therapy). The nebulizations were indicated 4 times a day for 10 days. They were performed by placing 5 ml of electrolyzed saline in the nebulizer chamber (Nebucor, type MOD. P-100; Neb S.A. de C.V) and continuing the nebulization until the content was used up (10-15 min). The nebulizations were performed following the recommendations of the American College of Allergy, Asthma and Immunology (35), the British...
Lung Foundation (36), the Asthma Society of Ireland (37) and the British Thoracic Society (38).

The IV dosing began with a dose within a safe range, previously established in a phase I clinical trial conducted at the Instituto Estatal de Cancerología de Colima for the treatment of chikungunya (manuscripts in preparation; clinical trial registration number RPCEC00000226. The initial applications were 15 ml (dose level 2) once a day for 7 days, with successive increases to 20 ml/day (dose level 3), 30 ml/day (dose level 4), 40 ml/day (dose level 5), 50 ml/day (dose level 6), 80 ml/day (dose level 7) and 150 ml/day (dose level 8). All applications were made every 24 h for 7 days or 10 days only if diarrhea, myalgia, arthralgia or body temperature >37.5°C was present on the seventh day of treatment. Dose level 5 was the exception, where applications were made every 12 h for 3 (dose level 5.1), 6 (dose level 5.2) or 9 days (dose level 5.3) (Fig. S1). Nebulizations with electrolyzed saline solution were always added to all IV treatment regimens. The dose-limiting toxicity was not achieved at any dose level.

The electrolyzed saline solution was diluted in one-third of its volume with physiological saline solution (0.9% of NaCl) (1 ml physiological solution for every 2 ml electrolyzed saline), immediately prior to its application, for the case of dose levels 2-6. The solution was administered IV as a bolus (passing it in 1-2 min). For dose levels 7 and 8, 100 ml of normal saline solution (0.9% of NaCl) were withdrawn from a 250-ml bottle and the appropriate volume of electrolyzed saline for each regimen was added, under sterile conditions using a Class-II laminar flow hood BSL-2. The whole solution mixture was administered in 1 h, with applications once a day using a heparinized peripheral venous catheter for its intermittent use.

When COVID-19 symptoms of nausea, vomiting and/or diarrhea occurred, 30 ml oral electrolyzed saline was added, 4 times a day, for as long as gastrointestinal symptoms lasted, plus 2 more days after the symptoms disappeared. The oral route of electrolyzed saline has been shown to be safe and has been used to treat epidemic diarrhea virus infection in preclinical trials (39,40). In patients with oropharyngeal ulcerations and/or intense throat irritation (causing intense pain), the indication was to gargle with 10 ml electrolyzed saline 6 times a day and swallow the solution after gargling with it. This was performed for the number of days necessary for the pain to decrease to 4 or less on the 0-10 visual analog scale (VAS). The oral pathway was indicated in 17 patients and gargling was indicated in 25. These indications were added during the protocol to rapidly evaluate them without compromising the original trial outcomes, a procedure that has been considered adequate in previous scientific reviews (41). The indication was based on intended uses previously authorized (local treatment of throat infections and sore) by the Mexican Federal Commission for the Protection against Sanitary Risks (COFEPRIS) of a similar product (Estericida Bucofaríngeo, Reg. No. 1003C2013 SSA; Esteripharma).

Usual medical care. The patients receiving only usual medical care continued with the usual treatment prescribed by their family physician or specialist. Usual care is the care the targeted patient population would be expected to receive as part of normal practice (42). This is a valid strategy as a reference treatment in clinical trials, including various therapy trials against COVID-19 (43-45). It consisted of the administration of paracetamol, NSAIDs, steroids, azithromycin, chloroquine, ivermectin, and/or antiviral drugs, anticoagulants, etc.; the patients were instructed to return to the emergency service if there was respiratory difficulty or worsening of symptomatology. The researchers did not intervene in drug prescription or lifestyle indications (usual medical care).

Outcome measures and follow-up. There were 3 co-primary endpoints. The first was the number of patients with disease progression, defined as hospitalization or death. The second primary endpoint was the patient acceptable symptom state (PASS), defined as the value of symptoms the patient considered to be well-being thresholds of pain and function. In the present study, the most widely used anchoring question to identify PASS cut-off points was incorporated, which as follows: ‘Taking into account all your daily activities, do you consider your current state satisfactory in relation to pain level and functional impairment?’ with response options being ‘Yes’ or ‘No’ (46-48). Treatment success was defined as no disease progression or a PASS according to the answer in the affirmative test on days 1 to 20 of follow-up. The third endpoint was the change from the baseline in the patient overall self-assessment or the severity score, which was determined by the response to the following question: ‘Considering all the ways in which illness and health conditions may affect you at this time, please indicate how you are doing?’ with the response options measured on the 0-10 VAS, from ‘very well’ (score of 0) to ‘very poorly’ (score of 10) (49). This question was validated using the Routine Assessment of Patient Index Data 3, previously used to determine the activity of autoimmune diseases, degenerative diseases, such as osteoarthritis (50) and infectious diseases with a strong component of general malaise, such as chikungunya fever (51). That endpoint is similar to the symptom severity score (self-assessed using a 10-point VAS) recently used in a clinical trial that evaluated the efficacy of hydroxychloroquine in non-hospitalized patients with COVID-19, where 0 indicated ‘no symptoms’ and 10 indicated ‘severe symptoms’ (52). The patients were also classified at baseline according to disease severity, as directed by the World Health Organization (WHO) interim clinical management guidance; as mild, moderate, severe or critical disease (53). In addition, the concepts of asymptomatic patients (0 major symptoms and 0 minor symptoms) and pauci-symptomatic patients (0 major symptoms and 1-2 minor symptoms) were considered, as previously defined (major symptoms: Fever >37.8°C and new persistent cough; minor symptoms: Hoarse voice, non-persistent cough, sore throat, runny or stuffy nose, shortness of breath, wheezing, headache, muscle aches, nausea and/or vomiting and/or diarrhea and loss of sense of taste or smell) (54).

The secondary endpoints were changes from the baseline in different types of body pain (arthralgia, myalgia, headache and sore throat), or more precisely, the difference from the values at enrollment on all days of follow-up. Pain was measured on the 0-10 VAS (55). Intensity of pain was recorded, from ‘no pain’ (score of 0) to ‘worst pain imaginable’ (score of 10) (55,56). Patients completed the previously validated fatigue VAS (scale of 0-10) (57), which poses the questions of: ‘How much of a
problem has unusual fatigue or tiredness been for you today’)
and was anchored from 0 (fatigue is not a problem) to 10
(fatigue is a major problem). Daily coughing episodes were
reported by the patient on a numerical scale from 0 to 20. If
there were more than 20 episodes, they were registered as 20.
Dyspnea was determined once a day through the Borg scale,
from 0 to 10, according to which 0 indicates no dyspnea and
10 extremely severe dyspnea (58). Nausea, vomiting, diarrhea,
dizziness, conjunctivitis, rhinorrhea, exanthema, skin rash
and loss of sense of smell or taste were recorded as present or
absent for each day of follow-up. Adverse events were moni-
tored by the researchers through anamnesis and abnormal
routine laboratory test results. Follow-up was performed for at
least 20 days or until an endpoint was reached (cure or death).
Daily follow-up was suspended in the hospitalized patients,
and from the day of hospital admission, their registers were
considered lost data and were not considered in the analysis
from that day forward, with the exception of the PASS, the
result of which was reported as a negative acceptable symptom
state from then onwards. However, the general aspects of those
patients were registered, such as hospitalization and outcome
(cure or death).

Serial detection of SARS-CoV-2. In 10 patients from the
experimental group treated with electrolyzed saline, naso-
pharyngeal and oropharyngeal samples were collected with
swabs in 2.5 ml of viral transport medium, immediately prior
to starting treatment and on days 2, 4, 6 and 14, and stored
at -80˚C until processing. Viral RNA was isolated utilizing
TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according
to the manufacturer's protocol and SARS-CoV-2 testing was
performed through SYBR green-based reverse transcrip-
tion-quantitative (RT-q) PCR using the previously described
methodology (59). That procedure was not performed on any
of the patients in the control group.

Evaluation of hematologic and serologic parameters. In
the experimental group, changes in hematologic parameters
were evaluated at baseline, at 48 h (day 2) and on days 4,
6, 9 and 14. The complete blood count was evaluated using
Sysmex XP-300 (Roche®) equipment, the biochemical tests
for kidney function and liver function were performed using
Cobas c111 (Roche®) equipment and the serum concentration
of testosterone and cortisol was determined by immunofluo-
rescence with the iCHROMA (Boditech Med Inc.) equipment.
The testosterone/cortisol ratio was calculated by dividing
the two hormone levels, both expressed in nm/l (60). Thirty
patients with any type of steroidal or hormonal treatment were
excluded from this analysis. Systemic inflammation markers
(erythrocyte sedimentation rate and C-reactive protein)
were also evaluated and rapid staining of blood smears
with staining kits (Hycel) were performed to quantify the
following: i) Reactive lymphocytes, also called virocytes;
ii) large granular lymphocytes, a representation of NK cells
or cytotoxic T lymphocytes; iii) activated monocytes; and
iv) monocytes with aberrant nuclei (clumped chromatin) and
basophilic cytoplasm (14,61,62).

Blinding. Only the researchers that evaluated treatment
effectiveness through the VAS, PASS and other endpoints
instruments answered by the patients, as well as those that
performed the statistical analyses, were blinded. The personnel
who provided the treatments were different from the personnel
in charge of evaluating the effectiveness of the treatments.

Sample size. The sample size calculation was based on the
number of patients that had disease progression (hospitalization
or death). Progression in 10% of patients in the experimental
group and 35% of subjects in the control group was predicted.
Those figures were based on local data from the Mexican city
of Colima, according to which 43% of confirmed patients
were hospitalized, according to health authority reports (63).
A total of 32 patients from each group were needed to reach
the required statistical power (0.8) when the statistical analysis
was performed at the level of a one-tailed alpha-value of 0.05.
At the end of the study, the statistical power for detecting
a difference between two distinct groups was calculated
(one-tailed alpha=0.05), utilizing the number of patients with
disease progression, resulting in 99.2%.

Statistical analysis. Values are expressed as the mean ± stan-
dard deviation (for data with a normal distribution), median
with 25 and 75th percentiles (interquartile range) for data
with a non-normal distribution or percentages. Normality
of distribution of data was first determined using the
Kolmogorov-Smirnov test and the equality of variances
was confirmed using Levene's test. Parametric data with a
normal distribution [e.g., body mass index (BMI) or age] were
compared between groups utilizing Student's t-test. Categorical
variables were compared using the Fisher's exact test or likelihood ratio χ² test. To compare continuous vari-
ables with a non-normal distribution or data in ordinal scale
between two groups, the Mann-Whitney U-test was applied to
independent samples and the Wilcoxon signed-rank test was
applied to matched samples. For the oxygen saturation param-
eter, the change from baseline was used to observe the absolute
differences between the evaluation periods, calculated through
the value after intervention minus the value at baseline, in
each patient, which is an acceptable manner for analyzing
trial results with baseline and after the beginning of treat-
ment measurements (64). To test for a significant difference
in means over time in blood parameters, repeated-measures
ANOVA was used, followed by Dunnett's post-hoc test (any
time-point vs. baseline). The Jonckheere-Terpstra test was
used to determine differences in symptom severity between
dose levels on different days, followed by pairwise compar-
sions between groups using Dunn's test. Kaplan-Meier analyses
were performed to compare survival and the log-rank test was
applied to determine significant differences between groups.
Binary logistic regression analyses were employed to deter-
mine the probability of hospitalization or achieving PASS on
day 5 (binomial outcome: Yes or no) with the experimental
treatment, compared with the usual medical care. Data were
summarized as relative risk (RR) with 95% confidence
interval (CI) and P-value, adjusted for age, sex, BMI, baseline
of oxygen saturation (SpO2), diabetes, hypertension, progres-
sion time, baseline severity and other relevant variables.
Binomial regression is considered the most adequate choice
for estimating RR in multivariate analyses (65-67). Pearson's
correlation coefficients (r) were calculated for bivariate
correlation between numeric and normally-distributed parameters (C-reactive protein, monocytes, platelets, lymphocytes, cortisol and testosterone-cortisol ratio); while Spearman's rank correlation coefficients (r) were generated when any of the above parameters was correlated with the patient symptom severity score (ordinal scale). Significant correlations were discussed based on the P-value.

The statistical analysis was performed using the SPSS version 20 software (IBM Corp.), with the exception of the number needed to treat (NNT), which was calculated using MedCalc v17.7.2 software (MedCalc Software bvba), and sample size and statistical power, which were calculated using the online calculator software by HyLown Consulting LLC to compare 2 proportions: 2-sample, 1-sided (http://powerandsamplesize.com/Calculators/Compare-2-Proportions/2-Sample-1-Sided) (68). P<0.05 was considered to indicate statistical significance. Sample size and statistical power were calculated for a one-tailed test. The remaining analyses were two-tailed tests.

Results

Patients and symptoms. A total of 242 patients were randomized and screened. Finally, 113 patients in the experimental group and 104 patients in the control group agreed to participate in the study. In the experimental group, 3 patients discontinued the intervention, leaving this group with 110 patients for the analysis (Fig. S1). Gender Ratio in the analyzed patients was 101.88 male per 100 female subjects. The mean ages of the experimental and control patients were 45.5±14.1 and 41.8±15.4 years old, respectively (P=0.073) (Table I). The major clinical characteristics and prescribed drugs are presented in Table I, exhibiting homogeneous characteristics between the groups (experimental vs. control) at the beginning of the study. The symptoms at baseline were also similar (Table SI).

The clinical severity distribution of all patients with SARS-CoV-2 infection in the present study according to the WHO interim clinical management guidance (53) was as follows: Mild (79.9%), moderate (8.9%) and severe (11.2%). The median reported symptom severity score, according to a self-assessment 10-point VAS, was 7 (interquartile range, 5 to 8), and the median number of COVID-19-compatible symptoms was 8 (interquartile range, 7 to 9 symptoms). None of the patients was asymptomatic or pauci-symptomatic, since all of them required specialized therapy with a healthcare professional. Therefore, in spite of the absence of any clinical or imaging signs suggestive of pneumonia, the patients were symptomatic.

Evaluation of clinical improvement and disease progression. The results were analyzed through two data grouping strategies. The control group (usual medical care) was compared with the experimental group, which included all dose levels of the experimental therapy. The other analyses compared the different dose levels of therapy between one another and with the control group, to determine the most efficacious therapeutic dose. In the control group, 19.2% of the patients had disease progression (hospitalization or death), compared with 7.3% of the patients receiving the experimental therapy, with a statistically significant difference in the Kaplan-Meier analysis with log-rank test (P=0.008). Fig. 1A shows that the group of patients that received electrolyzed saline had fewer hospitalizations, compared with the patients that received only the usual medical care. Regarding only the patients that were hospitalized, the time interval from inclusion in the study to hospitalization was lower in the control group compared with that in the experimental therapy group (4.5±1.3 days vs. 7.0±4.0 days, respectively; P=0.018) (see Table II). Death occurred in 8.7% of all the patients in the control group and 1.8% of the patients in the experimental group (P=0.025, Kaplan-Meier analysis with log-rank test) (Table II). Fig. 1B shows the proportion of patients achieving PASS. The mean time to PASS in the control group was 9.0±0.6 days, compared with 5.1±0.4 days in the experimental therapy group (P<0.001, Kaplan-Meier analysis with log-rank test). With respect to the different treatment schemes with electrolyzed saline, their effect on the severity of symptoms was dose level-dependent, with IV + nebulized administration being better than nebulized administration alone, but nebulized administration was better than usual medical care alone (Fig. 2).

The multivariate analysis indicated that in patients who received the experimental treatment, in addition to usual medical care, the risk of becoming hospitalized was reduced by 89% (adjusted RR=0.11, 95% CI: 0.03-0.37, P<0.001), the risk of death was reduced by 96% (adjusted RR=0.04, 95% CI: 0.01-0.42, P=0.007) and the probability of achieving an acceptable symptom state on day 5 was 18-fold higher (adjusted RR=18.14, 95% CI: 7.29-45.09, P<0.001), compared to usual medical care alone. The analysis also indicated the relationship between baseline characteristics of patients (such as sex, age, relevant comorbidities) and the probability of achieving an acceptable symptom state, or being hospitalized, or dying from COVID-19. It was observed that the presence of diabetes, advanced age or an SpO₂<94% were factors associated with an increased risk of being hospitalized or dying from the disease (Table SII). With the experimental therapy, the NNT to prevent hospitalization of a patient was 8.3 (95% CI: 4.7-32.6), one out of two patients treated with electrolyzed saline achieving an acceptable symptom state on day 5 or earlier (NNT=2.4; 95% CI: 1.90-3.52).

When the patients were classified according to the severity of their disease (Table II), it was observed that for patients with mild disease, treatment with electrolyzed saline significantly reduced the time to reach an acceptable symptom state compared with usual medical care alone (4.2±0.4 days vs. 7.2±6 days, P<0.001). Furthermore, for patients with moderate/severe disease, electrolyzed saline combined with usual medical care vs. usual medical care alone achieved a large decrease in the proportion of hospitalized patients (19% vs. 88%) and deaths (7.7% vs. 41%; Table II).

Progression of signs and symptoms. Table SI provides an analysis of the symptoms with respect to their presence or absence at the beginning of the study and throughout the follow-up. The number of patients with those symptoms at baseline did not differ between groups (except for sore throat and nausea, which were higher in the control group). The number of patients with fatigue, myalgia, fever, vomiting, conjunctivitis, dizziness, anosmia and/or ageusia was significantly reduced in the experimental group compared with the control
group during the follow-up time, but not at the baseline. This suggests that a proportion of patients were spared of certain symptoms during their illness due to the experimental treatment. With respect to patients with one particular symptom, the last day from enrolment they presented with fever (0.7±1.1 vs. 2.1±1.5, P<0.001), headache (3.7±3.5 vs. 7.3±4.3, P<0.001), fatigue (6.0±3.8 vs. 8.6±4.3, P<0.001), malaise (4.4±3.5 vs. 6.0±3.4, P=0.001), retro-orbital eye pain (2.1±2.9 vs. 3.9±3.6, P=0.001), chills (1.6±2.3 vs. 2.9±3.1, P=0.003), rhinorrhea (2.8±3.4 vs. 4.5±4.6, P<0.001), nausea (1.8±2.5 vs. 4.4±4.5, P<0.001), vomiting (0.7±2.0 vs. 1.7±2.4, P=0.026), dizziness (1.6±2.2 vs. 3.8±4.6, P<0.001), conjunctivitis (0.9±2.2 vs. 3.0±4.1, P<0.001), anosmia (5.7±3.2 vs. 8.9±4.8, P<0.001), ageusia (3.6±3.4 vs. 7.7±5.2, P<0.001) or diarrhea (4.0±3.1 vs. 5.9±3.9, P=0.025) was significantly lower in the experimental group vs. the control group (Table SI).

A quantitative analysis of the symptom severity score (patient overall self-assessment) was performed and score/values on various scales (10-point VAS) for fatigue, headache, sore throat, retro-orbital eye pain, myalgia, body temperature (degrees centigrade) and oxygen saturation (SpO2) exhibited a significant improvement in the experimental group at 24 h from the start of treatment (day 1) as compared with the control group. There was also a decrease in cough and heart rate on day 3 and arthralgia on day 9 (Table SIII).

Oral administration of electrolyzed saline to treat gastrointestinal symptoms was indicated in 17 patients, while 25 were prescribed gargling to treat a sore throat. All of these patients

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Control (n=104)</th>
<th>Experimental (n=110)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex (%)</td>
<td>52.9</td>
<td>46.4</td>
<td>0.412*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>41.8±15.4</td>
<td>45.5±14.1</td>
<td>0.073*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.6±4.7</td>
<td>28.6±5.1</td>
<td>0.136*</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>15.4</td>
<td>17.3</td>
<td>0.717*</td>
</tr>
<tr>
<td>High blood pressure (%)</td>
<td>15.4</td>
<td>18.2</td>
<td>0.715*</td>
</tr>
<tr>
<td>Asthma (%)</td>
<td>2.9</td>
<td>7.3</td>
<td>0.216*</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>11.5</td>
<td>12.7</td>
<td>0.837*</td>
</tr>
<tr>
<td>Progression timea</td>
<td>4.1±2.6</td>
<td>4.7±3.6</td>
<td>0.142*</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>37.3±1.0</td>
<td>37.4±0.8</td>
<td>0.718*</td>
</tr>
<tr>
<td>%SpO₂</td>
<td>95.1±2.8</td>
<td>94.3±3.1</td>
<td>0.077*</td>
</tr>
<tr>
<td>SpO₂ &lt;94% (%)</td>
<td>35.6</td>
<td>41.8</td>
<td>0.400*</td>
</tr>
<tr>
<td>Degree of dyspnea</td>
<td>1.2±1.5</td>
<td>1.2±1.4</td>
<td>0.956*</td>
</tr>
<tr>
<td>Symptom severityd</td>
<td>6.8±2.2</td>
<td>6.4±2.3</td>
<td>0.153*</td>
</tr>
<tr>
<td>Number of symptomsf</td>
<td>8 (7-9)</td>
<td>8 (6-9)</td>
<td>0.109*</td>
</tr>
<tr>
<td>Disease severityWHO (%)</td>
<td>83.7</td>
<td>76.4</td>
<td>0.390*</td>
</tr>
<tr>
<td>Mild</td>
<td>6.7</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>9.6</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>Treatments</td>
<td>2.8±1.6</td>
<td>2.7±1.5</td>
<td>0.822*</td>
</tr>
<tr>
<td>Paracetamol (%)</td>
<td>56.7</td>
<td>50.0</td>
<td>0.522*</td>
</tr>
<tr>
<td>NSAIDs (%)</td>
<td>57.7</td>
<td>60.0</td>
<td>0.291*</td>
</tr>
<tr>
<td>Ivermectin (%)</td>
<td>9.6</td>
<td>13.6</td>
<td>0.373*</td>
</tr>
<tr>
<td>Chloroquine (%)</td>
<td>7.7</td>
<td>3.6</td>
<td>0.325*</td>
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<tr>
<td>Antibiotics (%)</td>
<td>45.2</td>
<td>45.5</td>
<td>0.368*</td>
</tr>
<tr>
<td>Antivirals (%)</td>
<td>22.1</td>
<td>14.5</td>
<td>0.345*</td>
</tr>
<tr>
<td>Antihistamines (%)</td>
<td>14.4</td>
<td>13.6</td>
<td>0.591*</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>30.8</td>
<td>27.3</td>
<td>0.479*</td>
</tr>
<tr>
<td>Anticoagulants (%)</td>
<td>14.4</td>
<td>11.8</td>
<td>0.430*</td>
</tr>
<tr>
<td>Vitamins (%)</td>
<td>16.3</td>
<td>13.6</td>
<td>0.476*</td>
</tr>
</tbody>
</table>

* Determined by Fisher’s exact test; Student’s t-test; days from appearance of the first symptoms; Symptom severity score (patient overall self-assessment) using a 10-point visual analog scale; Mann-Whitney U-test; number of Coronavirus disease 2019-compatible symptoms, expressed as the median and interquartile range; likelihood ratio χ² test. WHO, disease severity as directed by the World Health Organization (53); BMI, body mass index, underweight (<18.5), normal (18.5‑24.9), overweight (25.0‑29.9) and obese (≥30.0); SpO₂, oxygen saturation determined by a pulse oximeter on the right-hand middle finger; NSAIDs, nonsteroidal anti-inflammatory drugs; antivirals, oseltamivir or amantadine; antibiotics, azithromycin, clarithromycin or levofloxacin.
reported a reduction or disappearance of symptomatology within 24-48 h of administration.

**SARS-CoV2 detection during treatment with electrolyzed saline.** Serial virus detection in nasopharyngeal and oropharyngeal samples at baseline and on days 2, 4, 6 and 9 was performed in 10 patients. As presented in Table III, >50% of patients were negative for the virus on day 4, with only a 20% of positive patients on day 6 and 0% on day 9. In the majority of cases, the test for the virus was negative on the days after having achieved a PASS. Of note, patient P30 achieved a PASS on days 3-5, but reported an unacceptable state on day 6 and a PASS on day 7 and thereafter. This suggests that a PASS does not always accompany the elimination of the virus (positive patients up to day 6) and that there may be a relapse of symptoms. Patient P29 achieved a PASS on day 2, was negative for the virus until day 6, when she was once again positive. Patients P29 and P30 were a couple who were living together, without implementing any physical distancing measures during follow-up, signifying that the probable cause of positivity on day 6 of P29 was due to transitory reinfec- tion or contamination derived from living with a patient still presenting with viremia (P30).

**Inflammatory and immune response markers.** The erythrocyte sedimentation rate was a parameter that remained elevated during the entire follow-up (Table SIV), with no significant differences between the baseline value and the 14 days of follow-up included. This was due to the fact that the maximum value reached by each patient exhibited marked variations over the days of follow-up. There was a significant decrease in C-reactive protein (CRP) 48 h after starting the treatment, with average reductions of 43 and 73% at 48 h and 4 days after the beginning of treatment, respectively (Table SIV). Considering the baseline CRP values and symptom severity score (possible score of 0-10 resembling very well to very poor) as 100% and the relative value on the subsequent days of evaluation, there was a significant correlation between CRP and the clinical progression of the patients (r=0.301, P<0.001). A greater decrease in CRP was associated with a greater reduction in the patient symptom severity score (reduced severity) (results not shown).

In relation to the baseline level of hematopoietic cells, there was a significant increase (within normal values) of total leukocytes on days 6, 9 and 14. The quantity of total lymphocytes gradually increased on days 2 and 4, until reaching significantly elevated levels on day 6 (Table SIV). The reactive lymphocytes exhibited a significant elevation on day 2 of follow-up, reducing and losing its statistical significance with respect to the baseline value on subsequent days. The quantity of large granular lymphocytes (a representation of NK cells) began to rise gradually, with a mean of 65±33x10^3/µl at baseline, until they were significantly elevated on day 6, with 155±78x10^3/µl (P=0.006), after which they began to decrease again. The quantity of total monocytes exhibited a tendency to gradually decrease, with no significant differences. However, the aberrant monocytes (larger cells, with clumped chromatin and basophilic cytoplasm) decreased significantly, with a mean of 450±357x10^3/µl at baseline, to 229±232x10^3/µl after 48 h (P=0.003). That decrease was sustained during the entire follow-up. The activated monocytes exhibited no significant changes with respect to baseline values during the follow-up. Another change was an increase in platelets, which, although they remained within normal ranges, they rose consistently throughout the follow-up, having significantly high values on days 6-14 (Table SIV).

The quantity of total monocytes correlated with the CRP levels (r=0.466, P<0.001). Of note, the quantity of aberrant monocytes correlated with the patients’ overall self-assessment score (symptom severity score; r=0.478, P=0.001),signifying that the more the aberrant monocytes decreased, the better the patient felt (results not shown). The gradual and significant increase of platelets after treatment correlated with several beneficial aspects, such as increased lymphocytes and clinical

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**Figure 1.** Kaplan-Meier curves indicating the progression of patients. (A) Shows that the group of patients that received electrolyzed saline had fewer hospitalizations (7.3%), compared with the patients that received only usual medical care (19.2%) (P=0.008). (B) Shows the proportion of patients achieving PASS. The mean time to PASS in the control group was 9.0±0.6 days, compared with 5.1±0.4 days in the experimental therapy group (P<0.001). The log-rank test was applied to compare curves.
improvement of the patients, given that the quantity of platelets correlated with the total lymphocytes (r=0.341, P=0.004) and with the patients’ overall self-assessment score (r=−0.398, P=0.001) (results not shown).

Testosterone and cortisol levels. The concentration of cortisol significantly decreased on day 2. On the other hand, the testosterone concentration increased, although there was no statistical significance. A significant increase in the testosterone-cortisol ratio was present on days 2 and 4 (Table SIV). The gradual and significant decrease in cortisol after treatment correlated with the decrease in CRP values (r=0.202, P=0.033), and with the increase in lymphocytes (r=−0.319, P=0.001), monocytes (r=−0.251, P=0.005) and platelets (r=−0.172, P=0.046), whereas the increase in the testosterone-cortisol ratio correlated with the decrease in activated monocytes (r=−0.272, P=0.019) (results not shown).

Adverse events and toxicity. A total of two patients did not tolerate the nebulization due to a burning sensation in the throat and stopped using it on the second day but continued with IV applications. In addition, four patients reported transitory dizziness lasting for 10 min after the IV application of the experimental solution; this was self-limited and managed by lying down. Furthermore, five patients reported mild pain in the first 5 cm of the vein path where the solution was applied after the entire treatment scheme. This mild pain was self-limited and not accompanied by any other signs or symptoms; it disappeared within 1 to 2 days after the end of the treatment. No other adverse events were reported. There were no abnormal or unexpected alterations due to COVID-19 in the serum levels of liver enzymes (ALT, AST, lactate dehydrogenase and ALP), bilirubin, albumin, glucose, creatinine, uric acid, urea or complete blood count (Table SIV).

Table II. Outcomes in the experimental and control groups of patients with Coronavirus disease 2019 according to WHO disease severity classification.

### A. All patients

<table>
<thead>
<tr>
<th>Item</th>
<th>Experimental (n=110)</th>
<th>Control (n=104)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days until PASS</td>
<td>5.1±0.4</td>
<td>9.0±0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PASS on day 5 (%)</td>
<td>79.8</td>
<td>39.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hospitalized (%)</td>
<td>7.3</td>
<td>19.2</td>
<td>0.008</td>
</tr>
<tr>
<td>Days to be hospitalized</td>
<td>7.0±4.0</td>
<td>4.5±1.3</td>
<td>0.018</td>
</tr>
<tr>
<td>Death (%)</td>
<td>1.8</td>
<td>8.7</td>
<td>0.024</td>
</tr>
<tr>
<td>Days to death</td>
<td>19.5±2.1</td>
<td>18.5±10.1</td>
<td>0.902</td>
</tr>
</tbody>
</table>

### B. Mild disease

<table>
<thead>
<tr>
<th>Item</th>
<th>Experimental (n=84)</th>
<th>Control (n=87)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days until PASS</td>
<td>4.2±0.4</td>
<td>7.2±6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PASS on day 5 (%)</td>
<td>84.5</td>
<td>46.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hospitalized (%)</td>
<td>3.6</td>
<td>5.7</td>
<td>0.380</td>
</tr>
<tr>
<td>Days to be hospitalized</td>
<td>6.2±3.8</td>
<td>4.0±0.7</td>
<td>0.250</td>
</tr>
<tr>
<td>Death (%)</td>
<td>0.0</td>
<td>2.3</td>
<td>0.257</td>
</tr>
<tr>
<td>Days to death</td>
<td>NA</td>
<td>27.5±3.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

### C. Moderate and severe disease

<table>
<thead>
<tr>
<th>Item</th>
<th>Experimental (n=26)</th>
<th>Control (n=17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days until PASS</td>
<td>7.8±1.4</td>
<td>18.5±1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PASS on day 5 (%)</td>
<td>65.4</td>
<td>5.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hospitalized (%)</td>
<td>19.2</td>
<td>88.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Days to be hospitalized</td>
<td>7.8±4.3</td>
<td>4.7±1.4</td>
<td>0.025</td>
</tr>
<tr>
<td>Death (%)</td>
<td>7.7</td>
<td>41.2</td>
<td>0.012</td>
</tr>
<tr>
<td>Days to death</td>
<td>19.5±2.1</td>
<td>15.2±8.9</td>
<td>0.548</td>
</tr>
</tbody>
</table>

Severity of disease was according to the WHO interim clinical management guidance (53). P-values were determined using Fisher’s exact test, except days for PASS, which was compared using log-rank test for comparison of Kaplan-Meier analysis. PASS, patient acceptable symptom state; WHO, World Health Organization. Days to be hospitalized and days to die: Time elapsed since the patient begins his treatment within the study until his hospitalization or death occurs. NA: Not analyzed because n=0 in one group.
In ambulatory patients with COVID-19 receiving the usual medical care, additional administration of electrolyzed saline reduced the probability of disease progression (hospitalization and death) by 89%, compared with ambulatory patients treated with usual medical care alone. Different signs and symptoms, such as fatigue, headache, sore throat, retro-orbital eye pain, myalgia, body temperature and oxygen saturation, improved significantly after the first 24 h of experimental therapy.

By adding neutral electrolyzed saline to the usual medical care, it was possible to significantly reduce the time to reach an acceptable state of symptoms in all patients, particularly in those with mild and severe disease. The greatest benefit of the treatment is observed in patients with moderate/severe disease, where a major change was observed in the proportion of patients who were hospitalized (19% vs. 88%) or died (7.7% vs. 41%), compared with the patients under usual medical care alone. The treatment was more effective when high doses (≥30 ml) of IV electrolyzed saline were administered. All dose levels of electrolyzed saline were significantly better in reducing the severity of symptoms than the usual medical care alone and the higher dose levels (dose level 7 + 8) were significantly better than just the nebulizations (dose level 1). The beneficial effects of the administration of electrolyzed saline may generally be associated with the mechanisms related to the following: i) Reduction of inflammatory processes; and ii) elimination of the virus by the immune system and by direct contact with the electrolyzed saline. The proposed mechanism of action is illustrated in Fig. 3.

The improvement of signs and symptoms correlated with a significant reduction of systemic inflammation, with a >40% decrease of CRP levels at 48 h after starting treatment. There was also a correlation between CRP levels and the quantity of monocytes. Said reduction, particularly of aberrant monocytes, was significant at 48 h and lasted to the end of follow-up, strengthening the hypothesis of the modulating effect of the systemic administration of electrolyzed saline on inflammation, reflected in the clinical improvement of the patients. In the early stage of COVID-19, CRP levels have previously been indicated to reflect the extent of lung lesions and disease severity, providing an important clinical evaluation index (69). Monocytes and pulmonary monocytes have a key early role in the progression to severe COVID-19 by promoting a cytokine storm, ARDS and disseminated peripheral tissue damage (14). The aberrant monocytes that decreased after the experimental treatment were larger than normal monocytes, with clumped chromatin and basophilic cytoplasm (62). Morphologically altered monocytes, particularly larger ones, are associated with a hyperinflammatory gene expression profile and with admission to intensive care units in patients with type 2 diabetes with COVID-19 (70). By contrast, with the reduction in the quantity and relative percentage of aberrant monocytes seen after the experimental treatment, the number of normal monocytes increased. Patients with a high number of normal monocytes have a better outcome, with earlier recovery and discharge from hospital (71). This result has been postulated to be relatively specific for COVID-19, as a similar pattern in patients with other viral illnesses, such as H1N1, influenza, HIV or hantavirus, has not been observed (71).

In relation to improved immune function, through the administration of electrolyzed saline, a gradual increase in total lymphocytes and large granular lymphocytes (a representation of NK cells) was observed, reaching a significantly elevated level on day 6. Lymphocytes have a crucial role in virus clearance after a viral infection. On the one hand,
NK cells eliminate virally infected cells via degranulation, receptor-mediated apoptosis and antibody-dependent cell-mediated cytotoxicity (72). On the other hand, the humoral immune response, primarily mediated by the production of antibodies by plasma B cells (B lymphocyte-derived cells), has a role in the neutralization of the virus (73). In line with the results of the present study, the lymphocyte count and the number of NK cells have been postulated to correlate with disease severity and may serve as a tool for identifying patients with a more severe clinical presentation of SARS and COVID-19 (61,69,74). A lymphocyte count of <1.5x10⁹/l may be useful in predicting the severity of clinical outcomes (75).

Figure 3. Proposed mechanism of action of the electrolyzed saline in patients with Coronavirus disease 2019. The systemic effect, generated mainly by intravenous application, has an immunomodulatory effect that reduces inflammation, with a reduction in aberrant and activated monocytes, as well as an increase in lymphocytes that help eliminate the virus. An increase in platelets and the testosterone-cortisol ratio, with a reduction in cortisol, contribute to this process. A local effect in the airways and digestive tract generates an anti-inflammatory, analgesic and tissue regeneration effect, with the inactivation of the virus by contact. All of these mechanisms contribute to clinical improvement.

Table III. SARS-CoV-2 detection over time in nasopharyngeal samples of 10 patients in the experimental group.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Dose level (years)</th>
<th>Progression time (days)</th>
<th>Score</th>
<th>WHO symptom score</th>
<th>Number of symptoms</th>
<th>Days until PASS</th>
<th>SARS-CoV2 detection result (days)</th>
</tr>
</thead>
</table>

Percentage of positivity (%)  

1 Patient code, followed by the letter ‘M’ for male, or ‘F’ for female. a Progression time, days from the appearance of the first symptoms; b symptom score (patient overall self-assessment), using a 10-point visual analog scale, from ‘very well’ (0) to ‘very poorly’ (10); c disease severity was defined according to the WHO (53); d number of Coronavirus disease 2019-compatible symptoms; e partner of P30, living together during entire follow-up. PASS, patient acceptable symptom state; WHO, World Health Organization; Neg., negative; Pos., positive; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.
Even though T lymphocytes were not specifically identified in the present study, the large granular lymphocytes observed are a type of T lymphocyte (14,61,62). Previous studies have indicated that the time of recovery of the T-lymphocyte count was fairly consistent with the clinical course (73). Patients with severe disease, but who recovered, the value of T lymphocytes was reported to begin to increase after 15 days of treatment, finally returning to normal levels after 25 days of treatment (73). By contrast, the level of T lymphocytes in severely ill patients and that finally deceased, continued to fall until they succumbed to the disease (73). That behavior concurred with the variation in the number of the large granular lymphocytes observed in the present study, in which that special type of lymphocyte increased on day 6 of treatment, in accordance with the clinical improvement of the majority of patients, and began to decrease in quantity on day 9. The speed with which the process of elevation and reduction in those cells took place should be considered.

Another relevant aspect was the constant and significant increase in platelets after treatment with electrolyzed saline. Yang et al (76) recently demonstrated an association between reduced platelets and mortality in patients with COVID-19. Yang et al (76) correctly interpreted those results as follows: i) A ‘higher’ platelet count for an illness as severe as COVID-19 is unusual and likely points towards liver activation and thrombopoietin release; ii) the lung-specific entry of SARS-CoV-2 suggests that the lung megakaryocytes, in response to liver thrombopoietin, locally produce a large number of platelets to help with the defense of the host; iii) the reduction of platelets in patients with severe disease may be due to the fact that the platelets are being consumed to form pulmonary thrombi, which occurs when multiple efforts (including those of the platelets) to stop the infection have not succeeded and blocking the viral invasion has become necessary; and iv) Yang et al (76) also indicated that mortality decreased with the increase of the platelet count, suggesting the thrombotic process has abated and platelets are no longer consumed into the clot. In addition, platelets also have an anti-inflammatory function by regulating macrophage activity, regulatory T cells and secreting pro-resolving mediators (77). All of those observations concur with the results of the present study, according to which the increase in platelets correlated with an increase in total lymphocytes and clinical improvement in the patients (a lower patient overall self-assessment score).

Cortisol and testosterone are hormones related to immune system regulation (78). The increase in testosterone detected in the present study (although not statistically significant) is in agreement with the result of a recent study reporting that low testosterone levels are associated with immune system deficiencies and greater severity of COVID-19 (79). Likewise, low levels of cortisol, as detected in the present study, correlated with increased lymphocytes, which may contribute to a better antiviral response by the body. It has recently been indicated that high cortisol levels are associated with a greater risk of death of patients with COVID-19 (80). Similarly, the present study reported an increase in the testosterone-cortisol ratio on days 2 and 4 after the beginning of treatment. This is a parameter not previously studied in patients with COVID-19, to the best of our knowledge. This increase was correlated with a reduction in activated monocytes, which may help reduce the systemic inflammatory process. Monocyte activation was abnormal and contributes to the COVID-19 cytokine storm by releasing massive amounts of pro-inflammatory cytokines (14,81).

The influence of testosterone and cortisol on monocytes has been previously reported. In patients with diabetes with hypogonadism, testosterone therapy reduced inflammatory activation of monocytes (82). It has also been indicated that cortisol signaling through the mineralocorticoid receptor, under oxidative stress, may promote monocyte inflammatory activation (83,84); thus, a reduction in cortisol would also be favoring the reduction of activated monocytes, particularly in the context of rising testosterone levels. Furthermore, based on the assumption that free testosterone is a marker of anabolism, while cortisol is indicative of catabolism, it has been suggested that an increase in the testosterone-cortisol ratio is favorable for protein anabolism (60,85), which may be beneficial in patients with COVID-19.

Electrolyzed saline, also known as electrolyzed water, has important antiseptic properties (86) and may be used directly on contaminated tissues or fluids (87,88). Thus, in addition to the immunomodulatory effect produced when administered systemically, it may inactivate the new coronavirus by degradation of the envelope and nucleocapsid proteins (89,90), when administered locally, without dilution to the lungs and throat, via nebulization and/or gargling, as it has been previously demonstrated for multiple viruses (87-90). However, the present study was the first to reveal the remarkable immunomodulating effect of electrolyzed saline administered systemically at the proper concentration of active species of chlorine and oxygen, acting to control and limit COVID-19 disease. Of note, all of the results of the present study concur with the proposed mechanism of rapid elimination of the virus from the respiratory tract, occurring within days, with negative virus test results in 60 and 80% of the patients on days 4 and 6, respectively.

Local administration of electrolyzed saline to the throat to control pain or its oral intake to control the gastrointestinal symptoms of nausea, vomiting or diarrhea, were successful in reducing or eliminating said symptomatology within 24 to 48 h, which is in accordance with previous preclinical studies (39,91). In fact, the company supplying the product utilized in the present study (Esteripharma S.A. de C.V) offers products for intranasal (EstériFlu®) and buccopharyngeal (Estericide® Bucofaringeo) applications, as antisepsics that inactivate viruses and eliminate bacteria. However, it is likely that electrolyzed saline, besides having a direct effect on the SARS-CoV-2 virus in the throat, also has an analgesic and regenerative effect on the epithelium at the local level (91). The oral route for electrolyzed saline has already been demonstrated to have no adverse effects in preclinical trials (40). Utilized in pigs to treat porcine epidemic diarrhea virus infection, the symptom duration in infected pigs was markedly shortened and symptom severity was also reduced, producing a much higher survival rate (39). The oral route for aqueous H₂, a component of electrolyzed saline, has potent local and systemic anti-inflammatory effects, along with regulating effects on the immune system (30), which may be involved in the mechanism for improving gastrointestinal symptoms.

The administration of electrolyzed saline has been indicated to have positive regulatory effects on the immune
Evidence of a direct impact of ROS on the life cycles of viruses is scarce and controversial. Numerous lines of evidence suggest that marked signs of increased production of ROS accompany all respiratory viral infections, which are associated with potentially pathologic processes including cytokine production, inflammation and cell death (92). However, none of the published data are based on direct measurement of ROS levels, but rather on their indirect determination (e.g. quantification of oxidated metabolites, which although is an accepted technique to evaluate ROS concentration, it continues to be an indirect determination) (92). In accordance with the results of the present study, the view that ROS contribute to the suppression of certain respiratory infections through the induction of innate immune responses, including T-cell receptor signaling and T-cell activation, is posited (92).

Examples of mechanisms that support the administration of ROS as beneficial in the fight against viral infections are as follows: i) Influenza virus enhances interferon λ1 (IL29) and λ2/3 (IL28A/IL28B) production via ROS (93). ROS scavenging or suppression of ROS production leads to the inhibition of IFNλ synthesis and secretion, and in turn, the enhancement of viral replication (92); ii) signal transducers and activators of transcription (STAT) activation has been indicated to be a relevant event in the response against different viruses (94). ROS formation is involved in STAT activation and the subsequent interferon regulatory factor 1 (IRF-1) and IRF-7 gene expression (95). IRF-1 has been indicated to have a role in shaping innate and adaptive antiviral immunity by inducing the expression of IFN-stimulated genes and mediating signals downstream of IFN-γ (95), contributing to the clinical improvement of patients with viral infection (96).

Antioxidant therapies are also known to ameliorate and improve disease outcomes (92). Since electrolyzed saline also contains small amounts of molecular H₂, additional antiviral and anti-inflammatory effects, associated to antioxidant mechanisms, may be expected (97). Treatment with molecular antioxidants reduces intracellular levels of influenza virus polymerase, providing a possible mechanism of viral titer reduction in response to antioxidant treatment (98). Additionally, it has been demonstrated that small antioxidant molecules, specifically molecular hydrogen, produce anti-inflammatory effects over multiple COVID-19 target organs, such as the lung, kidney, liver and brain, when compromised by acute and/or chronic diseases (97,99-102). For instance, a study suggested that intraperitoneal administration of hydrogen-rich saline to rats with ischemia/reperfusion-induced acute kidney injury, prevented fibrosis damage and improved renal function (99). The use of a hydrogen-enriched solution during hemodialysis therapies in patients diminished pro-inflammatory markers and prevented complications related to oxidative stress (100).

In conclusion, IV or nebulized administration of electrolyzed saline markedly reduced the symptomatology and risk of disease progression in ambulatory patients with COVID-19. Its administration was well-tolerated and there were no important adverse effects. The treatment effect was mediated by the reduction of inflammation and the apparently increased antiviral immune response, induced by the active species of oxygen and chlorine from the electrolyzed saline that appeared to mimic the effect of physiologic ROS. Further studies are required to confirm those results.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

IDE, JGE, VM and JMG designed the study and wrote the manuscript. IDE, BAPM and JP conceived the novel application of electrolyzed saline. CEBS, KAMR, CMR, MDM, EMZ, JATV, OGDE, JDE, MWG, HRGS, PDM, VM, PJMD, HPM, JMIV and LGG visited the ambulatory patients and administered their medication. CEBS, KAMR, DAMG, CMR, JATV, AEHR, IRPR, DTJ, IGV and VOR performed the biochemical and molecular analyses. VM, FEG, FRL, MJHM, JGE, LMFR, SAZF, PJMD, HPM, JMIV, FGA, LDL, HPGS, MAMH and EPDC performed the clinical evaluations of the patients. EBV, MLMF, MR, GGS, CRMP and IDE designed and performed the statistical analysis. BAPM and ACL coordinated the production and quality control processes of the experimental therapeutic product. EDJM coordinated and authorized the recruitment of patients at the INSABI Poliforum hospital. JDE was the administrative coordinator of the clinical trial. IDE and JGE checked and approved the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study (registered 2020-01-05) was approved by the ethics committee of the School of Medicine of the Universidad de Colima (Colima, Mexico) and written informed consent was obtained from all participants. All procedures performed in the present protocol were in accordance with the Declaration of Helsinki and the clinical trial was registered as TX-COVID19: RPCEC00000309 in the RPCEC database (05/05/2020).

Patient consent for publication

Not applicable.

Competing interests

BAPM and ACL declare that they work for the company Esteripharma, who provided the neutral electrolyzed saline administered in this trial. The company owns a patent for the synthesis of the electrolyzed saline, but had no role in the study design, data collection and analysis or decision to publish the manuscript. Those authors did not participate in the study design, data collection or data analyses. The other authors declare that they have no competing interests.

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