

Nebulized enriched heparin improves respiratory parameters in patients with COVID-19: a phase I/II randomized and triple-blind clinical trial.

This supplement contains the following items:

1. Supplementary protocol (Page 2-7)
2. Summary of changes in the protocol from the original protocol (Page 8)

Supplementary Trial protocol

Inclusion criteria

Eligibility criteria are as follows:

- Sign and agreed to the Free and Informed Consent Form ¹;
- Aged between 18 and 90 years old, regardless of ethnic origin or gender;
- Been diagnosed with COVID-19 confirmed by RT-PCR of a nasopharyngeal swab;
- Presented symptoms of COVID-19 for a period of less than 14 days before inclusion in the study;
- Under hospital treatment with a gas exchange ratio ($\text{paO}_2/\text{pFiO}_2$) above 200, characterizing mild hypoxemia.

Exclusion Criteria

Exclusion criteria are as follows:

- Disagree with the terms of the study;
- Moderate or severe respiratory failure requiring ICU admission requiring invasive mechanical ventilation or positive pressure noninvasive ventilation (NIV);
- Pregnant or puerperium;
- Presented an unfavorable clinical evolution requiring orotracheal intubation within 48 hours after inclusion in the study;
- Hematological diseases;
- Coagulation disorders;
- Previous use of anticoagulants in therapeutic dosages;
- Previous heparin-induced allergy, thrombocytopenia or current thrombocytopenia with a count of less than 50,000 platelets/mm³. Have an allergy to heparin or a history of heparin-induced thrombocytopenia, thrombocytopenia with a count below 50,000 platelets/mm³.

Discontinuation Criteria

Discontinuation criteria are as follows:

- Voluntary withdrawal of consent by the participant, regardless of the reason;

- Loss to follow-up, for any reason, during the study;
- Occurrence of any type of severe adverse events, defined in accordance with the standards of the Common Terminology Criteria for Adverse Events (CTCAE v5.0)².
- If COVID-19 is not confirmed by RT-PCR within 72 hours of the inclusion, the patient will be withdrawn from the study.

Complementary Methods

Cells treatment

The African green monkey kidney cell line (ATCC Vero CCL-81) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% sodium pyruvate (Sigma-Aldrich Co., Deisenhofen, Germany) and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C. 96-well plates were seeded with these cells and considered ready for use when they reached 90-100% confluency. A clinical isolate SARS-CoV-2/SP02/human/2020/BRA (GenBank accession number MT126808.1) was propagated in Vero cells, and the viral titer was determined by the 50% tissue culture infectious dose (TCID₅₀), as previously described by Araujo and collaborators³. We conducted all infection experiments in a biosafety level 3 (BLS-3) laboratory at the Institute of Biomedical Sciences at the University of São Paulo, São Paulo, Brazil, following laboratory biosafety guidelines related to the novel coronavirus (2019-nCoV) by WHO. Tests were performed with enriched heparin versus conventional unfractionated heparin and control. Each heparin was diluted in DMEM to a final concentration of 100 µg/mL.

Cell viability assays

Cell viability was evaluated using a colorimetric assay by quantifying lactate dehydrogenase (LDH) released into the culture supernatant from cells with damaged membranes, using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corp., Madison, WI). Detection was performed using a microplate reader (POLARstar® Omega, BMG LABTECH, Ortenberg, Germany)

at 492 nm. The activity of the released LDH was reported as a percentage of the total cellular LDH (measured after the complete lysis of control cells corresponding at the maximal amount that can be released by cells, therefore 100%). Cell viability was normalized to untreated cells. Viability below 70% was used as a threshold for cytotoxicity.

Antiviral activity of heparin during different time-of-addition

Vero CCL-81 cells were seeded in a clear-bottomed 96-well plate (5×10^4 cells/mL) and incubated for 24 h at 37 °C for cell adherence. Then, they were treated with 100 µg/mL of heparin at different stages of virus infection as described below. Three different time-of-addition were evaluated,5 compromising absorption (AD), post-infection (PI), and adsorption plus post-infection, named full-time (FT). For AD treatment, the heparin was added to the cell's monolayer for 1 h before virus infection and maintained for 1h for the viral attachment process. Then, the virus-heparin mixture was replaced with fresh DMEM until the end of the experiment. During the PI experiment, the virus was added to the cells to allow infection for 1 hour, and then virus-containing supernatant was replaced with 100 ug/mL of heparin-containing medium until the end of the experiment. For FT treatment, Vero CCL-81 cells were pretreated with heparin for 1 h before virus infection, followed by incubation with the virus for 1 h in the presence of the compound. Then, the virus mixture was removed, cells were and cultured with the same concentration of heparin-containing medium until the end of the experiment. For all experimental groups, cells were infected with the virus at a multiplicity of infection (MOI) of 0.02, and at 72 h.p.i. cell supernatants were collected for RT-qPCR, and cells were screened for the presence/absence of cytopathic effects (CPE) under an optical microscope (Olympus, Tokyo, Japan).

Cells nucleic acid extraction, Nasopharyngeal swab, and RT-qPCR for COVID-19.

For cells RT-qPCR evaluation was used the semi-automated NucliSENS® easyMag® platform (BioMerieux, Lyon, France), extraction of total nucleic acid was performed in cell culture supernatant, following the manufacturer's instructions. The quantification of viral RNA was done using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Weiterstadt, Germany) on an ABI 7500

SDS real-time PCR machine (Applied Biosystems) using primers and probe for E gene⁴. Numbers of RNA copies/mL were quantified using a specific in vitro-transcribed RNA quantification standard, as described previously⁵.

Samples from patients' nasopharynx were collected using a swab. RNA was extracted from the swab and RT-qPCR was performed using the Applied biosystems 7500 Real Time PCR system (Thermo Fisher Scientific, Massachusetts, USA) to detect SARS-CoV-2 viral RNA. Viral load quantification was performed using the TaqMan™ SARS-CoV-2 Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), which follows real-time quantitative amplification methodology and has a detection limit of 10 copies/mL. The results were expressed in terms of positivity or negativity and the viral load was quantified by comparison with a synthetic RNA calibration standard, using the standard curve generated by the manufacturer. Viral load results were expressed in copies/mL, and subsequently transformed into logarithms for statistical analysis.

Complete Blood Count (CBC)

Blood samples were analyzed using flow cytometry, impedance, spectrophotometry and parameters calculated by the Yumizen H2500 hematological analyzer (Horiba, Kyoto, Japan). Were considered normal values: hemoglobin of 13.5 to 17.5 g/dL, hematocrit of 41 to 53%, MCV of 80 to 98 μmm^3 , RDW of 10 to 20%, platelets of 140 to 440 $\times 10^3/\text{mm}^3$, leukocytes (white blood cells) from 4 to 11 $\times 10^3/\text{mm}^3$, lymphocytes from 23 to 33 % and neutrophils from 53 to 67 %.

Coagulation profile

The coagulation analysis methods were used using Destiny Max analyzer (Tcoag, Ireland) to determine the aPTT and INR ratio levels. The value considered normal for aPTT was < 1.25 and for INR < 1.2 .

D-Dimer

Blood d-dimer quantification was performed using the CS2500 automated analyzer (Sysmex Corporation, Kobe, Japan), following the standards

established by the manufacturer. The normal value used to interpret the results was < 500 ng/dL.

Blood gas analysis

Blood samples were collected through manual aspiration and stored in 1 mL syringes. Oximetric parameters were evaluated in whole blood using a RAPIDPoint® 500 analyzer (Siemens, Erlangen, Germany). To interpret the results, reference values were used for pH between 7.35 - 7.45, pO₂ 80 - 100 mmHg, pCO₂ 35 - 45mmHg and bicarbonate between 22 - 26 mEq/L.

Biochemical exams

Biochemical analyzes of serum samples were performed to determine the levels of urea, creatinine, C-reactive protein, amylase, total proteins and their fractions (albumin and globulin). The quantifications were performed using colorimetric and potentiometric methods on an automated biochemical analyzer VITROS® XT 7600 (Ortho Clinical Diagnostics, New York, USA). The references values were: urea (19 to 42 mg/dL), creatinine (0.66 to 1.25 mg/dL), C-reactive protein (< 1 mg/dL), total proteins (6.3 to 8.3 g/dL), albumin (3.5 to 5.0 g/dL) and amylase (25 to 125 U/L).

Inflammatory cytokines

Cytokines were quantified using the commercial kit LEGENDplex™ COVID 19 Cytokine Storm Panel 1 (14-plex), which allows the simultaneous quantification of 14 different cytokines, including IL-6, MCP-1 (CCL2), G-CSF, IFN-α2, IL-2, IFN-γ, IL-7, IL-1RA, IL-8 (CXCL8), TNF-α, IP-10 (CXCL10), MIP-1α (CCL3), RANTES (CCL5) and IL-10. The reagents, standard curve and washing buffer solution were prepared following the manufacturer's instructions. Plasma samples were previously diluted (1:2) in Assay Buffer. In a 96-well microplates were added 25 µL of samples or each point on the standard curve, with 25 µL of assay buffer in the samples wells, and 25µL of matrix B in the standard curve wells. Next, capture bead mix (25µL) was added to all wells. The plates were sealed and protected from light for incubation under gentle agitation for 2 hours at room temperature. Subsequently, they were centrifuged for 5 minutes at 250 xg, and inverted to remove the supernatant, adding 200 µL of wash buffer (1x) to

each well, followed by incubation under shaking for 1 minute to repeat the washing process. After washing twice, 25 μ L of detection antibody was added to all wells, the plates were sealed and protected from light for incubation under gentle agitation for 1 hour at room temperature. After this period, 25 μ L of SA-PE reagent were added to each well and incubated for 30 minutes at room temperature, under slight agitation, protected from light. The process of centrifugation and removal of the supernatant was repeated, then adding 200 μ L of wash buffer to each well, incubating for 1 minute, carrying out a two-step washing process. Samples were resuspended in 150 μ L of buffer and transferred from the plate to falcon tubes for reading on the FACSCalibur-4 color cytometer (Becton Dickinson). The mean fluorescence intensity of each analyte is directly proportional to the cytokine concentration and the results were expressed in pg/mL. Data were analyzed using the LEGENDPlex v8.0 software.

Chest Computed Tomography

To perform the chest computed tomography, the Toshiba Activion 16 tomograph (Toshiba Medical Systems Corporation, Tochigi, Japan) was used with a specific lung evaluation protocol for patients with suspected or confirmed COVID-19. The protocol included thin 1 mm axial sections, without contrast, from the apex of the lungs to the bases, with multiplanar reconstruction in axial, coronal and sagittal projections. The images were analyzed by a single specialized radiologist. In the evaluation of the chest tomography, the radiological parameters of presence or absence of Ground-glass opacification, consolidation, linear opacities and congestion were considered. Furthermore, a percentage score (0 to 4) was used to measure lung impairment in relation to abnormal areas, being: 0 – absence of lung impairment; 1 – less than 25% of the lung area impairment; 2 – between 25% and 50%; 3 – between 50% and 75%; and 4 – above 75% of the lung area impairment.

Summary of Changes in Protocol

The trial protocol for this study was previously published as follows in Bertanha et al. (2021)¹. However, some details from the initial protocol have changed as follows:

Changes made	Excluded	Included
Eligibility criteria	<ul style="list-style-type: none"> - Include patients with a strong suspicion of COVID-19, according to compatible clinical and radiological findings. - Demand for supplemental oxygen therapy (O₂) less than 5 L/min. 	
Exclusion criteria	COVID-19 vaccinated patients.	Patients who received the first dose of the COVID-19 vaccine.
Outcomes	Primary outcome: Efficacy related to the proposed treatment, by the analysis of the SARS-CoV-2 viral load of the participants treated for 7 days, by the sequential assessment of RT-PCR in the nasal swab.	Secondary outcome: Efficacy related to the proposed treatment, by the analysis of the SARS-CoV-2 viral load of the participants treated for 7 days, by the sequential assessment of RT-PCR in the nasal swab.
Statistic plan	Multivariate analysis will be evaluated with regression Cox analysis and long-term clinical analyzes will be compiled on Kaplan–Meir curves.	<ul style="list-style-type: none"> - Qualitative variables: Chi-squared tests. For paired data, the McNemar test or Chi-square test of independence. - Quantitative variables: unpaired t-tests or paired t-tests for parametric distribution. For the longitudinal analysis comparing moments D0, D2, D5, and D7, One-way ANOVA, followed by Tukey's post-test was applied for parametric distributions, and the Kruskal-Wallis test was applied for non-parametric distributions, followed by Dunn's post-test.

References

1. Bertanha, M. *et al.* Nebulized enriched heparin to treat no critical patients with Sars-Cov-2. *Medicine (Baltimore)*. **100**, e28288 (2021).
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5. Machado, R. R. G. *et al.* Inhibition of Severe Acute Respiratory Syndrome Coronavirus 2 Replication by Hypertonic Saline Solution in Lung and Kidney Epithelial Cells. *ACS Pharmacol. Transl. Sci.* **4**, 1514–1527 (2021).