Development, Implementation, Pharmacokinetic and Safety Evaluation of an Immunotherapeutic Treatment for COVID-19: Double-blind Randomized Placebo-controlled Trial

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Abstract: Passive immunotherapy has been evaluated in many infections. The present study aims to evaluate purified $F(ab')_2$ fraction of equine hyperimmune IgG (anti-SARS-CoV-2) in the treatment of coronavirus lung disease. Patients with coronavirus disease of 2019 (COVID-19) with World Health Organization (WHO) score 3, 4 or 5 up to 72 hours of evolution from the onset of symptoms were included. They were randomly assigned to anti-SARS-CoV-2 or placebo. Follow-up was performed for 28 days to assess efficacy, safety, pharmacokinetics, detection of anti-horse antibodies, circulating cytokines and determination of anti-SARS neutralizing activity. The 20 initial patients (44±14 years) were included. On the third day of treatment there was an improvement (P=0.02) in arterial saturation (95±1.6 vs. 93±2.5%) with increasing differences over time between treatments (day 8: 97±0.1 vs. 94±0.3%). The length of oxygen therapy treatment was 2±0.8 vs. 3±0.9 (0.048) in patients falling within WHO 5 category (no difference to WHO 4). Mean hospitalization was 13±2.5 vs. 14±0.8 days (P=0.095) and time to clinical improvement was 2±0.5 vs. 3±0.9 days (P=0.048) in patients with initial 5 WHO category, with no differences to patients who started with WHO stage 4. The time to nasal swab negativization was 10±2.1 vs. 12±0 day (P=0.015). No adverse reactions or intercurrences were detected. All patients presented heterophile antibodies without clinical correlate. The new treatment shows improvement in arterial saturation (days 3 to 12), and a decrease on detectable viral RNA (days 8 to 11) with good pharmacokinetic and safety profile.

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Introduction

The coronavirus pandemic caused damages in social, economic and health terms. Meanwhile, even after a decade of coronavirus research, there are still no licensed therapeutic agents. In early January 2020, Kruse (2020) advocated for therapeutic strategies in an outbreak scenario to treat the novel coronavirus originating from Wuhan. He recommended starting with options based on knowledge of immunology, to combat SARS-CoV-2 (severe acute respiratory syndrome linked to Coronavirus type 2) and treat patients under compassionate use, while conducting formal clinical trials, including the use of convalescent plasma as a potential therapy for COVID-19 (Bloch et al., 2020).

Passive immunotherapy is a well-established historical procedure introduced by von Behring in 1890, as a cure for diphtheria and tetanus using antibodies isolated from horse blood (Klein, 1894). Von Behring received the Nobel Prize in Physiology and Medicine in 1901 for his work. This approach was used with success in other major epidemics, such as the Spanish flu in 1918 (McGuire and Redden, 1918), the 1934 measles epidemic in the United States of America (le Fleming, 1937), most recently during the Middle East Respiratory Syndrome (MERS) epidemic in the Middle East in 2012 (Ko et al., 2018), SARS of viral etiology (Mair-Jenkins et al., 2015) and against Ebola in 2015 (Racine et al., 2019; Fischer et al., 2020). The strategy of using convalescent plasma in SARS-CoV-2 infection is based on the same therapeutic principle of passive immunization to neutralize the virus (Abolghasemi et al., 2020).

Immunology clearly demonstrates that antibodies in blood or plasma fraction recognize epitopes of pathogens (e.g. viruses). They neutralize or reduce the virus load along with cellular responses to prevent or eventually cure disease; therefore, antibodies are very efficient endogenous molecules to start the healing process in the human body.

For more than 100 years, the extraction and transference of specific antibodies directed against certain antigens to infected people as a passive immunization is an established therapeutic alternative for many diseases such as diphtheria, rabies, tetanus, and the Ebola virus (Fischer et al., 2020).

Although in some infectious diseases such as influenza, the most recent clinical studies did not show evidence of benefit with passive immunotherapy (Beigel et al., 2019; Davey et al., 2019). A subsequent analysis has shown that these studies presented some methodological and conceptual errors (Kanjilal and Mina, 2019). For example, both studies used material (immunoglobulins or plasma) from healthy individuals, with no history of recent infection, and therefore possibly possessing lower antibody titters, raising the necessity of the assessment of neutralizing activity. Beigel's study evaluated plasma defined as "high titter" (1:80) and Davey's study evaluated the use of high-titter immunoglobulin (quantified as amount of immunoglobulins or by affinity to viral components but not by neutralizing activity). The latter showed benefit against influenza B, against which it had higher affinity, showing that the immunoglobulin concentration is not a good unit of measurement to correlate with the neutralizing activity. Further, inhibition titters are generally considered protective if they are much higher than 1:80, with some evidence indicating that values higher than 1: 50,000 are necessary. For the periodic viral infections, the quality, rather than the quantity of antibodies, is more likely to drive efficacy (Kanjilal and Mina, 2019). Several studies have pointed out the importance of evaluating the preparations used in passive immunization through neutralization activity with a neutralizing titter ≥ 1:80 (FDA, 2023), being neutralization by ELISA IgG a valid substitute (Ko et al., 2018).

SARS-CoV-2 is a new coronavirus that poses a global threat and places unprecedented burdens on healthcare providers and the healthcare system. Until now, there is no specific and effective antiviral therapy against COVID-19 disease. From an immunological point of view, antibodies collected from patients who have recovered from COVID-19 show neutralizing activity (NAb). Passive immunization represents an alternative treatment until other drugs become available (Bloch et al., 2020). Meanwhile, the rebirth of passive immunization could be a bridge technology until effective drugs or active immunization are available (Chen et al., 2020; Duan et al., 2020; Keith et al., 2020; Roback and Guarner, 2020; Tanne, 2020; Tiberghien et al., 2020; Ye et al., 2020). Shen C. and colleagues from China were the first to report that convalescent plasma could be a treatment option for COVID-19 patients with respiratory failure (Bloch et al., 2020). Passive immunization improved the clinical situation in 5 patients where antiviral drugs or steroids were not effective. The patient's viral load decreased and became negative within 12 days after the transfusion (Bloch et al., 2020). One problem with this report was that all patients received antiviral drugs and steroids before receiving their convalescent plasma. The last treatment was done as compassionate use as no other treatment therapy worked. Therefore, the results are very difficult to interpret. Within days of this initial report, a case report from Korea showed that 2 elderly patients improved after convalescent plasma application (Ahn et al., 2020). One of the patients was a 71-year-old

man with no underlying medical conditions who was initially treated with antimalarial drugs and who needed respiratory assistance for severe pneumonia. His condition improved when he was treated with convalescent plasma from a young patient, along with steroids. The second patient, a 67-year-old woman, did not respond to initial treatments that included chloroquine, remdesivir, and oxygen therapy. She began to recover after receiving plasma and steroid therapy at the same time. A study (Duan et al., 2020) in 10 severely ill SARS-CoV-2 individuals who were transfused with 200 ml of convalescent plasma with specific neutralizing antibody titters greater than 1:640 in addition to standard supportive care, showed that the therapy was well tolerated, and it could improve clinical outcomes by neutralizing viremia. In a case series from Wuhan, 1–3 convalescent plasma infusions were also well tolerated and effective, in addition to being associated with negative pharyngeal swab (Klein, 1894; Bloch et al., 2020).

The Food and Drug Administration (FDA) has approved convalescent plasma as a treatment option for critically ill patients with COVID-19 (Tanne, 2020). The optimal dose and time point of application, as well as the clinical benefit of convalescent therapy, needs further investigation in larger, well-controlled trials. Even so, the administration of convalescent plasma has some drawbacks, such us availability of plasma and donors, the risks inherent in any transfusion (circulatory overload, acute lung injury, allergic and anaphylactic reactions, transmission of infections, non-hemolytic febrile reactions, red cell alloimmunization, transfusion hemolytic reaction), some donors develop low neutralizing antibody titters (material received from different individuals is not homogeneous). Similarly, there is no clear guideline of the minimum neutralizing value with verifiable clinical efficacy, although the FDA recommends: "When the measurement of neutralizing antibody titters is available, we recommend neutralizing antibody titters of 1:160. A 1:80 titter may be considered acceptable if an alternative matching unit is not available" (FDA, 2023).

High-dose polyclonal human immunoglobulin (25 grams per day) was also used, obtaining improved respiratory function in a case series (Cao et al., 2020). As an alternative approach (Wang et al., 2005; Luo et al., 2007; Zhou et al., 2007), antibodies of equine origin, with known safety and pharmacological properties (Klein, 1894; McGuire and Redden, 1918; Bal et al., 2015), present some advantages: greater availability, higher neutralizing titter, better homogeneity, avoiding the risk of transmission of various pathogens (human immunodeficiency virus – HIV, hepatitis B or C, etc.), and reducing the incidence of adverse reactions associated with the product after enzymatic digest which cleave the Fc fragment.

This study aims to evaluate the initial safety, pharmacokinetics, and efficacy of a hyperimmune equine serum with neutralizing activity against the SARS-CoV-2 virus.

Material and Methods

Trial design and investigational product

An adaptive phase 2/3, double-blind, parallel (1:1), placebo-controlled, multicenter clinical trial that analysed the pharmacokinetics, safety and efficacy of a sterile injectable solution product of purified $F(ab')_2$ fraction of horse IgG anti-SARS-CoV-2 (investigational product), manufactured under Good Manufacturing Practices (GMP) conforming to the guidelines recommended by ANMAT (National Regulatory Authority or National Drug Regulatory Agency), in the ANLIS Production Factory approved by ANMAT. The equine hyperimmune serum was generated from antigenic stimulation with the SARS-CoV-2 receptor binding domain (RBD) purified protein.

Participants

Hospitalized adult patients were eligible if they presented: (1) over 18 years old and under 80 years old; (2) positive results by RT-PCR for SARS-CoV-2; (3) clinical picture compatible with respiratory compromise in the form of pneumonia attributed to COVID-19 – stage 3, 4 or 5 according to the World Health Organization (WHO) scale – lasting up to 72 hours from the onset of symptoms to their evaluation to be incorporated into the study; (4) patients with good disposition towards the study and that signs the informed consent.

Exclusion criteria were: (1) patients with clinical disease corresponding to mild/asymptomatic forms (absence of radiological infiltrate and risk factors, with normal auscultation and arterial saturation of oxygen [$SatO_2$] greater than 95%); (2) patients with clinical disease corresponding to severe forms (severe pneumonia: presence of severity criteria [according to American Thoracic Society and Infectious Diseases Society of America – ATS/IDSA], one of two major or three minor criteria); (3) patients who have received other therapeutic strategies in the framework of an experimental study that make it difficult to evaluate the results obtained; (4) pregnant or lactating women; (5) women of childbearing potential not using an effective contraceptive method; (6) history of severe anaphylactic reaction with the administration of equine plasma; (7) patients with comorbidities that

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justify a risk of high mortality from causes independent of SARS-CoV-2 infection (e.g. stage IV cancer); (8) patients who do not consent to participate.

Although the clinical study is designed as multicenter, the initial phase of the study (first twenty subjects) was carried out in a single care center (data were collected in Emergency Department of Hospital Santojanni).

Interventions

The "treatment group" received the administration of two doses of 10 ml of an investigational product (sterile injectable solution with neutralizing activity of SARS-CoV-2 not less than 1/5120), by slow intravenous infusion (10 ml diluted in 100 ml of physiological solution, administered during 50 to 60 minutes by slow drip), at time 0 when incorporated into the study – initial dose – and after 48 hours – second dose.

The "control group" received the administration of 10 ml of a sterile injectable saline or physiological solution with no-neutralizing activity of SARS-CoV-2 (also manufactured under Good Manufacturing Practices conforming to the guidelines recommended by ANMAT [National Regulatory Authority], in the ANLIS Production Factory approved by ANMAT), administered in similar conditions.

Outcomes

The primary objective was to demonstrate the efficacy and safety of the purified $F(ab')_2$ fraction of equine hyperimmune serum (anti-SARS-CoV-2). The primary efficacy endpoint was the change in time needed to clinical improvement, during 28 days after the assignment (time).

Secondary outcome measures include: (1) change in the number of patients in each (WHO) ordinal scale category (0 to 8 being 0 better and 8 worse) (days 7, 14 and 21 post-inclusion); (2) change in mortality rate (28 days); (3) change in mechanical ventilation requirement rate (28 days); (4) change in duration of oxygen treatment requirement (28 days); (5) change in length of hospitalization (28 days); (6) change in frequency of nosocomial infections (28 days); (7) change in lymphocyte cell count (28 days); (8) change in viral RNA negativization rate on nasopharyngeal swab test (at 7, 14, 21, and 28 days); (9) description of adverse events type and frequency (28 days); (10) requirement of additional treatments for adverse drug reactions (28 days); and based on quantification of purified $F(ab')_2$ anti-SARS-CoV-2 on different times (basal, 1, 3, 6, 24, 48, 49 and 96 hours, and on days 7, 14, 21 and 28) describing the pharmacokinetics in terms of (11) area under the curve (AUC); (12) maximum plasma concentration (C_{max}); (13) elimination half-life (t1/2); (14) elimination constant (Ke) (16).

Safety outcome measures included: number and type of adverse events, seriousness classification occurred during the 28-day follow-up period, discontinuation or suspension of infusions, and laboratory values abnormalities.

Sample size

The original sample size was determined as 200 patients (100 individuals for each group), which will allow reaching a statistical power (1-beta) of 80% with a level of significance (2 tails) of alpha = 0.05, to detect an 8-day change in time to clinical improvement in the intervention group (Grasselli et al., 2020; Onder et al., 2020). The study will be a phase 2/3 adaptive research. First, 20 subjects (1:1 ratio) were staggered, one patient at a time, so that no new patient should be included until at least 24 hours after the second administration of the last patient included. The Data Monitoring Committee for Patient Safety (CMDSP) review the safety data and interim analysis was proposed after 20, 50, 100 and 150 patients have been included in the study (given the 1:1 randomization in blocks of 10, it will correspond to the incorporation of 25, 50, and 75 patients for each branch). The current manuscript presents the partial results corresponding to the first 20 subjects of the adaptive study, where the pharmacokinetic study was carried out.

Randomization

Sequence generation: permuted block randomization to receive active treatment (purified $F[ab']_2$ fraction of equine hyperimmune serum with anti-SARS-CoV-2 neutralization activity) or placebo was performed for each block of 10 patients with a mixed 1:1 allocation. Randomization was performed through an allocation system based on random manual draw. Based on randomization, the study medication was prepared in such a way that each treatment (numbered from 1 to 200) had an indistinguishable label in terms of its content (active treatment or placebo) and with a label that clearly details the clinical study for which it will be used, the name of the producing laboratory and the number of the patient who should receive it. The patients were incorporated successively following the numerical order and receiving the treatment that was previously assigned by their number through a random mechanism.

Allocation concealment mechanism: treatment allocation was kept hidden from the professionals involved in the treatment of the patients. An envelope sealed with security sealing wax was kept in the health center guarded by a person not linked to patient care

so that in the event of an eventual need to unmask treatment in an emergency, it could be carried out.

Implementation: random allocation sequence was generated by independent personnel caring for patients. The blinded enrolment and allocation of treatment was carried out by the research team.

Blinding

The treatment was blinded for the patients, the researcher, his team and all the professionals involved in the care of the patients, as well as the personnel who carried out the determinations in laboratory samples. Active treatments (investigational product: sterile injectable solution with purified $F[ab']_2$ fraction of equine hyperimmune serum with anti-SARS-CoV-2 neutralization activity) and placebo (control solution: sterile injectable saline or physiological solution without neutralizing activity for SARS-CoV-2) were prepared in similar volumes, vials of the same characteristics and labelled in such a way that the presence or absence of the active treatment cannot be differentiated.

Ethics and trial evaluation

The study was approved by the hospital's independent ethics committee (registry identifier PRIISA.BA 2578), authorized by the National Drug Regulatory Agency (ANMAT DI-2021-2196-APN-ANMAT#MS), registered in the National Registry of Health Research (identifier: RENIS IS003268), and clinicaltrials.gov (NCT04913779).

Study procedures

After the detection of a case that meets the inclusion criteria, information was presented to the candidate in a clear way, with simple language, providing space for questions and giving the necessary time to make the decision to enter the study or not.

In the case of accepting to participate, and after signing the informed consent, a sequential number of participations in the study was assigned, immediately correlative to the last assigned number, which would determine, according to the previously designed randomization table, the treatment (blind) received for each patient. The treatment was blind for the patient, investigator and the treating medical team, who only had access to the medication previously labelled for that assigned patient number. Baseline clinical and laboratory variables were obtained and lung X-ray and/or tomography performed. Treatment was administered as infusion of a vial of 10 ml of treatment diluted in 100 millilitres of physiological solution to pass intravenously in a slow drip for 1 hour. The vial could correspond (blinded treatment) to 10 ml of placebo or control solution or 10 ml of investigational product. A second dose of treatment, with the same posology, was administered 48 hours after the first. Once the first administration was finished, the participating research subject was checked again frequently during the following 6 hours and the corresponding samples were extracted from the first 20 subjects (at time 0 min, 1 h, 3 h, 6 h, 24 h, 48 h, 49 h, 96 h, 7 days, 14 days, 21 days and 28 days) for kinetic analysis. After the treatment, the participating research subject was closely monitored by the research staff evaluating: 1) clinical status of the patient according to ordinal point scale, vital signs and general clinical assessment, laboratory analysis, existence of intercurrences/complications and their description, and development of adverse events (with special emphasis on the appearance of symptoms compatible with anaphylaxis and/or later serum sickness). At 24 h, 48 h, 49 h, 96 h, 7 days, 14 days, 21 days and 28 days, a blood sample was drawn to measure the neutralizing activity of SARS-CoV-2 and to quantify horse $F(ab')_2$ in the first 20 study participants. On days 7, 14, 21 and 28, if the patient was still hospitalized, the detection of viral RNA detected in the nasopharyngeal swab sample was evaluated. If a patient is discharged from hospital before day 28, the day was recorded, and the follow-up continued by telephone (landline/cell phone), mail and/or personal (home visit) aimed at maximizing the permanence of the subject in the study in order to record the evolution, clinical status, and adverse reactions at least until day 28 after the start of treatment.

Determination of pharmacokinetic parameters

Plasma concentration of the $F(ab')_2$ equine therapeutic product ("P") was monitored at basal conditions (pre-infusion) and at different times post-first infusion (1 h, 3 h, 6 h, 24 h, 48 h) and post second infusion (49 h and 7, 14, 21 and 28 days) in all patients. To this aim, a double antibody ELISA was carried out. High binding ELISA polystyrene plates (Corning) were coated with 2 µg of rabbit anti-equine IgG unconjugated as capture antibody (Novus Biologicals™) at pH 9.0 during 2 hours at 37 °C. Plates were blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 12 h at 37 °C and washed. As standard, "P" was loaded in a range of concentrations between 51,870 and 0.405 mg/l, which fit the required linearity of the assay (r^2>0.98). Standard and samples were loaded in duplicate and incubated during 1.5 h at 37 °C. Then, plates were washed with PBS-Tween 0.05% and incubated with rabbit anti-equine IgG, human-adsorbed, horseradish peroxidase (HRP) conjugated as detection antibody (Novus

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Biologicals™). Tetramethylbenzidine (TMB, Invitrogen) was used as developer and HCl 1N as stopper. Finally, optical density was assessed at 450 nm. The standard (St) curve was plotted as the best fit curve of absorbance for 5 serial sample dilutions. Plasma equine $F(ab')_2$ concentrations were interpolated from the St curve and the mean results of two independent assays were expressed as mg/l. Data processing was performed using the GraphPad Prism program, version 6.0 for Windows.

From the results at the different sampling times, the following variables were calculated: AUC_{0-t}: area under the serum concentration curve between time 0 and time t (last extraction performed); $AUC_{0-\infty}$: area under the serum concentration curve, resulting from the extrapolation between time 0 and time ∞; C_{max}: maximum serum concentration; T_{max}: time in which the maximum serum concentration is reached.

Immunogenicity assessment

The detection of circulating antibodies anti horse $F(ab')_2$ and their titters were carried out in all patients at basal conditions, 14 and 28 days post first infusion employing an indirect ELISA. High binding ELISA polystyrene plates (Corning) were coated at pH 9.0 and 37 °C during 2 hours with 2 µg of an intermediate pepsin digestion product with the purpose of detecting antibodies anti equine $F(ab')_2$ but also with specificity towards possible traces of contaminants. After blocking with 3% skim milk in PBS at 37 °C for 2 h and washing with PBS, diluent (in blank wells) or samples were loaded in duplicate from 1/150 to 1/2,400 dilutions (range that fit the linearity of the assay with a r^2>0.98) and incubated at 4 °C overnight. After washing with PBS-Tween 0.05%, goat anti human IgG, horse-adsorbed, HRP conjugated (Invitrogen) was added. TMB (Invitrogen) was used as developer and HCl 1N as stopper and finally optical density was assessed at 450 nm. Then, the best fit curve of absorbance/dilutions was plotted for each sample. Titters of antibodies anti equine $F(ab')_2$ were expressed as the mean AUC of two independent assays. Data processing was performed using the GraphPad Prism program, version 6.0 for Windows.

Cytokine plasma levels

Plasma concentrations of IL-6, TNFα and IFNα were assessed by a magnetic bead multiplex assay employing the human cytokine panel HCYTOMAG-60K, in a Magpix® equipment (all Merck-Millipore). The assay was performed according to the manufacturer's instructions. Standard curves and samples were tested in duplicate. The standard curve detection ranged from 3.2 to 10,000 pg/ml for all analytes. Standards were plotted and concentrations were determined using xPONENT® software version 4.2 and expressed in pg/ml.

Statistical methods

The original sample size was determined as 200 patients (100 individuals for each group or arm), which will allow reaching a statistical power (1-beta) of 80% with a level of significance (2 tails) of alpha = 0.05, to detect a change of 8 days in time to clinical improvement in the group subject to the intervention, assuming that the control group will have an average time of 20 days and 60% will achieve clinical improvement.

Interim analysis for safety and efficacy was planned when: 20, 50, 100, and 150 patients were achieved. The patient safety control committee evaluates the result of each analysis performed to recommend the continuation or interruption of the study.

The epidemiological characteristics of the included patients were described according to the classic methodology of descriptive statistics. The discrete variables were described in percentages with 95% CI (confidence interval) and the continuous ones in means ± standard deviation.

The primary analysis of efficacy was carried out considering intention to treat and will include all patients who have been incorporated into the study and assigned to any of the therapeutic arms. Time to clinical improvement was assessed after all patients had reached day 28.

The secondary objectives related to the proportion of patients in each category of the WHO ordinal scale, the negativization of the swab sample by PCR for SARS-CoV-2 and mortality, were analysed using a Kaplan-Meier graph and compared with a log rank test.

The variables: duration of oxygen therapy (quantified in days required to supply oxygen to achieve arterial saturation greater than 92%), duration of hospital stay (quantified in days from diagnosis of SARS-CoV-2 infection to hospital discharge), and lymphocyte count (integer continuous numerical variable) were expressed as means ± standard deviation. The results obtained with the different treatments were compared with the chi-square test and with the *t*-test.

The frequency of adverse reactions was presented as the absolute risk of adverse reactions associated with each treatment and broken down into serious and non-serious reactions. If the number allows it, they were subclassified according to Medical Dictionary for Regulatory Activities (MedDRA) and discriminated by Class of Organs and Systems, High Level Grade Term and High Level Term. The adverse reactions that had required medical treatment were described individually, and based on their number, they

were grouped to quantify the risk associated with the therapeutic conduct taken as absolute risk for each treatment and relative risk.

The biological activity was evaluated by quantification of the serum neutralization *in vitro* with samples of serum extracted from the first research subjects recruited at the different times. The serum neutralizing activity was analysed as a function of time, obtaining the following pharmacokinetic parameters for each treatment: C_{max}, T_{max}, AUC_{0-t} (28 days), $AUC_{0-\infty}$, C_{max} (maximum activity equivalent to the maximum concentration found) and T_{max} (time in which maximum activity was obtained), will be obtained directly from the original data set, and AUC_{0-t} was calculated using the linear trapezoidal rule. $AUC_{0-\infty}$, was calculated as AUC_{0-t} + Ct/Ke, where Ct was the last concentration measured and the elimination rate constant, Ke, was calculated by linear regression of the log-linear portion of the serum neutralization-time curve. The half-life (t1/2) will be calculated as ln(2)/Ke.

Statistical comparisons of the titter of heterophilic antibodies at basal conditions between "study" and "control" groups were analysed applying unpaired two-tailed *t*-test using the nonparametric Kruskal-Wallis test, followed by the Mann-Whitney post-test to compare pairs. Data were expressed as medians with interquartile ranges. Statistical comparisons of titter of heterophilic antibodies and cytokine levels among time of study were analysed by one-way ANOVA and Tuckey's multiple comparison test. All analyses were performed using GraphPad Prism 6.0 software package (San Diego, CA, USA) for Windows. A P-value < 0.05 was considered statistically significant.

Results

Clinical and laboratory results

Twenty patients were recruited between 06/15/21 and 08/16/21 (Figure 1). In all cases, the patients were evaluated and received a talk with information about the clinical study individually, they were given a copy of the Informed Consent Form (ICF), they were given at least 30 minutes to read the information from the ICF, and they were offered a space to ask questions and clear up doubts and queries, and they were allowed to reflect on it. Subsequently, the level of understanding of the information provided was evaluated. No patient presented vulnerability criteria. All of them presented a positive result of detection of the SARS-CoV-2 viral genome by PCR in a nasal swab sample at the time of signing the ICF.

The patients did not present significant differences in the general characteristics, vital parameters, laboratory, symptoms at the time of inclusion, and clinical stage according to the WHO score (Table 1).

Monitoring of vital parameters (systolic and diastolic blood pressure, heart rate, respiratory rate, and

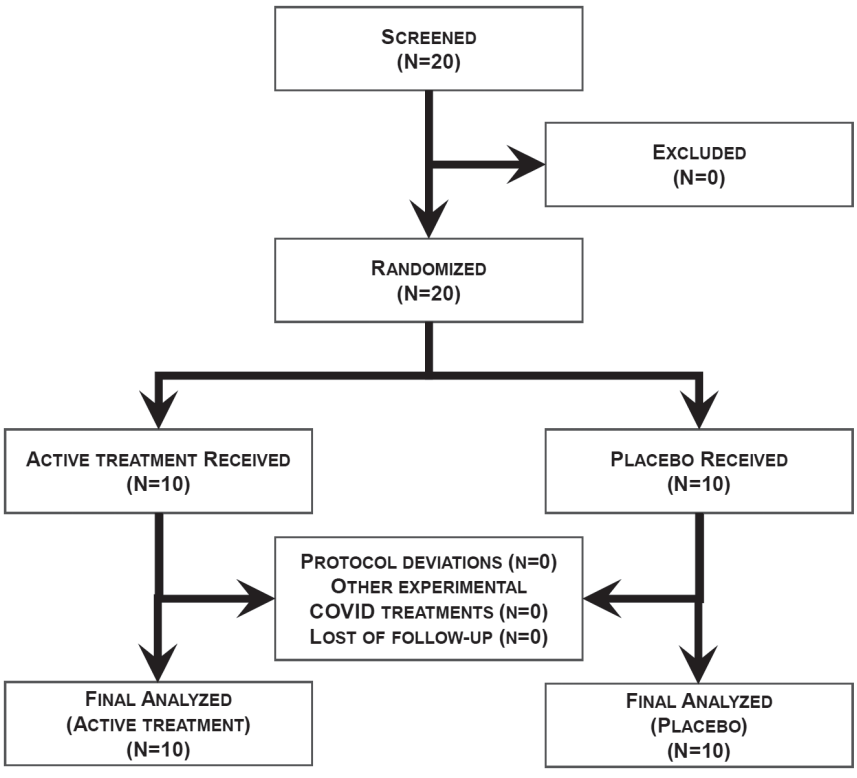

Figure 1: Consort flow-chart.

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Table 1: Sample characteristics

		Total sample (N=20)	Treatment (N=10)	Control (N=10)
General	Age (years)	44 ± 14 (18–73)	44 ± 13 (26–73)	43 ± 15 (18–60)
	Gender (female)	♀ 6 (30%)	♀ 5 (50%)	♀ 1 (10%)
	Weight (kg)	84 ± 16 (54–109)	80 ± 17 (56–103)	88 ± 15 (54–109)
	Height (cm)	172 ± 9 (151–186)	168 ± 9 (151–185)	176 ± 7 (164–186)
	BMI (kg/m^2)	28 ± 5 (20–38)	28 ± 6 (20–38)	29 ± 5 (20–34)
Vital signs	SBP (mm Hg)	113 ± 12 (100–135)	118 ± 13 (100–135)	109 ± 10 (100–125)
	DBP (mm Hg)	76 ± 13 (60–100)	80 ± 13 (60–100)	72 ± 12 (60–90)
	HR (bpm)	84 ± 16 (60–104)	87 ± 18 (60–104)	81 ± 14 (60–104)
	RR (bpm)	19 ± 3 (16–24)	19 ± 3 (16–24)	20 ± 4 (16–24)
	Temperature (°C)	37 ± 1 (36–38)	37 ± 1 (36–38)	37 ± 1 (36–38)
	$SatO_2$ (%)	89 ± 2 (85–91)	89 ± 2 (86–91)	89 ± 2 (85–91)
Laboratory tests	HCT (%)	35 ± 3 (30–38)	35 ± 3 (32–38)	34 ± 2 (30–38)
	WBC (/mm^3)	7824 ± 1115 (5280–9300)	7888 ± 1213 (5280–9000)	7760 ± 1069 (6240–9300)
	PLT (×10^3/mm^3)	232 ± 70 (124–318)	230 ± 77 (124–318)	235 ± 67 (126–309)
	Glucose (mg/dl)	103 ± 9 (91–119)	101 ± 9 (91–116)	106 ± 10 (91–119)
	Urea (mg/dl)	32 ± 6 (20–41)	31 ± 7 (20–41)	33 ± 5 (26–40)
	Creatinine (mg/dl)	1.0 ± 0.1 (0.8–1.2)	1.0 ± 0.1 (0.8–1.2)	1.0 ± 0.1 (0.9–1.2)
	Prothrombine time (%)	84 ± 12 (71–103)	81 ± 12 (71–103)	87 ± 13 (72–103)
	aPTT (s)	36 ± 6 (28–45)	36 ± 6 (28–45)	36 ± 5 (30–45)
Symptoms	Cough (N, %)	12 (60%)	5 (50%)	7 (70%)
	Fever (N, %)	15 (75%)	8 (80%)	7 (70%)
	Odynophagia (N, %)	16 (80%)	9 (90%)	7 (70%)
	Dyspnoea (N, %)	13 (65%)	6 (60%)	7 (70%)
	Ageusia (N, %)	15 (75%)	7 (70%)	8 (80%)
	Anosmia (N, %)	16 (80%)	9 (90%)	7 (70%)
WHO score	5	9 (45%)	4 (40%)	5 (50%)
	4	11 (55%)	6 (60%)	5 (50%)
	3	0 (0%)	0 (0%)	0 (0%)

BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HR – heart rate; RR – respiratory rate; $SatO_2$ – arterial oxygen saturation; HCT – hematocrit; WBC – white blood cell count; PLT – platelet count; aPTT – activated partial thromboplastin time; WHO – World Health Organization

temperature) did not show changes of significant clinical interest. Arterial oxygen saturation showed higher values in the treated group between the third and twelfth days (Figure 2). The mean values were compatible with the absence of oxygen therapy requirement from day 3 in the treated group, which was reached in the control group from day 5.

On biochemical parameters, the treatment group presented increased lymphocyte count (day 4), decreased concentration of ferritin (days 4 and 5), and decreased D-dimer (day 4). No other significant changes were observed (Table 2). The PCR for SARS-CoV-2 test on nasal swab showed an earlier negative result in the treated patients, presenting a decrease in it on day 8 to 11, in such a way that the patients treated with anti-SARS-CoV-2 presented a 50% negativization of the swabs, compared to the control group (Table 3).

Individuals in the treatment group presented more frequently lower WHO clinical categories in the 3rd to 5th days of treatment (all the individuals in the treatment group had a WHO score of 3 on day 4, while 30% of the control sample still had a WHO score of 4). Hospitalization time did not present significant differences between the treated groups, although a lower proportion of hospitalized patients can be observed in the treatment group at any time of the study (Table 4).

There were no cases of altered consciousness, impaired ventilatory mechanics, inotropic drug

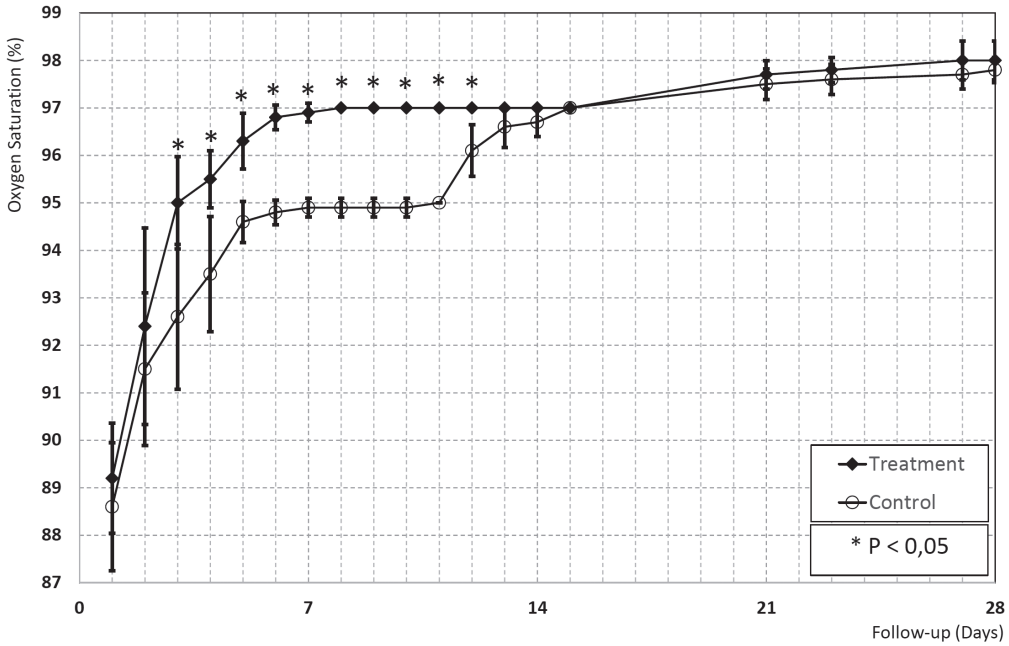

Figure 2: Arterial oxygen saturation follow-up.

Table 2: Laboratory result during follow-up

		Total sample	Treatment (10)	Control (10)	P
Day 01	Lymphocytes (/mm^3)	1060 ± 239 (600–1300)	1020 ± 215 (700–1300)	1100 ± 267 (600–1300)	0.47
	Neutrophils (/mm^3)	2025 ± 297 (1500–2400)	1970 ± 323 (1500–2400)	2080 ± 274 (1600–2400)	0.42
	Urea (mg/dl)	29 ± 7 (20–43)	29 ± 8 (20–40)	29 ± 7 (20–43)	0.95
	LDH (IU/l)	269 ± 86 (100–390)	276 ± 71 (120–370)	262 ± 102 (100–390)	0.73
	CRP (mg/l)	0.35 ± 0.16 (0.13–0.57)	0.29 ± 0.15 (0.13–0.55)	0.40 ± 0.15 (0.13–0.57)	0.11
	Ferritin (ng/ml)	360 ± 79 (210–480)	360 ± 68 (290–480)	360 ± 92 (210–470)	1.00
Day 04	D-dimer (ng/dl)	273 ± 108 (110–460)	276 ± 117 (120–460)	269 ± 104 (110–430)	0.89
	Lymphocytes (/mm^3)	1305 ± 267 (800–1700)	1460 ± 152 (1300–1700)	1100 ± 283 (800–1400)	**0.04***
	Neutrophils (/mm^3)	1885 ± 243 (1600–2300)	1960 ± 288 (1600–2300)	1740 ± 167 (1600–2000)	0.18
	Urea (mg/dl)	33 ± 8 (20–44)	35 ± 6 (27–42)	32 ± 10 (20–43)	0.65
	LDH (IU/l)	213 ± 66 (110–340)	188 ± 52 (110–240)	256 ± 98 (120–340)	0.21
	CRP (mg/l)	0.19 ± 0.07 (0.10–0.28)	0.22 ± 0.04 (0.15–0.26)	0.22 ± 0.08 (0.13–0.28)	0.85
	Ferritin (ng/ml)	345 ± 104 (210–490)	254 ± 77 (210–390)	442 ± 61 (340–490)	**<0.01***
	D-dimer (ng/dl)	213 ± 89 (110–370)	152 ± 28 (110–180)	316 ± 55 (250–370)	**<0.01***
Day 05	Lymphocytes (/mm^3)	1330 ± 258 (900–1700)	1400 ± 274 (1000–1700)	1260 ± 251 (900–1500)	0.42
	Neutrophils (/mm^3)	1920 ± 244 (1600–2300)	1820 ± 192 (1600–2100)	2020 ± 268 (1600–2300)	0.21
	Urea (mg/dl)	32 ± 8 (22–44)	27 ± 5 (22–33)	37 ± 7 (29–44)	0.04
	LDH (IU/l)	203 ± 48 (130–320)	204 ± 11 (190–220)	202 ± 71 (130–320)	0.95
	CRP (mg/l)	0.15 ± 0.06 (0.10–0.26)	0.13 ± 0.03 (0.10–0.19)	0.18 ± 0.07 (0.10–0.26)	0.20
	Ferritin (ng/ml)	341 ± 93 (230–480)	270 ± 39 (230–320)	412 ± 74 (290–480)	**0.01***
	D-dimer (ng/dl)	192 ± 81 (110–320)	152 ± 33 (110–190)	232 ± 97 (110–320)	0.12
Day 09	Lymphocytes (/mm^3)	1650 ± 280 (1300–2200)	1850 ± 265 (1600–2200)	1517 ± 214 (1300–1900)	0.06
	Neutrophils (/mm^3)	1910 ± 311 (1500–2400)	1700 ± 231 (1500–1900)	2050 ± 288 (1600–2400)	0.08
	Urea (mg/dl)	32 ± 7 (25–44)	39 ± 6 (30–44)	28 ± 4 (25–34)	**0.01***
	LDH (IU/l)	186 ± 67 (100–290)	145 ± 47 (100–190)	213 ± 68 (130–290)	0.12
	CRP (mg/l)	0.16 ± 0.03 (0.11–0.21)	0.15 ± 0.04 (0.11–0.19)	0.17 ± 0.04 (0.11–0.21)	0.60
	Ferritin (ng/ml)	276 ± 52 (200–340)	295 ± 65 (200–340)	263 ± 43 (210–340)	0.38
	D-dimer (ng/dl)	134 ± 25 (100–180)	150 ± 22 (130–180)	123 ± 23 (100–160)	0.11

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		Total sample	Treatment (10)	Control (10)	P
Day 10	Lymphocytes (/mm^3)	1750 ± 284 (1300–2100)	1883 ± 223 (1500–2100)	1550 ± 265 (1300–1900)	0.06
	Neutrophils (/mm^3)	1960 ± 227 (1600–2400)	2050 ± 226 (1800–2400)	1825 ± 171 (1600–2000)	0.13
	Urea (mg/dl)	29 ± 8 (21–43)	30 ± 8 (21–43)	28 ± 10 (22–42)	0.67
	LDH (IU/l)	153 ± 31 (100–210)	142 ± 27 (100–170)	170 ± 32 (140–210)	0.17
	CRP (mg/l)	0.16 ± 0.03 (0.11–0.19)	0.16 ± 0.04 (0.11–0.19)	0.16 ± 0.02 (0.13–0.18)	0.88
	Ferritin (ng/ml)	258 ± 44 (200–330)	242 ± 26 (220–290)	283 ± 59 (200–330)	0.17
	D-dimer (ng/dl)	76 ± 24 (50–120)	75 ± 23 (50–110)	78 ± 30 (50–120)	0.88
Day 22	Lymphocytes (/mm^3)	1925 ± 240 (1600–2300)	1940 ± 196 (1700–2300)	1910 ± 288 (1600–2300)	0.79
	Neutrophils (/mm^3)	2035 ± 289 (1500–2400)	1970 ± 298 (1500–2400)	2100 ± 279 (1700–2400)	0.33
	Urea (mg/dl)	33 ± 8 (20–44)	33 ± 10 (20–44)	34 ± 6 (26–44)	0.83
	LDH (IU/l)	151 ± 33 (100–190)	156 ± 31 (110–190)	145 ± 35 (100–190)	0.46
	CRP (mg/l)	0.15 ± 0.02 (0.11–0.19)	0.14 ± 0.03 (0.11–0.19)	0.15 ± 0.02 (0.11–0.17)	0.56
	Ferritin (ng/ml)	194 ± 29 (150–240)	191 ± 29 (150–240)	196 ± 30 (150–240)	0.71
	D-dimer (ng/dl)	74 ± 15 (50–90)	71 ± 15 (50–90)	77 ± 16 (50–90)	0.40
Day 28	Lymphocytes (/mm^3)	1950 ± 278 (1600–2300)	1950 ± 284 (1600–2300)	1950 ± 288 (1600–2300)	1.00
	Neutrophils (/mm^3)	2030 ± 303 (1600–2400)	2010 ± 338 (1600–2400)	2050 ± 280 (1700–2400)	0.78
	Urea (mg/dl)	31 ± 8 (20–44)	36 ± 7 (25–44)	26 ± 5 (20–35)	**<0.01***
	LDH (IU/l)	145 ± 31 (100–190)	145 ± 38 (100–190)	144 ± 24 (110–180)	0.94
	CRP (mg/l)	0.14 ± 0.03 (0.10–0.19)	0.13 ± 0.03 (0.10–0.19)	0.15 ± 0.02 (0.11–0.17)	0.07
	Ferritin (ng/ml)	197 ± 30 (150–240)	207 ± 24 (170–240)	187 ± 33 (150–240)	0.14
	D-dimer (ng/dl)	71 ± 15 (50–90)	71 ± 14 (50–90)	71 ± 16 (50–90)	1.00

*statistically significant differences; LDH – lactate dehydrogenase; CRP – C-reactive protein

Table 3: Percentage of positivity of nasal swabs throughout the follow-up

Day	Total	Treatment	Control
Basal	20 (100%)	10 (100%)	10 (100%)
4	20 (100%)	10 (100%)	10 (100%)
8	15 (75%)	5 (50%)	10 (100%)
12	0 (0%)	0 (0%)	0 (0%)
15	0 (0%)	0 (0%)	0 (0%)
21	0 (0%)	0 (0%)	0 (0%)
28	0 (0%)	0 (0%)	0 (0%)

requirements, mechanical respiratory support requirements, or other criteria for moving to an intensive care unit in any of the groups arms.

No adverse reactions were reported in any of the research subjects studied through the 28-day follow-up. Adverse reactions were actively investigated through questioning, laboratory controls, and spontaneous reports, not registering any type of event.

There were no differences regarding the total duration of hospitalization, time to clinical improvement or time required for supplemental oxygen therapy. A shorter time was found for nasal swabs to become negative in the treatment group. A disaggregated analysis based on severity at patient admission showed that those with greater severity (WHO score 5) had less time to obtain clinical improvement, and less time on oxygen therapy (Table 5).

Neutralizing activity

The samples obtained from blood after the administration of the product did not present detectable neutralizing activity by means of the method used in any of the post-administration times.

Safety controls

Patients were evaluated by physical examination and questioning daily during hospitalization and periodically

Table 4: WHO clinical category classification and hospital discharge follow-up comparison between groups

Day	Treatment				Control			
	hospitalized			ambulatory	hospitalized			ambulatory
	WHO 5	WHO 4	WHO 3	WHO <3	WHO 5	WHO 4	WHO 3	WHO <3
Basal	4 (40%)	6 (60%)	–	–	5 (50%)	5 (50%)	–	–
1	4 (40%)	6 (60%)	–	–	5 (50%)	4 (40%)	1 (10%)	–
2	1 (10%)	5 (50%)	4 (40%)	–	1 (10%)	7 (70%)	2 (20%)	–
3	–	2 (20%)	8 (80%)	–	1 (10%)	5 (50%)	4 (40%)	–
4	–	–	10 (100%)	–	–	3 (30%)	7 (70%)	–
5	–	–	10 (100%)	–	–	1 (10%)	9 (90%)	–
6	–	–	10 (100%)	–	–	–	10 (100%)	–
7	–	–	10 (100%)	–	–	–	10 (100%)	–
8	–	–	10 (100%)	–	–	–	10 (100%)	–
9	–	–	10 (100%)	–	–	–	10 (100%)	–
10	–	–	10 (100%)	–	–	–	10 (100%)	–
11	–	–	6 (60%)	4 (40%)	–	–	10 (100%)	–
12	–	–	5 (50%)	5 (50%)	–	–	10 (100%)	–
13	–	–	5 (50%)	5 (50%)	–	–	9 (90%)	1 (10%)
14	–	–	5 (50%)	5 (50%)	–	–	9 (90%)	1 (10%)
15	–	–	4 (40%)	6 (60%)	–	–	2 (20%)	8 (80%)
16 to 28	–	–	–	10 (100%)	–	–	–	10 (100%)

WHO – World Health Organization

Table 5: Time to different parameters of clinical improvement

		Total	Treatment	Control	P
Length of hospitalization (days)	total	13 ± 1.9 (10–15)	13 ± 2.5 (10–15)	14 ± 0.8 (12–15)	0.095
	WHO 5	14 ± 1.8 (10–15)	13 ± 2.4 (10–15)	14 ± 0.5 (14–15)	0.209
	WHO 4	13 ± 2.1 (10–15)	13 ± 2.7 (10–15)	14 ± 0.9 (12–14)	0.389
Time to clinical improvement (days)	total	8 ± 5.4 (2–15)	8 ± 5.7 (2–15)	9 ± 5.4 (2–14)	0.968
	WHO 5	3 ± 0.9 (2–4)	2 ± 0.5 (2–3)	3 ± 0.9 (2–4)	**0.048**
	WHO 4	13 ± 2.1 (10–15)	13 ± 2.7 (10–15)	14 ± 0.9 (12–14)	0.389
Time to nasal swab negativization (days)	total	11 ± 1.8 (8–12)	10 ± 2.1 (8–12)	12 ± 0.0 (12–12)	**0.015**
	WHO 5	11 ± 1.8 (8–12)	10 ± 2.3 (8–12)	12 ± 0.0 (12–12)	0.182
	WHO 4	11 ± 1.9 (8–12)	10 ± 2.2 (8–12)	12 ± 0.0 (12–12)	0.076
Length of oxygen therapy (days)	total	3 ± 1.2 (2–6)	3 ± 0.8 (2–4)	4 ± 1.3 (2–6)	0.057
	WHO 5	3 ± 0.9 (2–4)	2 ± 0.5 (2–3)	3 ± 0.9 (2–4)	**0.048**
	WHO 4	4 ± 1.3 (2–6)	3 ± 0.8 (2–4)	4 ± 1.6 (2–6)	0.247

WHO – World Health Organization

(every 3 to 7 days) during the outpatient period until 28 days' post administration.

Neither the physical examination, interrogation nor laboratory showed elements compatible with adverse events that motivated his report.

There were no cases of complications, hemodynamic decompensation, deterioration of consciousness, deterioration of respiratory mechanics, or any other cause that could motivate the transfer of a patient to the intensive care unit.

This behaviour did not present differences between both therapeutic groups.

Pharmacokinetics analysis

The 10 patients in the treated group presented detectable plasma concentrations of equine IgG after

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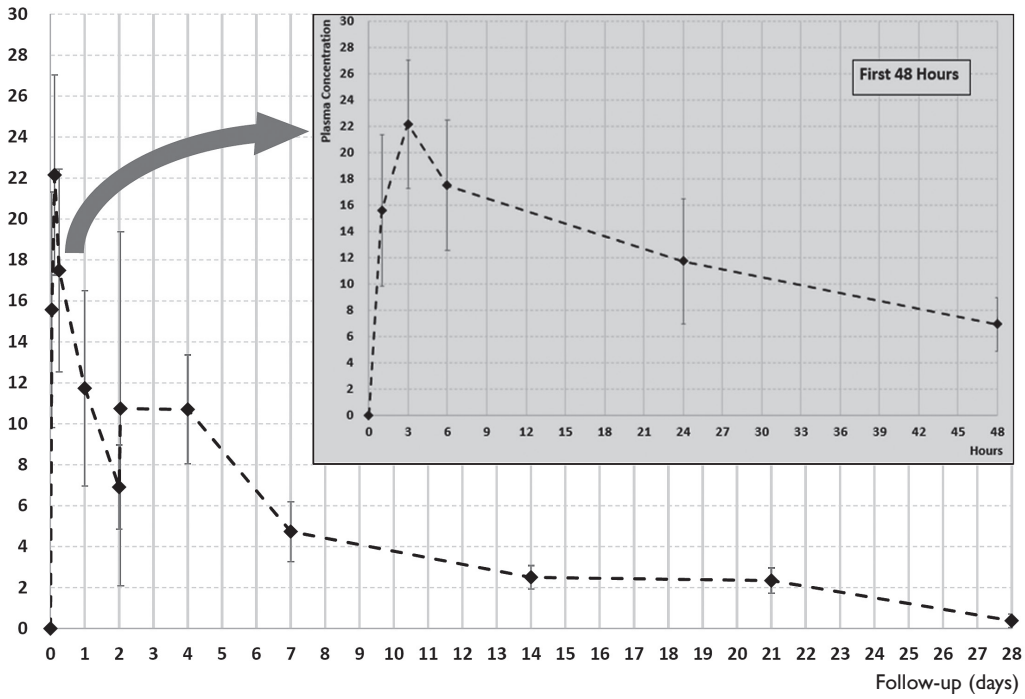

Figure 3: Mean plasma concentration over time.

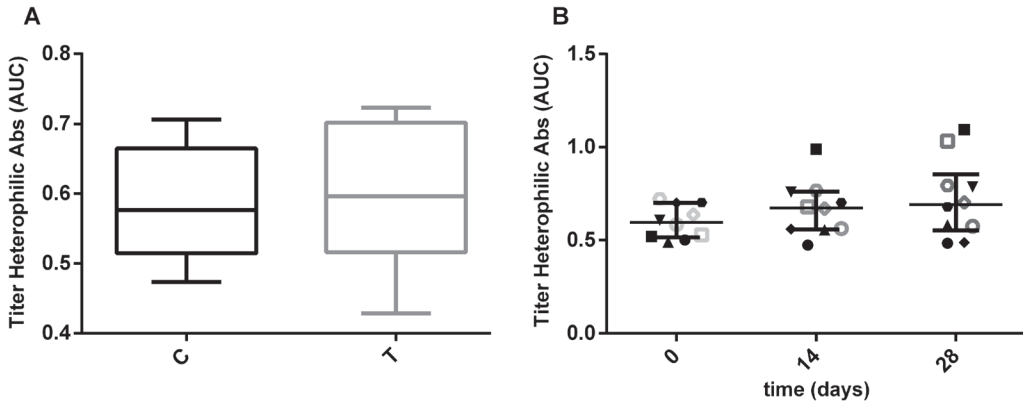

Figure 4: Titter of human heterophilic antibodies against equine immunoglobulins (HHA-HI) in the basal samples.

Table 6: Comparative evolution of heterophilic antibodies titter

	All patients	Control	Treatment				
	basal	basal	basal	day 14		day 28	
Individuals	20	10	10	10	Δ%	10	Δ%
Mean	0.5917	0.5833	0.6002	0.6724	18.67	0.7223	23.48
SD	0.0816	0.0777	0.0886	0.1472	35.80	0.2105	44.70
SEM	0.0182	0.0246	0.0280	0.0465	11.32	0.0666	14.14
Min	0.4734	0.4734	0.4900	0.4748	–20.00	0.4848	–30.50
25% percentile	0.5196	0.5145	0.5162	0.5598	–4.10	0.5540	–3.25
Median	0.5832	0.5764	0.5962	0.6748	5.60	0.6913	10.00
75% percentile	0.6736	0.6645	0.7016	0.7616	37.43	0.8543	45.73
Max	0.7231	0.7062	0.7231	0.9893	89.90	1.0940	110.00
Inferior limit 95% CI	0.5536	0.5278	0.5367	0.5671	–6.94	0.5717	–8.50
Superior limit 95% CI	0.6299	0.6389	0.6636	0.7777	44.28	0.8728	55.46

SD – standard deviation; SEM – standard error of the mean; CI – confidence interval

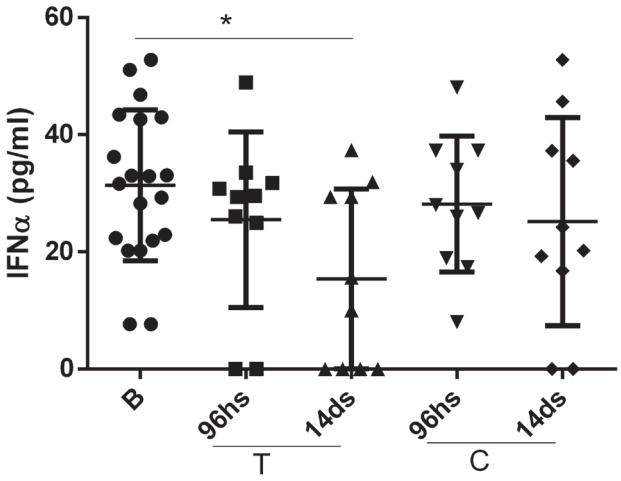

Figure 5: α-IFN concentration compared between groups throughout time.

its administration and up to 21–28 days (Figure 3). The therapeutic product was undetectable in all patients from the control group.

In the treated group, the maximum concentration (C_{max}) was reached between 1 and 3 hours (T_{max}) after the start of administration, with an average C_{max} value of 27.21 ± 7.16 (16.80 to 36.53) mg/ml. The AUC_{0-t} until the last detectable concentration (21 days for 6 patients and 28 days for 4 patients) was 2,903 ± 608 (1,897–3,804) mg/l×h, with a residual area of 22.33 ± 31.67 (0–81.10) mg/l×h and the $AUC_{0-\infty}$ of 1,925 ± 609 (1,897–3,830) mg/l×h. The Ke was quantified at –0.018 ± 0.007 (–0.007 to –0.027), correlating with a terminal elimination half-life (t1/2) of 47 ± 22 (26 to 94) hours (4 or 5 plasma concentrations were used as minimum to Ke estimation). The total clearance was 1.435 ± 0.501 (0.394 to 2.329) ml/min. The apparent volume of distribution (Vd) was 5.33 ± 2.29 (2.36 to 10.62) liters.

Immunopharmacology

The results obtained showed the presence of human heterophilic antibodies against horse IgG (HHA-HI) in the basal samples of the 20 patients analysed (treatment and control groups). Relative titter expressed as mean AUC value of two assays was 0.59 ± 0.08 (0.47 to 0.72). There was no difference between the control and treatment groups (Figure 4, Table 6).

The equine anti-$F(ab')_2$ antibody titter in the patients who received the product was studied as a function of time (Figure 5). Statistical analysis showed no significant differences between study times using ANOVA and Tuckey's multiple comparisons test (Table 6).

Table 7 shows the individual results at each time expressed as the average AUC value of two measurements, and the percentage of the variation of AUC with respect to baseline (Δ%), where: $\Delta\% = ([AUC_{Time} - AUC_{Basal}]/AUC_{Basal})\times 100$. Results showed that 5/10 patients maintained (±10%) post-infusion the levels of anti-equine antibodies detected in basal conditions (P1, P4, P16, P17, and P19), 2/10 (P12 and P13) showed a mild increase around 19–30%, 2/10 showed a greater increase around 95–100% (P7 and P8) and 1/10 (P14) diminished 30% the titter of anti-equine antibodies.

Determination of cytokine plasma levels

Plasma concentrations of IL-6, TNFα and IFNα were assessed at t = 0, t = 96 h and t = 14 days in all patients. The kinetics of plasma IFNα levels may

Table 7: Individual variation of the titter of heterophilic antibodies anti horse over time

Patient code	Basal	14 days		28 days	
	AUC	AUC	Δ%	AUC	Δ%
P01	↓ 0.5019	↓ 0.4748	–5.3	↓ 0.4848	–3.2
P04	0.5843	0.5626	–3.7	0.5760	–1.4
P07	0.5210	↑ 0.9893	+89.9	↑ 1.0940	+110.0
P08	0.5300	0.6790	+75.0	↑ 1.0335	+95.0
P12	↓ 0.4800	↓ 0.5569	+13.7	0.5830	+19.0
P13	0.6081	0.7595	+24.9	0.7863	+29.3
P14	0.7009	0.5608	–20.0	↓ 0.4880	–30.5
P16	0.6387	0.6706	+5.0	0.7032	+10.1
P17	↑ 0.7035	0.7029	+1.0	0.6794	–3.4
P19	↑ 0.7231	↑ 0.7679	+6.2	0.7945	+9.9

The titter of human heterophilic antibodies anti horse IgG (HHA-HI) was expressed as the mean area under the curve (AUC) of the best fit curve of absorbance for 5 serial sample dilutions in indirect ELISA. Arrows point out values below or above of interquartile range. (↑) greater than 75% percentile; (↓) lesser than 25% percentile

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Table 8: Cytokines determination over time

		α-IFN	IL-6	α-TNF
All (N=20)	basal	31.36 + 12.89 (7.67–52.78)	1.03 + 1.10 (0.40–5.00)	8.60 + 3.09 (4.00–13.79)
Treatment (N=10)	96 h	25.50 + 14.96 (0.00–48.94)	0.63 + 0.11 (0.50–0.88)	8.46 + 2.87 (4.00–12.74)
	14 days	15.38 + 15.37 (0.00–37.40)	0.94 + 0.60 (0.45–2.02)	8.08 + 2.81 (4.00–12.10)
Control (N=10)	96 h	28.16 + 11.61 (8.00–48.08)	0.79 + 0.54 (0.40–2.01)	7.31 + 3.04 (4.00–10.74)
	13 days	25.18 + 17.75 (0.00–52.78)	1.95 + 0.56 (0.40–1.95)	7.22 + 3.43 (4.00–11.33)

mirror the course of the patient's innate antiviral response. Having discriminated between treated and control patients from previous pharmacokinetic (PK) studies, plasma IFNα levels among baseline (B), treated (96 h and 14 days) and controls (96 h and 14 days) were compared. The population distribution of the results is shown in Table 8 and Figure 5. Results showed that the basal levels of IFNα from both groups of patients were similar. In addition, IFNα levels peaked at t = 0 (oscillating between 7.660 pg/ml and 52.779 pg/ml), while for others, it peaked at t = 96 h (maximum value in the treated group: 48.936 pg/ml and in the control group: 48.08 pg/ml). Despite these variations, plasma levels of IFNα in the treated group decreased significantly after 14 days with respect to the baseline ($P<0.05$). The concentration of IFNα remained constant along the study.

The plasmatic levels of IL-6 and TNFα represent markers of systemic inflammatory response in severe cases SARS-Cov2 infection. Table 8 shows the population distribution of plasma levels of IL-6 and TNFα respectively. Data indicated that, in the present cohort, all patients showed low circulating levels of these cytokines, either at baseline or along the study in both groups.

Discussion

Passive immunotherapy is an old tool, and also a well-known, well-studied tool when a new therapy approach is needed to easy to develop a treatment on a larger scale, with properties that are largely known (Pan et al., 2020; Piccoli et al., 2020). This type of therapy includes different variants that can be classified into general groups: use of human convalescent plasma, use of animal polyclonal immunoglobulins, and specific monoclonal antibodies. Convalescent plasma has advantages (species similarity, source study feasibility, lower cost of obtaining), and disadvantages (risk of disease transmission, lower neutralizing titter, higher infusion volumes required, hemodynamic and hypersensitivity adverse effects). At the opposite pole, monoclonals offer great advantages (extremely high specificity and neutralizing power, lower volume requirement, lower risk of disease transmission), and disadvantages (selectivity of the neutralization point that can present mutations and therefore resistance, very high production cost, longer development time, less predictable pattern of adverse effects). Polyclonal antibodies of equine origin present an intermediate pattern between both groups, providing great advantages such as high specificity and neutralizing activity, lower infusion volumes required, combined with the possibility of blocking several points (and less possibility of resistance or phenomena of escape), and lower production cost (compared to monoclonals), possibility of rapid development and scaling of the product. For these reasons, this tool should be taken into account whenever a pathogen spreads rapidly, generating an epidemic and/or pandemic outbreak faster than the development capacity of other new specific therapies (Casadevall, 2002; Hussen et al., 2020).

Although the strategy of using this type of tool has been initially developed by several research groups (250 MEDLINE publications linking the MeSH ("Immunoglobulin Fragments" [Mesh] AND "SARS-CoV-2"[Mesh]), few groups have reached the clinical phase, and none to our knowledge have simultaneously reported pharmacokinetics, clinical effects, immunological effects, and inflammatory markers (Piechotta et al., 2020). This manuscript shows the pharmacological properties of a biological drug based on $F(ab')_2$ fragments of polyclonal antibodies of equine origin with anti-SARS-CoV-2 neutralizing activity, describing pharmacokinetics, clinical efficacy, neutralizing activity, effect on neutralization of nasal swabs, correlation with cytokine concentration, safety and adverse event profile, and immunopharmacology.

The study was able to demonstrate a reduction in time needed to clinical improvement, during 28 days after the assignment in the WHO category 5 subgroup of this small sample of 20 initial patients. The results in this subgroup (statistically significant despite the small initial number of patients) adds important evidence in favour of passive immunotherapy in patients with

severe COVID-19 disease. These findings are in line with what was previously reported, according to which passive immunization strategies seem to provide improvement only in patients with severe symptoms, where it has also been shown that it is necessary to report more data to develop sufficient evidence to make recommendations (Piechotta et al., 2020; Libster et al., 2021).

There was no evidence of shorter duration of oxygen therapy, but there was evidence of improvement in arterial saturation measured between days 3 and 12. This contrasts with studies that showed effects in reducing the duration and severity of some episodes, although our work did not analyse a population with prolonged COVID (Cimellaro et al., 2022).

Time to viral clearance (negative SARS-CoV-2 RT-PCR test) is a marker of great interest to assess the efficacy of antiviral treatment. To our knowledge, this is the first study to show (in a clinical study with $F[ab']_2$ products) a decrease in the proportion of patients with viral RNA detected on days 8 to 11 (Deng et al., 2023), which had been suggested in experimental models with other coronaviruses and in other infections (Zhou et al., 2007; Zhao et al., 2017).

Although, the effects on mortality, use of mechanical ventilation, and other complications were not evaluable due to the absence of similar outcomes in the small group studied, we study and found no evidence of a decrease in hospital stay, like previous studies (Deng et al., 2023).

Pharmacokinetic parameters, including maximum concentration and T_{max} (1 to 3 hours), AUC to last time (21 to 28 days) with low residual area, and terminal elimination half-life (t1/2 = 47 hours) are as expected and reported. Widely with this type of products, in the same way that they allow expecting a sustained therapeutic effect with the dosage regimen used (Deng et al., 2023).

Inflammatory parameters show a gradual post-treatment reduction that is greater for α-IFN in the treated group. Although IL-6 and α-TNF levels also decreased with treatment, the baseline determination was already low and did not correlate with previously reported cases of severity (>30 pg/ml) (Galván-Román et al., 2021). From another point of view, cytokine concentrations are an additional safety parameter, showing that the patients did not present an increase in the profile of pro-inflammatory cytokines analysed (IL-6, TNFα) as a consequence of the administration of the research product (behaving similarly to the untreated control group). While the group of treated patients presented a significant decrease in IFNα plasma levels 14 days after starting treatment, which suggests a favourable evolution of the infectious process (Krämer et al., 2021).

It should be remembered that the research product (obtained from plasma horses) was processed to remove the Fc portion of IgG by pepsin digestion, followed by partial purification of the $F(ab')_2$ fragment, a proven technology used in anti-venom $F(ab')_2$ products with no records of hypersensitivity issues and decades of excellent safety records. The safety of the treatment was actively monitored and showed no differences between arms, similar to studies performed with plasma and monoclonal antibodies (Bal et al., 2015; Deng et al., 2023).

The presence of human heterophile antibodies against horse immunoglobulins (HHA-HI) in healthy individuals, not previously exposed to equine serum derivatives, has been previously demonstrated (Hennig et al., 2000; Herrera et al., 2005; Ayres et al., 2006). The HHA-HI are natural antibodies, capable of reacting against the $F(ab')_2$ fragment and whole horse IgG and correspond mainly to the IgG class (León et al., 2008; Sevcik et al., 2008; Pan et al., 2020). Previously, HHA-HI titter was compared between healthy volunteers and patients who received IgG or $F(ab')_2$ equine antivenom without finding differences before and after immunotherapy or between both groups (Piechotta et al., 2020). Albeit another study (Casadevall, 2002) reported titter increase at 14 and 28 days post immunization, the literature agrees that HHA-HI levels would not be related to anaphylactoid reactions. The increase in anti-equine antibodies in the detected cases does not induce an increase in the formation of immune complexes in a clinically relevant manner. Regarding the expression of the HHA-HI titter, it is worth mentioning that the cited works compare levels of HHA-HI at a single dilution of study serum, while in the present report, the HHA-HI titter is expressed in a more stringent, as AUC from a range of sample dilutions from 1/150 to 1/2,400. The presence of circulating HHA-HI reported in the present study is widely accepted in the literature and agrees with the data reported here on the 20 study patients. Although most patients do not change their HHA-HI levels after the administration of equine $F(ab')_2$, according to the results reported in this study, the production of anti-equine antibodies responds to the individual behaviour of each patient. Lastly, the existence of circulating HHA-HI constitutes a factor to be considered in the interpretation of the results of the study of the plasmatic concentration kinetics of equine $F(ab')_2$ in patients as well as in the neutralizing activity data observed in the same samples.

The emergence of strains with mutations in the spike protein has brought much concern, being able to affect the efficacy observed with monoclonal antibodies and vaccines, although possibly with less possibility of affecting the efficacy of preparations of

polyclonal origin. Even with the current availability of efficacious vaccines, the possibility of having equine antibody products may provide a therapeutic option to save lives in cases of patients with immunosuppression, insufficient immunization, and severe clinical pictures.

Hyperimmune $F(ab')_2$ concentrates are manufactured in existing facilities, using a long-proven platform technology, which could help accelerate the health system response facing an outbreak. The regulatory approval pathway could be simple, considering the existence of antivenoms regularly produced under the same conditions (same active pharmaceutical ingredient).

Limitations of the study

The hyperimmune equine $F(ab')_2$ developed provides high neutralization titters against previous known SARS-CoV-2 strains, meanwhile new strains appear periodically, so neutralizing potency should be reassessed regularly.

Until now, our study used a very small group of patients, appropriate for the pharmacokinetic evaluation and a number of inflammatory mediators and viral presence; however, it remains to continue with the trial and evaluate the effects in a larger number of patients with a multicenter design.

Conclusion

The study was able to include 20 initial patients and verify that although the primary objective has not yet been achieved, the disaggregated analysis shows that it would have been achieved in the WHO category 5 subgroup.

Regarding the secondary objectives: with the evaluated treatment, an acceptable pharmacokinetic and safety profile was verified, and a lower proportion of patients in category 4 and 5 early (day 3 to 5). There was no evidence of shorter duration of oxygen therapy, but there was evidence of improvement in arterial saturation measured between days 3 and 12, and a decrease in the proportion of patients with viral RNA detected on days 8 to 11. The effects on mortality, use of mechanical ventilation, and other complications were not evaluable. There was no evidence of a decrease in hospital stay.

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