A combination treatment of IFN-α2b and IFN-γ accelerates viral clearance and control inflammatory response in COVID-19: Preliminary results of a randomized controlled trial

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Abstract

**Background:** There is in vitro evidence that a combination of IFN-α and IFN-γ acts synergistically to inhibit SARS-CoV replication. We conducted a randomized controlled clinical trial to evaluate the safety and therapeutic efficacy of administration of a combination of IFN-α and IFN-γ for COVID-19.

**Methods:** Adults with confirmed COVID-19 were randomized to receive either subcutaneous treatment with 3.0 MIU IFN-α2b and 0.5 MIU IFN-γ (study group), twice a week for two weeks, or an intramuscular injection of 3.0 MIU IFN-α2b (control group), three times a week for two weeks. Additionally, all patients received lopinavir-ritonavir (200/50 mg orally twice daily) and chloroquine (250 mg orally twice daily). The primary endpoints were time to viral clearance and the progression to severe COVID-19. The protocol was approved by the Ethics Committee from the Hospital and National Regulatory Agency. Informed consent was obtained from each participant.

**Results:** A total of 79 patients with laboratory-confirmed SARS-CoV-2 infection, including symptomatic and asymptomatic cases, met the inclusion criteria. Patients were randomly assigned to receive either subcutaneous treatment with 3.0 MIU IFN-α2b and 0.5 MIU IFN-γ or an intramuscular injection of 3.0 MIU IFN-α2b. There were 30 subjects in the study group and 33 in the control group. 23 (78.6%) of participants in the study group were immediately hospitalized, compared to 21 (63.6%) in the control group. The early application of Heberon Alpha R can protect the patients to enter in a more severe disease.

**Conclusions:** A combination treatment of IFN-α2b plus IFN-γ in COVID-19 cases accelerated viral clearance and control inflammatory response compared with treatment with IFN-α2b alone. None of the patients progressed to severe COVID-19. The early application of Heberon Alpha R can protect the patients to enter in a more severe disease.

**Trial registration:** The study was registered on April 2020 at: registroclinico.sld.cu/en/trials/RPCEC00000307.

**Abbreviations**

CQ: Chloroquine; CIGB: Center for Genetic Engineering and Biotechnology; CECMED: Center for the State Control of Medicines; CPP: Creatine Phosphokinase; CRFs: Registered in the Case Report Forms; CRP: C-Reactive Protein; ITT: Intention to Treat; MIU: Million International Units; NLR: Neutrophil Lymphocyte Ratio; PT: Prothrombin Time; SII: Systemic Immune Inflammation Index

**Background**

SARS-CoV-2 infection that results in COVID-19 have spread in 190 countries around the globe, resulting in 171.271 million infected cases and 3.571 deaths, with devastating social and economic consequences [1]. In the absence of a vaccine, there is an urgent need to develop strategies to limit virus infection. The first cases of COVID-19 in Cuba were confirmed on March 11, 2020: three tourists from the Italian region of Lombardy, who were immediately hospitalized [2]. In Cuba, 12.0% of people who have been diagnosed with COVID-19 are under the age of 20. 53.9% of patients are asymptomatic, and even in the most vulnerable persons, comprising the 80-year-old group, 51.7% of those infected present with no symptoms at virus confirmation [3].

Type I interferons (IFNs)-α/β [4] and IFN-γ [5,6] exhibit antiviral activities. IFNs exert both direct antiviral effects on different viruses at different stages of their replicative cycles and also elicit an immune response to clear virus [4]. Notably, the severity of COVID-19 correlates with the failure to initiate an IFN response to SARS–CoV–2 infection [7].

Cognizant that a number of antivirals are under evaluation globally in clinical trials, the Cuban Protocol for Management of COVID-19 [2], includes Heberon Alpha R (IFN-α2b) and other potential antiviral treatments including lopinavir/ritonavir (Kaletra) and chloroquine (CQ), administered during the symptomatic phase of disease. Cuban patients who are symptomatic and their close contacts are isolated in centers established for that purpose, and receive treatment. On confirmation of a positive SARS–CoV–2 test, cases are hospitalized and continue or start to receive Heberon Alpha R, Kaletra, and CQ, as established by Cuban Health Ministry guidelines. In a cohort of 761 confirmed SARS–CoV–2 infected individuals who received Heberon Alpha R plus Kaletra and CQ, 95.4% recovered fully from COVID-19, with only a 0.92% case fatality rate [8].

Early studies with combination treatments of a type I IFN and IFN-γ revealed synergistic inhibition of SARS CoV replication in vitro [9–12]. IFN-γ is a critical regulator linking the innate and adaptive immune responses [13]. Moreover, combination treatments with antivirals that target different stages of the virus replicative cycle [14–16] and IFN-α2b and IFN-γ may synergize further to accelerate viral clearance. Accordingly, we conducted a phase II randomized clinical trial to evaluate whether a combination of IFN-α2b and IFN-γ, in addition to standard of care would offer a therapeutic advantage in COVID-19 cases, promoting faster viral clearance and preventing progression to severe disease.

**Methods**

**Study design and patients**

Adult (19–82 yrs), PCR(+) confirmed SARS-CoV-2 infected cases were enrolled in this open-labeled, single center, prospective, randomized and controlled clinical trial at Military Central Hospital Luis Diaz Soto Hospital, Havana, Cuba. Patients were randomly assigned to receive either subcutaneous treatment with a co–hyphosphilized combination of 3.0 million international units (MIU) IFN-α2b and 0.5 MIU IFN-γ (HeberFERON®, Cuba Center for Genetic Engineering (CIGB), Havana, Cuba), twice a week for two weeks, or an intramuscular injection of 3.0 MIU IFN-α2b (Heberon® Alpha

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R, CIGB, Havana, Cuba), three times a week for 2 weeks. Additionally, all patients received standard of care, which included lopinavir/ritonavir (200/50 mg orally twice daily and chloroquine (250 mg orally twice daily). Patients were randomized individually to one of the two treatment arms by means of random computer-generated lists, with an allocation ratio of 1:1, with block sizes of six patients, based on a power calculation of 80%, and a level of confidence set at 95%, while also considering a dropout rate of 5%.

Heberon® Alpha R has been produced in Cuba by the CIGB for the past 34 years, with proven antiviral efficacy and an adequate safety profile [17]. HeberFERON® (IFN-α2b and IFN-γ2, co-lyophilized in the same vial) is produced at CIGB, and has been registered in Cuba since 2016 for the treatment of basal cell carcinoma [18].

Study execution conformed with the ethical principles of the Declaration of Helsinki and the International Council for Harmonization of Good Clinical Practice guidelines. No compensation was provided for enrollment in the trial. Patient personal data were protected. The authors were responsible for designing the trial and for collecting and analyzing the data. The authors assured the completeness and accuracy of the data collection and adherence to the protocol. The details of the trial are provided in the protocol that has been posted in TRIALS [19]. Primary endpoints were the time to viral RNA elimination from the start of treatment and the time to progression to severe COVID-19.

**Eligibility criteria**

A COVID-19 diagnosis was obtained by a positive real-time reverse transcription–polymerase chain reaction (RT-PCR) amplification of the viral E gene and then confirmation by amplification of the RdRP gene, from throat swab samples. Adults (≥18yrs), confirmed PCR(+) for SARS-CoV-2, with ECOG functional status ≥2 (Karnofsky ≥ 70%) and who signed informed written consent, were included. Patients with any of the following characteristics were excluded: decompensated chronic diseases at the time of inclusion (severe arterial hypertension, ischemic heart disease, diabetes mellitus, and a history of autoimmune disease), presence of hyperinflammatory response, known hypersensitivity to any of the components of the formulations under evaluation, pregnancy or lactation, and obvious mental incapacity to issue consent.

The clinical trial protocol was approved by the Ethics Committee on Clinical Investigation of Military Central Hospital Luis Diaz Soto, and the Center for the State Control of Medicines, Equipment and Medical Devices (CECMED) in Cuba. Patients were asked for written consent to participate after having been duly informed about the nature of the trial, objectives, benefits and possible risks. They were informed of their rights to participate or not, and to withdraw their consent at any time, without exposing themselves to any limitations on their medical care or other retaliation. The study was registered on April 2020 at: registroclinico.sld.cu/en/trials/ RPCEC00000307.

After a preliminary exploratory analysis of the outcomes of the first 79 patients, the monitoring board considered a preliminary report and early publication of the RT-PCR results from the 63 patients with available throat swabs, based on the significant effect of HeberFERON treatment on reduction of time to viral clearance. The trial has closed with 134 participants, from whom bio-samples are now being evaluated.

**Procedures**

Study participants received either 3.0 (MIU) IFN-α2b and 0.5 MIU IFN-γ (HeberFERON®), twice a week for two weeks, by subcutaneous injection, together with lopinavir–ritonavir 200/50 mg orally twice daily and CQ 250 mg, orally twice daily (treatment group), or 3.0 MIU IFN-α2b (Heberon® Alpha R), administered intramuscularly three times a week for two weeks, and lopinavir–ritonavir 200/50 mg orally twice daily and CQ 250 mg orally twice daily.

Demographic, clinical, laboratory, treatments and outcome characteristics of patients were extracted from medical record, registered in the case report forms (CRFs) and then entered in duplicate (independently by two operators) for the subsequent process of automatic comparison and cleaning of the database. Whereas operator blinding was not feasible, blinding was in place for laboratory SARS-CoV-2 RNA detection by RT-PCR, one of the endpoints of the study.

The hospital accepted patients from several zones in Havana City, and their RT-PCR test results were recorded in reference centers for SARS-CoV-2 infection, following the Cuban Ministry of Health guidelines for diagnostic testing. Patients were confirmed SARS-CoV-2 infected if they had two consecutive PCR(+) results, including the confirmatory test by RT-PCR targeting amplifications of E and/or RdRP genes. A cycle threshold up to value 40 was defined as positive.

Throat swab specimens were transported to a BSL2 certified laboratory at the CIGB for SARS-CoV-2 viral nucleic acid detection by RT-PCR, using the QIAamp Viral RNA Mini kit (Qiagen, USA), as per the manufacturer’s instructions. A multiplexed RT-PCR detection targeted the E and/or RdPR genes, using an EAV internal extraction control (TIB MOLBIOL Synthese labor GmbH, Berlin, Germany), as described for the Multiplex RNA Virus Master (Roche, USA).

Routine blood examinations were performed at the Clinical Laboratory of the Military Central Hospital Luis Diaz Soto and included whole blood counts, coagulation profiles, serum biochemical tests (including renal and liver function, electrolytes, and coagulation), C-reactive protein (CRP) and ferritin levels. All patients had a chest X-ray. The frequency of examinations was defined in the trial protocol and consisted of baseline (after randomization and before the administration of first dose of the IFNs) and weekly determinations, on days two and four of each week.

**Outcomes**

Primary outcomes included virologic and clinical evaluations. Throat swabs were collected at 48, 72, 96 and 120 hours post treatment onset and the presence of SARS-CoV-2
RNA assayed by RT-PCR. Time to viral clearance from start of antiviral therapy was evaluated; clinical evaluations included worsening of clinical symptoms and time to hospital discharge.

**Statistical analyses**

Quantitative variables were plotted as arithmetic means and standard deviations, and the median with IQR range. Absolute and relative frequencies (%) for qualitative variables were measured. The hypothesis test used was Fisher’s exact test. Time to viral clearance was analyzed using Kaplan–Meier plots and the comparisons used Mantel-Cox Log Rank and Gehan-Breslow-Wilcoxon tests. Changes in laboratory parameters were analyzed using a paired mixed model (which cannot adjust for missing values due to patient release from hospital). Correlations between time to viral clearance and laboratory parameters were studied using a two-tailed non-parametric Spearman correlation with a 95% confidence interval (CI). *p*<0.05 was considered statistically significant. All statistical analyses were performed using the Windows software package SPSS (version 25) and GraphPad Prism v8.0.

**Results**

Between Apr 11, 2020, and May 13, 2020, 144 PCR(+) COVID-19 cases were screened for trial eligibility (Figure 1). 57 patients were ineligible: one with icterus, one with chronic decompensating renal insufficiency, two unconfirmed SARS-CoV-2 PCR results, and 53 PCR(+) cases with persistent viral shedding beyond 21 days. Eight patients did not consent and were therefore excluded. 79 subjects met the inclusion criteria and were randomly assigned (1:1) to either the HeberFERON group (41 patients) or the Heberon Alpha R group (38 patients). 12 patients failed to start treatment, seven patients refused to start treatment, despite having consented, and five were excluded due to later ineligibility. Seven patients withdrew: three due to worsening respiratory symptoms (two in the HeberFERON group, with asthma as the underlying disease), three patients from the Heberon Alpha R group who remained PCR(+) on day 14 of treatment (administered HeberFERON outside of the clinical trial), and one patient who was pregnant, in the Heberon Alpha R group. Four patients were excluded: three in the HeberFERON group, based on PCR negativity prior to trial entry (1 detected pregnant, 3 positive PCR at day 14 and switched to HeberFERON out of the trial) and one worsening of symptom (42 discontinued interventions (switched to anti-anti-inflammatory treatment due to asthma symptoms worsening)).

**Figure 1:** Trial profile.
to start of treatment, and one in the Heberon Alpha R group who refused throat swab sampling. 34 patients received standard of care which included Heberon Alpha R (control group) and 33 patients were treated with HeberFERON plus standard of care.

In summary, 30 and 33 patients were analyzed by intention to treat (ITT) in the HeberFERON and Heberon Alpha R groups, respectively. Refer to Table 1 for patient characteristics. In the Heberon Alpha R group the median age was 31.0 years (IQR: 22-47), and for the HeberFERON group the median age was 42.0 years (IQR: 27-56) (p=0.023). The Heberon Alpha R cohort comprised 60.6% males (20/33) compared with 46.7% males in the HeberFERON group (14/30) (Table 1). These sex differences between HeberFERON and Heberon Alpha R groups were not statistically significant. More symptomatic patients (51.5%) were in the Heberon Alpha R group than in the HeberFERON group (40.0%), but this difference was also not statistically significant.

The median age of symptomatic subjects was higher in the HeberFERON group (50yrs [IQR: 28-67]) than in the Heberon Alpha R group (42yrs [IQR: 21-45]). In symptomatic patients, the sex distribution was not statistically significant in the whole cohort: 51.7% males, 48.3% females (p=0.093). However, 66.7% were females in the HeberFERON treated group compared with 35.3% in the Heberon Alpha R treated group. Patients in the HeberFERON group had symptoms for >7 days, compared with 35.3% in the Heberon Alpha R group, but this difference in symptom duration was not statistically significant. In asymptomatic patients a difference in sex distribution (p=0.002) was detected (Table 1).

The more common symptoms were fever and unproductive cough (16.4%), followed by headache (9.6%), weakness (8.4%), odynophagia and nasal secretions (5.4%), diarrhea, dyspnea, chills and general malaise (4.1%), and sore throat and myalgia

<table>
<thead>
<tr>
<th>Table 1: Study participants demographics and clinical characteristics.</th>
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</thead>
<tbody>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Median age (IQR) yr¶</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male (%)</td>
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<tr>
<td>Female (%)</td>
</tr>
<tr>
<td>Symptomatic (%)</td>
</tr>
<tr>
<td>Median age (IQR) yr</td>
</tr>
<tr>
<td>Male (%)</td>
</tr>
<tr>
<td>Female (%)</td>
</tr>
<tr>
<td>Mean time (SD) from symptom onset to randomization, days</td>
</tr>
<tr>
<td>Number of symptoms</td>
</tr>
<tr>
<td>&lt;3 (%)</td>
</tr>
<tr>
<td>≥3 (%)</td>
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<tr>
<td>Time from symptoms onset to start of treatment, days (%)</td>
</tr>
<tr>
<td>&gt;7 days</td>
</tr>
<tr>
<td>≤7 days</td>
</tr>
<tr>
<td>Asymptomatic (%)</td>
</tr>
<tr>
<td>Median age (IQR) yr</td>
</tr>
<tr>
<td>Male (%)</td>
</tr>
<tr>
<td>Female (%)</td>
</tr>
<tr>
<td>Coexisting conditions (%)</td>
</tr>
<tr>
<td>Any comorbidities</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Cardiac diseases</td>
</tr>
<tr>
<td>Asthma</td>
</tr>
<tr>
<td>Diabetes</td>
</tr>
<tr>
<td>Baseline Treatments (%)</td>
</tr>
<tr>
<td>Previous IFN-α-2b vials (3.0 MIU)</td>
</tr>
<tr>
<td>≥2 (%)</td>
</tr>
<tr>
<td>≥3 (%)</td>
</tr>
<tr>
<td>Lopinavir-ritonavir at baseline</td>
</tr>
<tr>
<td>Chloroquine at baseline</td>
</tr>
<tr>
<td>Antibiotic treatment at baseline</td>
</tr>
<tr>
<td>Vital signs</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg) (IQR)</td>
</tr>
<tr>
<td>Cardiac frequency (IQR)</td>
</tr>
<tr>
<td>Respiratory rate (breaths/minute) (IQR)</td>
</tr>
<tr>
<td>Temperature (°C) (IQR)</td>
</tr>
<tr>
<td>Radiological signs</td>
</tr>
<tr>
<td>RX Normal (%)</td>
</tr>
<tr>
<td>Abnormal (%)</td>
</tr>
</tbody>
</table>
(2.7%). 50.8% of all patients had co-morbidities, the most frequent of which were hypertension (22%), asthma (6.3%), diabetes and glaucoma (4.7%).

No other significant differences in symptoms, coexisting conditions, treatments, vital and radiological signs (Table 1), or laboratory results (Table 2), were observed between the treatment groups at baseline.

Adverse events were identified in 18 (31.5%) of 57 patients analyzed, eight (28.5%) in the Heberon Alpha R group and ten (34.4%) in the HeberFERON group (Table 3). The most common adverse events were headache (17.4%), nausea, hypertension, retro-orbital pain and burning eyes (3.5%). Nighty-four percent of adverse events were mild and none were severe. There were no differences between the incidence or duration of any of the adverse events between the treatment groups. No serious adverse events were reported and no patients died during the study.

We analyzed 63 patients with available throat swab samples. In the HeberFERON group 78.6% of patients were negative for the virus after four days of treatment, versus 40.6% of patients in the Heberon Alpha R group (p=0.004) (Table 3, Table 1S). HeberFERON eliminated SARs-CoV-2 in 95.8% of patients by day 5 post treatment onset (p=0.0479). The median time to viral clearance for the HeberFERON treated groups (Figure 2) was 3 days and for the Heberon Alpha R treated patients was 5 days (p= 0.002).

When viral clearance was evaluated at 96hrs after treatment onset, stratifying by the presence or absence of symptoms, 12 of 13 (92.3%) and 6 of 17 (35.3%) symptomatic patients were PCR(-) in the HeberFERON and Heberon Alpha R groups, respectively, (p=0.002) (Table 1). Moreover, the median time to viral clearance from onset of symptoms was 8 days for the HeberFERON treated cases compared with 14 days for the Heberon Alpha R cases (p=0.0182, HR 2.041, 95% CI 0.964-5.86) (Figure 1). Additional stratification of patients, based on start of treatment either within 7 days of symptom onset or after 7 days of symptoms, identified a better viral clearance for HeberFERON treated patients with less than 7 days of symptoms (Mantel Cox log-rank test, p=0.0007, HR 16.2; 95% CI, 3.243-80.97). Moreover, table refers that HeberFERON treatment was superior to Heberon Alpha R treatment when administered within 7 days of symptom onset (Mantel Cox log-rank test p=0.0002, HR 16.13; 95% CI, 3.653-71.22).

In asymptomatic patients, the rate of viral clearance was slower for patients in both treatment groups. However, HeberFERON treatment at 96hrs resulted in viral clearance in 11 of 16 patients (68.8%) compared with 7 of 15 (46.6%) in the Heberon Alpha R group (p=0.285) (Table 1). Worsening of respiratory symptoms was observed in 2 (6.6%) patients in the HeberFERON group and in one (3.3%) patient in the Heberon Alpha R group. Two male COVID-19 cases from the HeberFERON group experienced worsening respiratory symptoms. These patients were asymptomatic.

### Table 2: Laboratory baseline data.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HeberFERON</th>
<th>Heberon Alpha R</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell count (WBC), × 10⁹ per L</td>
<td>N</td>
<td>median (min-max)</td>
<td>N</td>
</tr>
<tr>
<td>Lymphocyte percentage (%)</td>
<td>30</td>
<td>6.4 (3.8-11.7)</td>
<td>32</td>
</tr>
<tr>
<td>Lymphocyte count, × 10⁹ per L</td>
<td>30</td>
<td>36.5 (11-57)</td>
<td>32</td>
</tr>
<tr>
<td>Monocyte percentage %</td>
<td>30</td>
<td>0.56 (0.051-131)</td>
<td>32</td>
</tr>
<tr>
<td>Granulocyte percentage %</td>
<td>30</td>
<td>50.5 (29-81)</td>
<td>32</td>
</tr>
<tr>
<td>Granulocyte counts, × 10⁹ per L</td>
<td>30</td>
<td>3.28 (1.421-6.58)</td>
<td>32</td>
</tr>
<tr>
<td>Platelet count, × 10⁹ per L</td>
<td>30</td>
<td>223 (117-351)</td>
<td>32</td>
</tr>
<tr>
<td>Platelet to Lymphocyte Ratio (PLR)</td>
<td>30</td>
<td>104.7 (34-327.7)</td>
<td>32</td>
</tr>
<tr>
<td>Neutrophils to Lymphocyte Ratio (NLR)</td>
<td>30</td>
<td>1.4 (0.5-6.8)</td>
<td>32</td>
</tr>
<tr>
<td>Neutrophil × lymphocyte: Systemic Immune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation Index (SII)</td>
<td>30</td>
<td>319.4 (115-2075)</td>
<td>32</td>
</tr>
</tbody>
</table>

on admission, both 33 years-old of age, with asthma as co-morbidity. One male received three doses of HeberFERON, the other, four. The male who received three doses of HeberFERON became PCR(−) at 48hr post onset of treatment, the other who received four doses, at 96hrs post onset of treatment. During the days of symptoms worsening, local climate conditions favoured exacerbation of their asthma. Anti-inflammatory treatment for asthma led to rapid resolution of asthmatic symptoms, by 48hr. In the Heberon Alpha R group an 80 year old symptomatic male with hypertension received only one dose of Heberon Alpha R. His respiratory symptoms worsened, requiring a transfer to the ICU. He became PCR(−) 20 days after symptoms worsening and was subsequently discharged.

By day 14, day of discharge, 100% of the patients had cleared virus in the HeberFERON group, compared with 91% in the Heberon Alpha R group (Table 3). Notably, no patient in either treatment group progressed to severe COVID-19 (Table 3). No differences in the evolution of vital signs were detected between groups (Table 3).

Close scrutiny of a number of clinical measures reveals differences between those patients that received IFN-α2b plus IFN-γ (HeberFERON) and those who only received IFN-α2b (Heberon Alpha R), along with standard of care, beyond faster viral clearance. Lymphocyte concentration was similar during the treatment time course for both cohorts of patients (Figure 3A). In the first seven days following treatment onset, those patients who received HeberFERON saw significant increases (p=0.0058) in the percentage of their lymphocytes that exceeded the increases observed for Heberon Alpha R patients (Figure 3B). For Heberon Alpha R treated patients, a significant increase in the percentage of lymphocytes was achieved by 2 weeks post treatment onset (p=0.011). For both the HeberFERON and Heberon Alpha R treated patients, treatment decreased circulating levels of neutrophils, significantly (p=0.0019). The data in Figure 3C and 3D suggest that the majority of Heberon Alpha R treated patients became neutropic.

Additionally, HeberFERON treatment significantly reduced the neutrophil:lymphocyte ratio (NLR) in the first week of treatment (p=0.0264), which was normalized by the end of the second week of treatment (p=0.0148). By contrast, the NLR remained low in Heberon Alpha R treated patients (p=0.0032) (Figure 4A). A measure of inflammation is the systemic immune inflammation index (SII), which considers platelet, neutrophil and lymphocyte levels. The SII remained within normal range for all patients treated with HeberFERON and Heberon Alpha R (Figure 4B). With the exception of one patient who received HeberFERON, ferritin levels remained within normal range (30–400μg/L) for all patients (Figure 4B). For those patients with elevated creatine phosphokinase (CPK) levels at start of treatment, they returned to normal range following both HeberFERON and Heberon Alpha R treatment (Figure 5C). Prothrombin time (PT) remained within normal range for all patients treated with HeberFERON and Heberon Alpha R (Figure 5D).

In a final series of analyses we examined whether the rate of viral clearance correlated with increases in lymphocyte levels, and reductions in serum factors associated with severe disease. (Table 4 and figure 6).
Figure 3: IFN effects on circulating levels of lymphocytes and neutrophils. (A) Absolute Lymphocyte count and (B) percentage of total leukocytes, (C) Absolute neutrophil count and (D) percentage leukocytes, in HeberFERON (blue) and Heberon Alpha R (red) treated patients prior to (0) and at weekly intervals post onset of treatment. The dotted lines for A and C indicate the upper range of normal (2 upper) and lower range of normal (2 lower), the dotted lines in the plots B and D indicate the normal range.

Figure 4: IFN treatment regulates inflammation in COVID-19. Effects of HeberFERON and Heberon Alpha R treatment on neutrophil-lymphocyte ratio (NLR) and platelet × neutrophil /lymphocyte (Systemic Immune inflammation Index: SII) during the course of treatment. Dotted lines indicate upper and lower limits of normal ranges.
In a final series of analyses we examined whether the rate of viral clearance correlated with clinical laboratory parameters. This correlation was analysed using a two-tailed non-parametric Spearman correlation with 95% confidence interval. Table 4 summarizes the parameters identified with significant direct or indirect relation with the reduction in the time needed to achieve a negative PCR result. We detected in the group of patients treated with HeberFERON® a direct correlation between lymphocytes concentration (p=0.0050) and PLR index (p=<0.0001) and viral clearance (Figure 6).

Discussion

Our study had several limitations. This trial was open label, without a placebo group and with unbalanced demographics (age) between treatment arms. In addition, throat swab sampling for detection of SARS CoV–2 does not reflect infection burden in the lower respiratory airways; one published report provided evidence for lower viral loads in throat swab specimens [20]. Irrespective of these limitations, the findings from this trial are that HeberFERON plus Kaletra and CQ treatment for COVID-19 is safe and results in faster viral clearance than treatment with Heberon Alpha R. Specifically; a combination treatment of IFN–α2b with IFN–γ promotes viral clearance more rapidly than IFN–α2b alone and exerts anti-inflammatory effect.

There is accumulating evidence that for COVID-19 there is an asymptomatic incubation period, with or without detectable viral RNA, followed by non-severe symptomatic conditions and detectable virus, which in some individuals progresses to a severe symptomatic stage with high viral load [21]. The
asymptomatic incubation period of 4–7 days is associated with aggressive transmission of the virus [22,23]. Added to this, some infected patients remain asymptomatic and yet are fully capable of transmitting virus [24–26]. Asymptomatic individuals pose the greatest threat to viral transmission, not only because they remain undetected in the general population, spreading infection, but also because there is evidence that viral shedding persists longer in asymptomatic versus symptomatic COVID-19 cases [27,28] this despite there being no evidence of higher viral loads in asymptomatic versus symptomatic COVID-19 cases [22,23]. We provide evidence of rapid viral clearance with HeberFERON treatment for both symptomatic and asymptomatic COVID-19 cases. By 48hrs after onset of treatment with HeberFERON, viral clearance was achieved in 45% of cases, and by day five in 96% of cases. Notably, a combination treatment of lopinavir/ritonavir, ribavirin and IFN-β did not achieve this rate of viral clearance [16], with an average of seven days to viral clearance, and the combination of lopinavir/ritonavir reportedly has no therapeutic advantage for course of disease [29,30]. Our results with Heberon Alpha R in combination with lopinavir/ritonavir and COX show viral clearance by day five in 76% of cases. In another exploratory clinical study, inhaled IFN-α2b treatment resulted in accelerated viral clearance in COVID-19 cases, on average seven days sooner than in those cases treated with the antiviral arbidol [31]. Recently, findings from a randomized controlled trial of treatment with Novaferon, a novel protein generated by shuffling the cDNAs from IFN-αs [12] revealed that Novaferon accelerated viral clearance by 3 days when compared with treatment with lopinavir/ritonavir [32]. Systemic viral dissemination is an important determinant in severe disease [33]. HeberFERON treatment, by accelerating viral clearance, may prevent disease progression, as noted in all COVID-19 cases in this trial.

The timing of initiation of antiviral therapy is a critical factor in the treatment of viral infections. With respect to SARS-CoV, no effect of several antiviral drugs was observed when the treatments were started 6–14 days after symptom onset [34] However, when SARS-CoV patients were treated with an IFN-α between 4–10 days post symptom onset, rapid resolution of disease was observed [35]. Early administration of antiviral medications may improve outcomes for COVID-19 cases [36]. Early IFN treatment was recommended for MERS [37] and late therapy (10–22 days) may contribute to poor
outcomes [38]. Certainly, rapid viral clearance may prevent the exacerbated inflammatory response associated with severe disease. The Cuban Protocol for Management of COVID-19 [2] enabled us to include patients within 7 days of symptoms onset. In the HeberFERON treated group, faster virus elimination was obtained in patients with less than 7 days of symptoms compared with those who had symptoms for longer than 7 days. Notably, HeberFERON treated patients showed faster viral clearance compared with Heberon Alpha R treated patients, when treatment was started within 7 days of symptom onset.

A recent open-label trial reported no therapeutic benefit when hospitalized adults patients with severe COVID-19 were treated with lopinavir/ritonavir. However, closer scrutiny of the data suggests some clinical improvement, particularly in those cases treated within 12 days of symptom onset [39]. The combination of lopinavir/ritonavir with other antiviral agents, as reported for the treatment of SARS, 39 MERS-CoV [40] and the trial reported herein, might enhance antiviral responses and improve clinical outcomes. Early treatment with lopinavir/ritonavir and IFN was associated with more favorable clinical outcomes than the use of lopinavir/ritonavir alone in COVID-19 patients [38]. The apriori time to start the antiviral treatment could be determinant in the decease outcomes.

Recent reports have provided evidence for a blunted type I IFN response to SARS-CoV-2 infection [23] and that genetic variant in TLR7 that limit an IFN response correlate with severe disease in adults with COVID-19 [41]. Most recently, studies have identified inborn errors of type I IFN immunity in a proportion of patients with severe COVID-19 [42] and the presence of autoantibodies against type I IFNs, also in a proportion of patients with life-threatening COVID-19 [43]. Viewed together, the rationale for HeberFERON therapy for COVID-19 to overcome this blunted IFN response becomes evident.

In COVID-19, lymphopenia has been associated with a restricted immune response in the lungs, and worsening disease [44]. Our data suggest that treatment with IFN-α2b or IFN-α2b plus IFN-γ maintained the absolute number of circulating lymphocytes and this may have contributed to rapid viral clearance. Indeed, there is evidence that IFN-α regulation of STAT4 in CD8+ T cells may drive antigen-specific responses required for viral clearance [45]. Additionally, IFN-γ could contribute to T cells homeostasis during infections, modulating tissue inflammation [46] and to protective immunity against COVID-19 [47].

Moreover, an antiviral activity associated to platelet has been described in patients with severe infection of the lung, by stimulation of immune cells and activating the complement system [48] a property that can explain the correlation between PLR index and the viral negativization in the patients treated with HeberFERON.

The presence of IFN-γ in the HeberFERON formulation, in addition to exerting strong immune regulatory functions, may also restrict angiotensin-converting enzyme 2 (ACE2) expression [49] a receptor for SARS-CoV-2 cell entry [50]. IFN-γ inhibits viral entry for several viral infections, including HCV and HIV [51] by controlling the expression and/or distribution of viral entry receptors. CQ may also limit cell entry, as several studies have shown that CQ lowers endosome pH that may then inhibit the fusion SARS-CoV with cell membranes, interfering with viral entry [52,53]. Moreover, CQ may diminish the IFN-γR1 turnover in the lysosomes, stimulating the IFN-γ mediated STAT-1 signaling [53]. Additionally, IFN-γ may organize an immune antiviral response in every organ and effectively clear virus-infected cells in whole organs and disseminated multi-organ invasion [55].

Intriguingly, there is evidence that type I IFNs upregulate expression of ACE2 [56] yet emerging evidences suggests that if this is the case, this does not appear to prevent the antiviral effects of IFN-α treatment in COVID-19 cases, and may confer protection from tissue damage [51,57].

A major concern relating to the use of IFN-α/β for the treatment of COVID-19, given that an exacerbated lung inflammatory response is associated with severe disease, is the notion that these IFNs will induce a cytokine storm to create an inflammatory response. In several published reports, where IFNs-α/β were used as antiviral therapeutics for SARS [35] ebola virus infection [58] and more recently, COVID-19 [29] there was no evidence of IFN-induced cytokine storms or exacerbation of inflammation. Indeed, when COVID-19 cases were treated with an inhaled IFN-α2b, evidence was provided for reductions in circulating levels of IL-6 and CRP, both biomarkers of inflammation [29].

As serum CRP levels are not affected by age, sex, and physical condition and levels correlate with degree of inflammation, 59 circulating CRP serves as a biomarker for severe pulmonary infection [60–62] In early stage COVID-19, increases in CRP levels may reflect lung lesions and disease severity. Treatment with just 2 doses of HeberFERON resulted in a significant reduction in circulating CRP levels. Other pro-inflammatory factors, such as neutrophils and the NLR index, were also regulated by HeberFERON treatment, remaining within normal ranges, as were ferritin levels, where elevated levels have been associated with severe disease. A proportion of COVID-19 cases have coagulation abnormalities and thrombotic microangiopathy, reflected in prolonged prothrombin times that are associated with decreased survival and increased need for intensive care [23, 63].

We provide evidence that treatment with IFN-α2b plus IFN-γ reduced prothrombin time. The inhibition of human platelet aggregation by CQ [64] may also contribute to anti-thrombotic effect in concert with HeberFERON, likely reducing the risk of disease progression.

About 15% of COVID-19 cases progress to severe disease, with a higher risk for patients over 65 years of age [22]. Extrapolating to this study, we would have anticipated that 9 of the 63 patients included in our study would have been expected to develop severe disease. However, no patient became severely
ill and there were no deaths. In a similar Cuban cohort of COVID-19 patients, treated early with IFN-α2b and standard of care, mortality rate was reduced to 0.9% [8].

It has been suggested that IFN treatment will only be effective in patients who lack co-morbidities [34,65] and that co-morbidities, such as diabetes, will diminish any response to IFN [64]. However, we provide evidence for rapid viral clearance, resolution of disease symptoms, and hospital discharge for all patients treated with HeberFERON, a cohort that included 57% of cases with existing co-morbidities. Indeed two diabetic patients in our cohort cleared virus by day 3 and before day 14 of treatment, respectively.

The favorable pharmacodynamics of HeberFERON treatment [18] are associated with a regimen that involves few doses and lower dosing of IFN-α2b and IFN-γ than is traditionally used when they are administered as monotherapies. The findings presented herein indicate that HeberFERON® was a safe, well-tolerated treatment, and superior to Heberon Alpha R in shortening the time to SARS-CoV-2 viral clearance in a cohort of both symptomatic and asymptomatic COVID-19 patients, ages 19 – 82 years. Viral clearance was achieved in 95% of patients within five days of treatment onset. Our findings indicate that this rapid viral clearance resulted in a blunted inflammatory response, suggested by reduced CRP, CPK, TP levels and NLR index. The combination of IFN-α2b plus IFN-γ while promoting host resistance to viral infection, control the inflammatory response which prevented disease progression in all patients.

Conclusions

The findings reported herein are the first to suggest therapeutic efficacy in COVID-19 for a combination treatment of IFN-α2b and IFN-γ with clear evidence of viral clearance, inflammatory control and no progression to severe disease or death. The use of HeberFERON combined with Kaletra and chloroquine could be a promissory approach to the early treatment of patients positive to SARS-CoV-2, symptomatic or not.

Declarations

The clinical trial protocol was approved by the Ethics Committee on Clinical Investigation of Military Central Hospital Luis Diaz Soto, and the Center for the State Control of Medicines, Equipment and Medical Devices (CECMED) in Cuba. Patients were asked for written consent to participate after having been duly informed about the nature of the trial, objectives, benefits and possible risks. They were informed of their rights to participate or not, and to withdraw their consent at any time, without exposing themselves to any limitations on their medical care or other retaliation.

Study execution conformed to the ethical principles of the Declaration of Helsinki and the International Council for Harmonization of Good Clinical Practice guidelines. No compensation was provided for enrollment in the trial. Patient personal data were protected. The authors were responsible for designing the trial and for collecting and analyzing the data.

Availability of data and materials

Qualified individuals may request access to the de-identified participant data, anonymized clinical study reports, informed consent forms, and related documents including the study protocol, through submission of a proposal with a defined research question to the corresponding author, Bello-Rivero Iraldo, provided that the necessary data protection and ethical committee approvals are in compliance with the trial registration. An agreement for transfer of these data will be required.

Authors’ contributions

LBR, JCR, LCC, EPC, STP, were the principal investigators that conducted the trial, recorded the clinical histories of patients, and supervised each team of health workers that managed COVID-19 cases; IEM, MDG, ASM, MGS, SMM, JPE, ABC, MVT, coordinated, executed and supervised the trial at the hospital; YDR, CMS, ICL were responsible for monitoring the trial and conducted quality checks, and interpretation of clinical data. DVB was responsible, executed and analyzed data from SARS-CoV-2 viral RT-PCR determinations wrote reports and contributed to proofreading the manuscript. HCR and JRFM were responsible for SARS-CoV-2 viral RT-PCR evaluations; MBR, CACH, and IMG evaluated and analyzed clinical laboratory data and contributed to proofreading the manuscript. MAV was responsible for data base and its quality, DBG was responsible for statistical analyses. GGN, HNC and FHB contributed to the design of trial protocol, VLMG supervised all the study steps for GCP. ENF analyzed the clinical and virologic data and significantly contributed to the writing of the final versions of the manuscript. IBR designed and wrote the protocol, supervised the trial, interpreted and discussed all the trial data and results, and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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