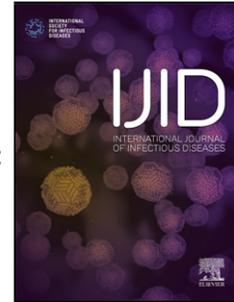# Journal Pre-proof

SARS-CoV-2 Clearance in COVID-19 Patients with Novaferon Treatment:
A Randomized, Open-label, Parallel Group Trial

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Highlights

- Novaferon considered as a potential antiviral drug for COVID-19

- Novaferon inhibited viral replication and protected cells from SARS-CoV-2 attack.

- Antiviral effects of Novaferon for COVID-19 patients observed in randomized trial

- Inhalation of Novaferon for COVID-19 treatment was safe.

Abstract

Background

The anti-viral effects of Novaferon, a potent antiviral protein drug on COVID-19 was evaluated in laboratory, and in a randomized, open-label, parallel group trial.

Methods

In laboratory, the inhibition of Novaferon on viral replication in cells infected with SARS-CoV-2, and on prevention of SARS-CoV-2 entry into healthy cells was determined. Antiviral effects of Novaferon in COVID-19 patients with treatment of Novaferon, Novaferon plus Lopinavir/Ritonavir, or Lopinavir/Ritonavir were evaluated. The primary endpoint was the SARS-CoV-2 clearance rates on day 6 of treatment, and the secondary endpoint was the time to SARS-CoV-2 clearance.

Results

Novaferon inhibited the viral replication(EC_{50}=1.02 ng/ml), and prevented viral infection(EC_{50}=0.10 ng/ml).Results from the 89 enrolled COVID-19 patients showed that both Novaferon and Novaferon plus Lopinavir/Ritonavir groups had significantly higher viral clearance rates on day 6than Lopinavir/Ritonavir group (50.0% vs.24.1%, p = 0.0400, and 60.0% vs.24.1%, p = 0.0053). Median time to viral clearance were 6 days, 6 days, and 9 days for three groups respectively, a 3-dayreductionin both Novaferon and Novaferon plus Lopinavir/Ritonavir groups compared with Lopinavir/Ritonavir group.

Conclusions

Novaferon exhibited anti-SARS-CoV-2 effects in vitro and in COVID-19 patients. These data justified the further evaluation of Novaferon.

Trial registration number: number ChiCTR2000029496at the Chinese Clinical Trial Registry(http://www.chictr.org.cn/).

Key words

COVID-19, SARS-CoV-2, Novaferon, Antiviral drug, Lopinavir/Ritonavir, Viral clearance, Aerosolized inhalation

INTRODUCTION

The deadly pandemic of COVID-19 caused by the infection of a novel coronavirus, SARS-CoV-2, represents a major health challenges around the world(WHO 2020; Zhu et al., 2020; Lu et al., 2020; Wu et al., 2020).The current failure on the containment of COVID-19was partially due to the lack of effective antiviral drugs for COVID-19. Such antiviral drugs, if administrated to early stage patients or to patients with mild and moderate illness, are reasonably expected to speed up the viral clearance. As a consequence, complete clearance of SARS-CoV-2 will lead to either the earlier recovery or to the reduction of the severe illness. In addition, the elimination of viral shedding following the viral clearance inpatients would also help to reduce viral transmission. Given the immediate availability and established safety profiles, approved antiviral drugs for other indications were repurposed in order to find effective anti-SARS-CoV-2 drugs in the shortest time possible.(Zhou et al.,2020).However, none of the tested or recommended antiviral drugs has been proved effective yet. Most published findings for the antiviral treatment of COVID-19 were based on the individual case reports or cellular antiviral results(Li et al.,2020;Holshue etal.,2020).Despite the lack of convincing evidence, Lopinavir/Ritonavir was quickly selected and recommended as an antiviral drug for COVID-19 in China since January. So far, only limited observations of Lopinavir/Ritonavir for coronavirus in SARS patients were reported(Chu et al.,2004).A recently completed trial of Lopinavir/Ritonavir

in patients with severe COVID-19 generated disappointed outcomes and revealed no significant antiviral effects(Cao et al.,2020).Health care workers have to rely on the supportive and symptomatic treatments to manage COVID-19 patients. Given the daily increase of confirmed COVID-19 cases and mortality, it is even more critical than two months ago to find antiviral drugs with efficacy supported by data from randomized clinical trials in COVID-19 patients(Xu et al., 2020;Zhang et al.,2020).

Novaferon as a novel antiviral protein drug which has been approved for treatment of chronic hepatitis B in China and exhibited broad-spectrum antiviral properties (unpublished data, available on requests) became an obvious candidate to be considered as a potential antiviral drug for COVID-19.Novaferon molecule is a non-natural protein consisting of 167 amino acids. According to the published information in a US patent (US 7,625,555 B2), this novel protein molecule was created by modified DNA shuffling technology using cDNA sequences of 12 human interferon subtypes as models, and named as Novaferon by its inventors(Wang et al., 2011).In addition to the human interferon-like physiological functions, Novaferon exhibits better antiviral activities that are at least 10 times more potent than human interferon alpha-2b(Li et al.,2014).Novaferon has been shown to enhance and improve the negative conversion of serum HBeAg in clinical studies (Daxianet al.,2015), and in April 2018, was approved in China for treatment of chronic hepatitis B by former CFDA (Chinese Food and Drug Administration). Novaferon protein's non-proprietary name was temporarily defined as "recombinant cytokine gene-derived protein injection" by Chinese Pharmacopeia Committee, and the recommended international non-proprietary name (rINN) by WHO is not available yet. For convenience purposes, Novaferon was used as the drug name in our study.

In the present study, we primarily attempted to observe the antiviral effects of NovaferononCOVID-19. We first determined whether Novaferon was able to inhibit

SARS-CoV-2 at cellular level, and subsequently conducted a randomized, open-label, parallel group trial to explore the antiviral effects of Novaferon in COVID-19patients by observing the SARS-CoV-2 clearance rates. The primary endpoint was the SARS-CoV-2 clearance rates on day 6, and the secondary endpoint was the median time toSARS-CoV-2 clearance after starting antiviral treatment. As a popular and recommended antiviral drug for COVID-19 in China, Lopinavir/Ritonavir was included in this study to serve as a control for comparison.

Methods

Production of Recombinant Novaferon Protein

Novaferon is a non-naturally existing protein molecule that is produced by recombinant technology via inserting a 498-nucleotides cDNA into *E. Coli*. The gene sequence (cDNA) encoding Novaferon protein molecule was created on the basis of modified DNA Shuffling technology to mimic the natural evolution of genome with intentions to invent novel protein molecules that have enhanced natural functions of the model proteins. In brief, cDNA sequences of over 12 human interferon subtype genes were selected as the model genes for DNA shuffling. These model cDNA sequences were cut into fragments by enzymes and then repeatedly amplified to induce randomized nucleotide-mutation of the cDNA fragments. The mutant cDNA fragments in the reaction system randomly and spontaneously connected with each other to form a huge mutant cDNA library. The clones of the newly formed cDNA sequences that encoded protein molecules with the broad-spectrum, enhanced antiviral and anti-proliferation activities were then screened and selected via a proprietary protein-screening method (High-efficient Protein Functional Screen System). After screening more than 100000 cDNA clones, a novel protein molecule has been identified to exhibit the enhanced potency against virus and tumor cells, and to possess the broad-spectrum antiviral and anti-proliferation properties as well. This novel

protein molecule was named as Novaferon subsequently. Novaferon is encoded by 498

nucleotides and composed of 166 amino acids with the following amino acid sequence:

CNLSQTHSLGSKRTLMLLAQMGKISLFSCLKDRHDFEFPQEEFDGNQFQKAQAIS

VLHELIQQTFNLFSTKESSAAWDEGLLDKFRTELYRQLNDLEACMMQEVGVEET

PLMNADSILAVKKYFQRITLYLMEKKYSPCAWEVVRVEIMRSLSFSTNLQKRLR

GKD. As a non-naturally occurring protein, Novaferon is not suitable to be classified

according to the classification terms of human interferon subtypes. The nucleotide and

amino-acid sequences of Novaferon are 89% (445/498) and 81% (135/166) homology to

human interferon α-2b which exhibit the best antiviral activities among all human

interferon subtypes (USPTO patent: US 7,625,555 B2) (Wang et al., 2011). Novaferon

drug used in this trial was manufactured in Qingdao city of Shandong Province by Genova

Biotech (Qingdao) Company Limited.

In vitro antiviral testes

All the in vitro experiments were conducted in a biosafety level-3 (BSL3/P3) laboratory at

Chinese Center for Disease Control and Prevention (Chinese CDC). We first determined

whether Novaferon inhibited the SARS-CoV-2 replication within the Vero E6 cells which

were already infected with SARS-CoV-2. Vero E6 cells (African green monkey kidney

cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA)

and maintained in Modified Eagle Medium (MEM; Gibco Invitrogen, Carlsbad, CA, USA)

with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, South Logan, UT)

at37 °C in the incubator with 5% CO_2. A clinically-isolated strain of SARS-CoV-2 (C-Tan-

nCov Wuhan strain 01) was propagated in Vero E6 cells before the experiments, and the

plaque assay was used to quantify the titre (plaque forming unit, PFU) of the SARS-CoV-

2. Blank Vero E6 cells in 96-well plates with a density of 1 x 10^4 per well were incubated

with C-Tan-nCov Wuhan strain 01 (100PFU) for 2 hours to induce the infection of the

cultured cells by SARS-CoV-2. After virus-containing supernatants were removed and cells rinsed, medium with various concentrations of Novaferon(0, 0.001, 0.01, 0.1, 1,10, or 100ng/ml)was added and the cells were then incubated for 24 hours to allow the viral replication. After 24 hours, 100 µL of supernatants from each well were collected, and the total viral RNA was extracted (Full-automatic nucleic acid Extraction System from TIANLONG) and measured. Takara Bio's One Step Prime Script Real-time PCR (RT-PCR) Kit (Perfect Real Time) was used to detect the copies of the virus RNA.

The Quantitative real-time PCR cycle threshold (Ct) was obtained, and the inhibition rates of virus replication by each Novaferon concentration were calculated. The Ct number of controls obtained in the absence of Novarferon was 22.6 and considered as 100%. The Ct numbers from SARS-CoV-2 infected Vero E6 cells with the addition of various concentrations of Novaferon were measured to calculate the inhibition percentages. The half-maximal effective concentration (EC_{50}) was calculated. The cytotoxicity of Novaferon on Vero E6 cells was assessed, and the half-maximal cytotoxic concentration (CC_{50}) of Novaferon on Vero Cells was determined by observing the cytopathic effects (CPE) of Novaferon. The selectivity index (CC_{50}/EC_{50}) was then calculated.

We further observed whether the previous treatment of Vero E6 cells with Novaferon protected the cells from viral entry through exposure of the pre-treated cells to SARS-CoV-2 later. Detailed operation procedures were identical to the above description, except that the step orders were changed to allow the observation of the preventive effects of Novaferon. Briefly, blank Vero E6 cells were incubated with series concentrations of Novaferon for 2 hours, and the supernatants containing Novaferon were then removed. The pre-treated Vero E6 cells were exposed to SARS-CoV-2 by incubation with C-Tan-nCov Wuhan strain 01 (100PFU) for 2 hours, and the supernatants containing SARS-CoV-2 were then removed. Fresh medium was added, and the cells were incubated for 48 hours. 100

μL of supernatants were taken from each well, and the total viral RNA in the supernatants was measured using the same methods described above. The Ct number obtained from Vero E6 cells without pre-treatment of Novaferon was considered as 100%, and the decreased Ct numbers obtained from the pre-treated Vero E6 cells with various concentrations of Novaferon were used to calculate the inhibition percentages. The preventive effects of Novaferon were then determined by observing the viral RNA reduction in the cells pre-treated with Novaferon. The EC_{50} of Novaferon for the observed preventive effects was decided accordingly.

Laboratory detection of SARS-CoV-2 nucleic acids by RT-PCR in nasopharyngeal swab samples

SARS-CoV-2 virus nucleic acids were detected by RT-PCR using SLAN-96P automatic medical PCR analysis system. The SARS-CoV-2 nucleic acid detection kit was obtained from Sensure Biotechnology Co. Ltd （Hunan province, China）, which has been approved for clinical test of SARS-CoV-2 by NMPA (National Medical Products Administration). The lowest detection limit (sensitivity)of this RT-PCR assay kitwas200 copies of SARS-CoV-2RNA in specimens. The specificity of this RT-PCR assay kit was determined by the failure of detecting viral RNA (cross-reaction) in specimens containing other coronaviruses, rotavirus, astrovirus, and adenovirus et al.

Procedures of the tests strictly followed the protocol of the kit. Samples from nasopharyngeal swab were collected in accordance with the standard procedures of the New Coronavirus Infection Pneumonia Laboratory Test Guide. FAM (ORF-1ab region) was used as fluorescent detection channel, and ROX (N gene) channels were used to detect the SARS-CoV-2 nucleic acids, while HEX channel was used as the internal standard. Cycle parameter steps were in the following order: 1）reverse transcription at 50℃for 30 minutes for 1 cycle; 2) pre-denaturation of cDNA at 95℃ for 1 minute for 1 cycle; 3)

denaturation at 95℃ for 15 seconds, and annealing, extension and fluorescence acquisition at 60℃ for 30 seconds for 45 cycles. 4) cooling at 25℃ for 10 seconds for 1 cycle. Positive results were determined by comparing between the Ct numbers of the testing samples and the standard Ct number that was 40 in this assay.

Clinical Study

Patients

The study was originally designed as a multi-center study across hospitals in Changsha city and in other cities of Hunan Province, China. However, per government order, all patients from hospitals in Changsha city had to be relocated to the First Hospital of Changsha, a designated treatment center for all COVID-19 patients in Changsha city, and hospitals in other cities of Hunan Province were not able to participate due to various reasons. The study was changed to a single center study. This study was approved by the ethics committee of the First Hospital of Changsha (file number KX-2020002) and was conducted at the hospital. The study was also registered at the Chinese Clinical Trial Registry (http://www.chictr.org.cn/), number ChiCTR2000029496.

Hospitalized COVID-19 patients with confirmed SARS-CoV-2 detection, clinically classified as moderate or severe, at the age over 18 years, and without comorbidity of severe heart, lung, brain diseases, were eligible for enrolling into this study. Moderate patients were defined as "patients with fever, symptoms of respiratory system and pneumonia changes in CT images, and severe patients were defined as "patients with any of the following: ① Respiratory distress, respiratory frequency ≥30/minute; ② Under rest status, arterial oxygen saturation (SaO$_2$)≤93%; ③Arterial partial pressure of oxygen(PaO$_2$)/fraction of inspired oxygen (FiO$_2$)≤ 300mmHg. In this study, we aimed to observe the moderate and severe COVID-19 patients as these patients would likely benefit more from antiviral treatments.

Trial design and treatments

This was a randomized, open-label, parallel group study. Patients eligible for the study were assigned, in a 1:1:1 ratio, to Novaferon, Novaferon plus Lopinavir/Ritonavir, or Lopinavir/Ritonavir group. A SAS generated simple randomization schedule was prepared by a statistician not involved in the trial. Based on the sequence that patients enrolled into the study, the patients were assigned to a treatment group which was implemented by a research assistant. Informed consents were obtained from all enrolled patients. Antiviral effects were assessed on day 3, day 6, and day 9 after starting drug administration.

The approved dosage of Novaferon for hepatitis B application is the daily injection of 10 μgof protein in 1.0ml volume per vial. Lopinavir/Ritonavir (Kaletra) was manufactured by AbbVie Inc. and each tablet contained 200mg of Lopinavir and 50mg of Ritonavir. The total daily doses (40 μg) of Novaferon were administered to patients twice per day by the oxygen-driven aerosolized inhalation for 15 minutes of 20 μg of Novaferon (2 x l ml vials) diluted with saline. For patients receiving Lopinavir/Ritonavir (Kaletra), 2 tablets were orally taken twice per day. The aerosolized inhalation was administrated to hospitalized patients in the negative-pressure wards at the designated COVID-19 center to minimize the risk of disease transmission.

Assessments

Samples of nasopharyngeal swab on day 3, day 6 and day 9 during the 7 to 10-day course of antiviral treatment were collected from the patients and tested for SARS-CoV-2 nucleic acids by RT-PCR. SARS-CoV-2 clearance in COVID-19 patients was defined as two consecutive negative-detection of SARS-CoV-2 RNA in nasopharyngeal swab samples with an interval of over 24 hours. Adverse events were monitored throughout the trial, reported and graded based on WHO Toxicity Grading Scale for Determining the Severity. The peak levels of SARS virus were around 10 days after onset and then the viral level

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began to decrease without effective antiviral treatment in SARS patients(Peiris et al., 2003).Considering the homology of gene sequences of SARS-CoV-2 and SARS was over 90%(Zhu et al., 2020), we assumed that the intervention of antiviral drugs in COVID-19 patients would likely enhance or shorten the time to viral clearance. In this regarding, the primary endpoint for this study was decided as the SARS-CoV-2 clearance rates in COVID-19 patients assessed on day 6 of antiviral treatment. The secondary endpoint was median time to SARS-CoV-2 clearance.

Statistical analysis

Statistical analysis was performed on an intent-to-treat basis, and all patients randomized and treated at least once with the study medications were included for the primary analysis. For patient demographics information and baseline disease characteristics, qualitative variables were compared among treatment groups with the use of Chi-square test, and quantitative variables were compared with the use of an ANOVA model. Only the overall differences among the three treatment groups were tested (based on null hypothesis, "all three groups were the same", against an alternative hypothesis, "at least one group was different")and therefore, no pairwise comparison was performed for baseline characteristics.

For the primary endpoint, SARS-CoV-2 clearance rate, estimates of the rates were calculated based on a binomial distribution. Difference between treatment groups was tested using the Chi-square test. To control the overall significance level for the study, the three pairwise comparisons for the primary endpoint were performed at the two-sided alpha = 0.05 using a closed testing procedure according to the following order:①Novaferon plus Lopinavir/Ritonavir vs. Lopinavir/Ritonavir alone; ②Novaferon alone vs. Lopinavir/Ritonavir alone; ③Novaferon plus Lopinavir/Ritonavir vs. Novaferon alone. For the secondary endpoint, time to SARS-CoV-2 clearance, median time for each group was

estimated with the use of the Kaplan–Meier method and treatment differences were tested using log-rank test. All tests were two-sided, with a p value of less than 0.05 considered to indicate statistical significance. Analysis was conducted using SAS V9.2.

For missing SARS-CoV-2 clearance status, Last Observation Carried Forward (LOCF) analysis was presented as the primary analysis. For purpose of sensitivity analyses, complete case analysis and worst case imputation methods were also performed. For the worst case imputation, missing SARS-CoV-2 status was replaced with 'positive'.

The planned sample size of 90 patients (30 patients per group) was not determined based on statistical consideration.

Adverse events were reported and graded using WHO Toxicity Grading Scale for determining the severity. Incidence of adverse events was summarized descriptively without a formal statistical test.

RESULTS

Inhibitory effects of Novaferon on SARS-CoV-2 at cellular level

Incubation of Novaferon (0.1□100ng/ml) with SARS-CoV-2-infected Vero E6 cells resulted in the dose-dependent reductions of the SARS-CoV-2 RNA that was released from the infected Vero E6 cells. The half-maximal effective concentration (EC_{50}) of Novaferon was 1.02 ng/ml. The tested Novaferon concentrations showed minimal cytotoxicity to Vero E6cells, and the half-maximal cytotoxic concentration (CC_{50}) was over 100ng/ml. The selectivity index (CC_{50}/EC_{50}) was over 98. These data indicated that Novaferon effectively inhibited the viral replication within SARS-CoV-2-infected cells. In addition, healthy Vero E6 cells that were previously incubated with Novaferon resisted the entry of SARS-CoV-2into cells, as indicated by the reduction of cellular viral RNA after Novaferon was removed and the treated cells were exposed to SARS-CoV-2 later. Novaferon exhibited

this preventive effect efficiently with the EC_{50}(0.1 ng/ml) lower than the EC_{50} for inhibiting SARS-CoV-2 replication in the infected cells (supplementary figure).These data suggested that Novaferon inhibited viral replication in SARS-CoV-2-infected cells and enabled healthy cells to resist the viral attack.

Clinical study

Patients and Treatments

As presented in Fig. 1, a total of 92 patients with moderate or severe illness were assessed for the eligibility criteria and 3 patients were excluded. 89 patients were randomized into the study from February 1 to 20, 2020. Of the 89 patients, 30, 30, and 29 patients were assigned into Novaferon group, Novaferon plus Lopinavir/Ritonavir group or Lopinavir/Ritonavir group respectively, among whom 84 were moderate illness and 5 severe illness. Supported by the government policy of reimbursing COVID-19-related expenses, patients were diagnosed, screened, and enrolled shortly after symptom onset. The median time (IQR) from symptom onset to antiviral drug administration were 4.0 days(3.0-6.5), 7.0 days(3.3-11.3), and 4.0days(3.0-6.0) in Novaferon group, Novaferon plus Lopinavir/Ritonavir group or Lopinavir/Ritonavir group respectively. Enrollment screening excluded patients with co-existing serve cardiac, kidney or liver diseases as described in exclusion criteria, and none of the enrolled patients has been given steroids treatment. The baseline demographic and clinical characteristics of the 89 patients were summarized in Table 1.Except some imbalances between the groups, there were no major differences between groups in demographic characteristics, baseline laboratory test results and disease severity at enrollment(Table 1).

Primary endpoint

Table 2summarized the complete RT-PCR test results of all 89 patients on day 3, day 6, and day 9 after starting drug administration. The negative results of SARS-CoV-2 nucleic

acid detection in the tested samples served as the indicator of in vivo SARS-CoV-2 clearance in patients. The SARS-CoV-2 clearance rates on day 3, day 6, and day 9 in three treatment groups were presented and compared (Table 2).On day 3, SARS-CoV-2 clearance rates were 16.7%(5/30) in Novaferon group, 36.7% (11/30) in Novaferon plus Lopinavir/Ritonavir group, and 10.3% (3/29) in Lopinavir/Ritonavir group respectively. SARS-CoV-2 clearance rate in Novaferon plus Lopinavir/Ritonavir group was significantly higher than in Lopinavir/Ritonavir group on day 3 (36.7%vs. 10.3%, p = 0.0175). No significant difference between Novaferon group and Novaferon plus Lopinavir/Ritonavir group was observed. On day 6, SARS-CoV-2 clearance rates in Novaferon group and Novaferon plus Lopinavir/Ritonavir group reached to 50.0% (15/30) and 60.0% (18/30) respectively, and were significantly higher than in Lopinavir/Ritonavir group (50.0% vs.24.1%, p = 0.0400, and 60.0% vs.24.1%, p = 0.0053, respectively). There was no statistically significant difference between Novaferon group and Novaferon plus Lopinavir/Ritonavir group, suggesting the similar extents of enhancedSARS-CoV-2 clearance on day 6 by Novaferon alone or together with Lopinavir/Ritonavir. On day 9, SARS-CoV-2 clearance rates were 56.7% (17/30) in Novaferon group, 70.0% (21/30) in Novaferon plus Lopinavir/Ritonavir group, and 51.7% (15/29) in Lopinavir/Ritonavir group. There were no statistically significant differences between the groups.

Secondary endpoint

The median time to SARS-CoV-2 clearance were 6 days, 6 days, and 9 days for Novaferon group, Novaferon plus Lopinavir/Ritonavir group, and Lopinavir/Ritonavir group respectively, indicating a 3-day reduction of time to SARS-CoV-2 clearance in both Novaferon and Novaferon plus Lopinavir/Ritonavir groups comparing with Lopinavir/Ritonavir group (Table 3).During the observation period, none of the moderate ill patients in Novaferon group and Novaferon plus Lopinavir/Ritonavir group progressed

to severe illness. In contrast, 4 moderate ill patients in Lopinavir/Ritonavir group progressed to severe illness.

Sensitivity analysis for missing data

Analyses based on both complete case analysis and worst case imputationforSARS-CoV-2 clearance rates showed little differences with the LOCF analysis, and the statistical conclusions for all the treatment comparisons remained the same.

Adverse Events

No severe adverse events (SAE) associated with the tested antiviral drugs were reported. The observed adverse events (AE) were grade1, or grade 2, and summarized in Table 4. No specific AEs were related to Novaferon treatment, and certain reported adverse events overlapped with the disease symptoms and laboratory findings.

Adverse events occurred in 25 of 30 (83.3%) patients in Novaferon group, 25 of 30 (83.3%) patients in Novaferon plus Lopinavir/Ritonavir group, and 26of 29 (89.6%) patients in Lopinavir/Ritonavir group. The most common adverse events were lymphopenia and loss of appetite in Novaferon group, lymphopenia and cough in Novaferon plus Lopinavir/Ritonavir group, and fatigue and lymphopenia in Lopinavir/Ritonavir group. The observed adverse reactions did not need extra medical interventions or cause termination of antiviral treatment.

Discussion

No matter whether exhibiting good, poor or none anti-COVID-19 effects, Lopinavir/Ritonavir in this study served as the control and allowed us to assess the antiviral effects of Novaferon by analyzing the differences between Novaferon and Lopinavir/Ritonavir. In this regarding, the significantly higherSARS-CoV-2 clearance rates on day 6 (the primary endpoint) inpatients with treatment of Novaferon alone or together with Lopinavir/Ritonavir comparing with Lopinavir/Ritonavir alone indicated that

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Novaferon indeed exhibited antiviral effectsinCOVID-19patients. The 3-day reduction of time to SARS-CoV-2 clearance in patients with Novaferon treatment further supported the antiviral effects of Novaferon.

As viral shedding in the early stages of COVID-19 represents a major challenge for controlling the transmission of SARS-CoV-2(Wölfel et al.,2020), the effective viral clearance in patients undergoing Novaferon treatment in the early course of disease was valuable in clinical setting. The negative detection of SARS-CoV-2 in samples from the respiratory system indicated the elimination of the viral shedding in patients. This in turn would contribute to the effective reduction of virus transmission by early stage patients who have been found to have the highest viral loads (Pan et al.,2020).

The antiviral effects of NovaferoninCOVID-19 patients were consistent with the laboratory findings. Inhibition of the viral replication by Novaferon at cellular level was very efficient as indicated by the low EC_{50}(1.02 ng/ml). More interestingly, healthy cells that were pre-treated by Novaferon obtained the ability, in the absence of Novaferon, to resist the viral entry into cells when the treated cells were exposed to SARS-CoV-2 later (EC_{50} 0.1 ng/ml). It might be worth to explore the potential use of Novaferon as a preventive agent for high risk population, especially for health care workers who have to routinely contact COVID-19 patients.

The viral loads in COVID-19 patients were reported to reach peak levels around 5□6 days after symptom onset (Pan etal.,2020)，the average time from the onset of symptoms to severe illness took about one week(WHO,2020).The early clearance of SARS-CoV-2 might help to shorten the disease course or to prevent the disease progress in patients with mile to moderateCOVID-19. Considering the high viral loads and peak levels of SARS-CoV-2were found in first week of symptom onset, the increased SARS-CoV-2 clearance rates on day 6 in COVID-19 patients with Novaferon treatment were

unlikely related to the natural disease course. Rather, the observed enhancement of SARS-CoV-2 clearance apparently reflected the antiviral effects of Novaferon in observed COVID-19 patients.

Limitations of this study

Our study has several limitations. First, all observations were done at one hospital in one city. Second, the sample size was relatively small and was not based on statistical consideration as limited by the availability of COVID-19 patients in Changsha city. Third, the unexpected difficulties associated with the COVID-19 outbreak compromised the quality of this study. For example, it is likely that adverse events were under-reported due to the emergent and risk situation. However, these limitations should not change the overall conclusion for this randomized trial because the antiviral assessments were strictly performed according to a vigorous standard.

Conclusions

Novaferon exhibited anti-SARS-CoV-2 effects at cellular level and in patients with COVID-19. Data obtained from this randomized, open-label, parallel group trial preliminarily demonstrated the anti-SARS-CoV-2 effects of Novaferon for COVID-19, and justified large-scale clinical studies to verify the efficacy of Novaferon as a potential antiviral drug for COVID-19.

Author Contributors:

Study design: Guozhong Gong,Wenjie Tan,Yuanlin Xie,Yongfang Jiang. Data collection: Fang Zheng, Yanwen Zhou, Zhiguo Zhou, Fei Ye, Baoying Huang, Yaxiong Huang, Jing Ma, Qi Zuo, Xin Tan, Jun Xie, Peihua Niu, Wenlong Wang. Data analysis: Yun Xu, Feng Peng, Ning Zhou, Chunlin Cai, Wei Tang, Xinqiang Xiao, Yi Li, Zhiguang Zhou. Writing: Yongfang Jiang, Yuanlin Xie, Wenjie Tan, Guozhong Gong. All authors read and approved the final manuscript. Fang Zheng, Yanwen Zhou, Zhiguo Zhou, Fei Ye contributed equally

to this work.

Competing interests:

The authors declare no competing interests.

Ethical approval:

The study was anonymous, and the protocol was approved bythe Ethics Committee of the First Hospital of Changsha, according to the Declaration of Helsinki, 2013. Written informed consent was obtained from all participants.

Declaration of interests
None

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Participants were assessed (n= 92)

Not meeting eligibility criteria (n= 3)

Randomized (n=89)

Allocated to Novaferon (n= 30)
♦ Received Novaferon (n= 30)

Allocated to Lopinavir/Ritonavir plus Novaferon (n= 30)
♦ Received allocated intervention (n= 30)

Allocated to Lopinavir/Ritonavir (n= 29)
♦ Received allocated intervention (n= 29)

Viral RNA test not done on day 6 (n= 2)

Viral RNA test not done on day 6 (n= 1)

Viral RNA test not done on day 6 (n= 2)

Analysed (n= 30)

Missing viral RNA outcome was imputed with Last Observation Carried Forward (LOCF) method

Analysed (n=30)

Missing viral RNA outcome was imputed with Last Observation Carried Forward (LOCF) method

Analysed (n= 29)

Missing viral RNA outcome was imputed with Last Observation Carried Forward (LOCF) method
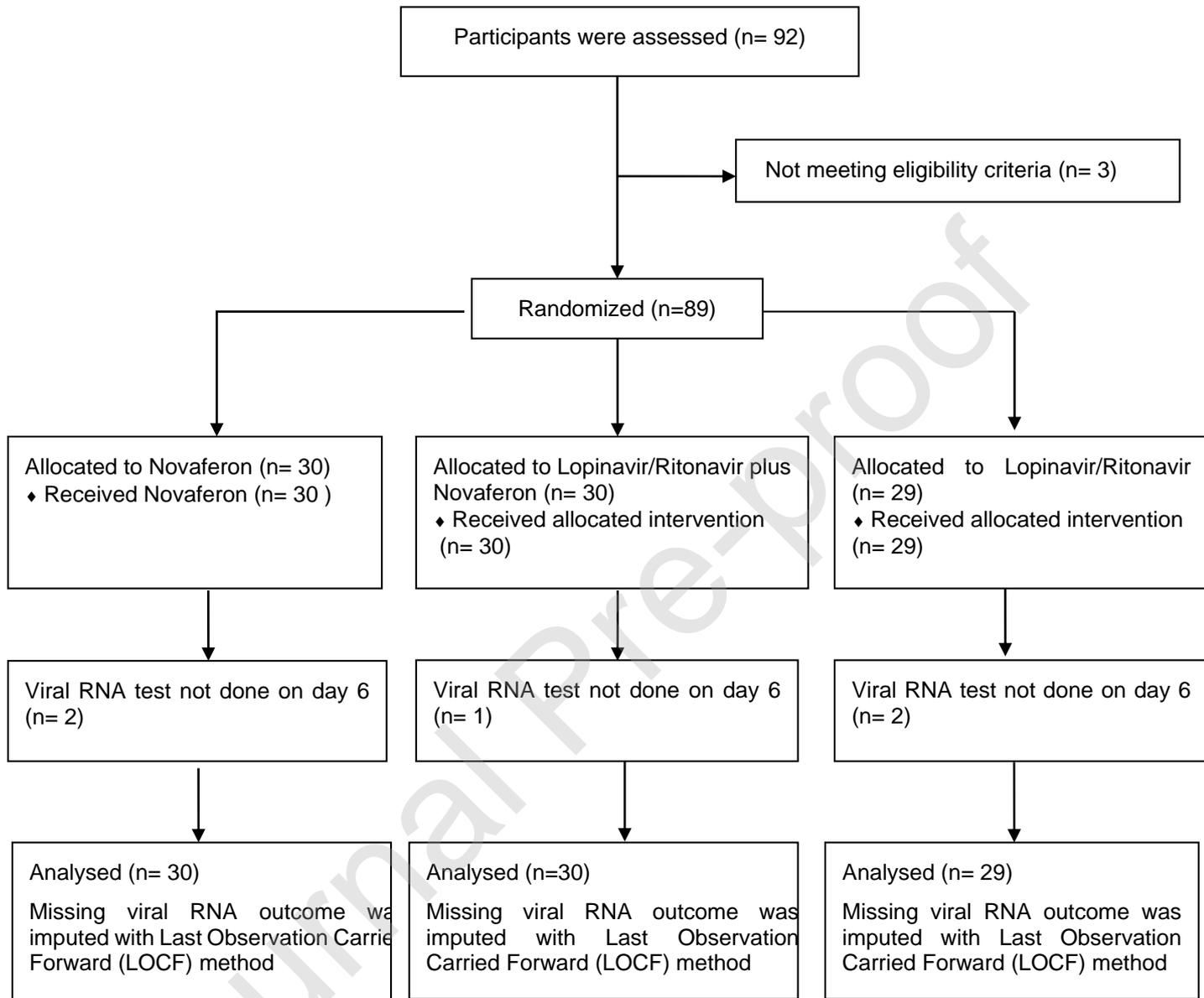
Figure 1. Randomization and Treatment Assignment

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Table 1. Demographics and Baseline Clinical Characteristics

Variables	Novaferon	LPV/r*+Novaferon	LPV/r
	N=30, n(%)	N=30, n(%)	N=29, n(%)
Age, Median (IQR)	46.5(40.0-63.8)	50.0(37.8-62.8)	37.0(26.0-54.0)
Male, n(%)	17(56.7%)	13(43.3%)	12 (41.4%)
Median time from symptoms to therapy, days（IQR）			
	4.0（3.0-6.5）	7.0（3.3-11.3）	4.0（3.0-6.0）
Moderate Cases, n(%)	28 (93.3%)	28 (93.3%)	28 (96.6%)
Severe cases, n(%)	2 (6.7%%)	2 (6.7%)	1 (3.4%)
Comorbidity, n(%)	7(23.33)	6(20.00)	5(17.24)
Diabetes	3(10.00)	3(10.00)	2(6.90)
Hypertension	2(6.67)	3(10.00)	1(3.34)
Coronary heart disease	1(3.33)	1(3.33)	1(3.34)
Chronic hepatitis B	1(3.33)	0(0)	1(3.34)
Chronic bronchitis	1(3.33)	0(0)	1(3.34)
Fever, n (%)	17 (56.67)	20 (66.67)	20 (68.97)
Cough, n (%)	16 (53.33)	16(53.33)	13 (44.83)
Fatigue, n (%)	6(20.00)	8(26.67)	12(41.38)
Sore throat, n (%)	3 (10.00)	2(6.67)	4 (13.79)
Headache, n (%)	3 (10.00)	2 (6.67)	2 (6.90)
Myalgia, n (%)	3 (10.00)	1 (3.33)	4 (13.79)

Dizziness, n (%)	1 (3.33)	3 (10.00)	1 (3.45)
Diarrhea, n (%)	1(3.33)	3(10.00)	1 (3.45)
Rhinorrhoea, n (%)	1(3.33)	1 (3.33)	1 (3.45)
Nausea, n (%)	0(0)	2(6.67)	1 (3.45)
Vomiting, n (%)	0(0)	2(6.67)	1 (3.45)
Dyspnea, n (%)	2(6.67)	2 (6.67)	1(3.33)
Loss of appetite, n (%)	0(0)	0(0)	4 (13.79)
Chill, n (%)	0(0)	0(0)	2 (6.90)
Leukopenia, n (%)	13(43.33)	10(33.33)	13(44.83)
Neutropenia, n (%)	11(36.67)	4(13.33)	6(20.69)
Lymphopenia, n (%)	4(13.33)	7(23.33)	6(20.69)
Thrombocytopenia,n (%)	4(13.33)	1(3.33)	2(6.90)
Hemoglobin decreased, n (%)	1(3.33)	2(6.67)	2(6.90)
ALT increased, n (%)	0(0)	2(6.67)	1(3.45)
AST increased, n (%)	1(3.33)	1(3.33)	3(10.35)

*LPV/ r: Lopinavir/Ritonavir.

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Table 2 Summary of SARS-CoV-2 clearance rates

	Novaferon	LPV/r*+ Novaferon	LPV/r	p value†		
				LPV/r vs. Novaferon	LPV/r vs. LPV/r + Novaferon	Novaferon vs. LPV/r + Novaferon
	(N=30)	(N=30)	(N=29)			
Day 3	16.7% (5/30)	36.7% (11/30)	10.3% (3/29)	0.4783	0.0175	0.0798
Day 6	50.0% (15/30)	60.0% (18/30)	24.1% (7/29)	0.0400	0.0053	0.4363
Day 9	56.7% (17/30)	70.0% (21/30)	51.7% (15/29)	0.7032	0.1502	0.2839

*LPV/ r: Lopinavir/Ritonavir.

†p values for comparisons between treatment groups using Chi-square test; At any visit, missing viral RNA outcome was imputed with Last Observation Carried Forward (LOCF) method.

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Table 3. Analysis for Time to SARS-CoV-2 Clearance

| | Novaferon | LPV/r*+ Novaferon | LPV/ r | p value | | |
				LPV/ r vs. Novaferon	LPV/ r vs. LPV/ r +Novaferon	Novaferon vs. LPV/ r +Novaferon
N(Censored)	30(13)	30(9)	29(14)			
Mean (days)	7.0	6.1	8.0			
Median(days)†	6	6	9	0.417	0.036	0.183

*LPV/ r: Lopinavir/Ritonavir.

†Median time for each group was estimated with the use of the Kaplan–Meier method and

treatment differences were tested using log-rank test.

Table 4．Summary of Common Adverse Events

Event	Novaferon (N=30) n (%)	Novaferon+LPV/ r(N=30)，n (%)	LPV/ r (N=29) n (%)
Any adverse event	25(83.3)	25(83.3)	26(89.6)
Lymphopenia	8(26.7)	14(46.7)	9(31.0)
Loss of appetite	8(26.7)	10(33.3)	9(31.0)
Cough	7(23.3)	14(46.7)	4(13.8)
Fatigue	7(23.3)	9(30.0)	10(34.5)
Neutropenia	5(16.7)	7(23.3)	7(24.1)
Dizziness	5(16.7)	5(16.7)	3(10.3)
Diarrhea	4(13.3)	4(13.3)	5(17.2)
Abdominal discomfort	4(13.3)	1(3.3)	4(13.8)
Anemia	3(10.0)	4(13.3)	3(10.3)
Sleep disorders	3(10.0)	3(10.0)	4(13.8)
Nausea	2(6.7)	5(16.7)	1(3.4)
Dyspnea	2(6.7)	2(6.67)	3(10.35)
Vomiting	1(3.33)	4(13.3)	1(3.3)
Hepatic injury	1(3.3)	3(10.0)	3 (10.3)
Chest tightness	1(3.3)	2(6.7)	6(20.7)

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