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Clinical Efficacy of Inhaled Nitric Oxide in Preventing the Progression of Moderate to Severe COVID-19 and Its Correlation to Viral Clearance: Results of a Pilot Study

Merlin Moni[1], Thushara Madathil[2], Dipu T. Sathyapalan[1], Veena Menon[3], Georg Gutjahr[4], Fabia Edathadathil[5], Deepthi Sureshkumar[6], Preetha Prasanna[1], Soumya Jose[3], Roshni Jerome[3], Ajai Krishnan[3], Indulekha C.L. Pillai[7], Geetha Kumar[7], Bipin Nair[7], Victor Nizet[8,9], Aveek Jayant[1,✉]

Abstract

Hypoxic patients with coronavirus disease 2019 (COVID-19) are at high risk of adverse outcomes. Inhaled nitric oxide (iNO) has shown anti-viral and immunomodulatory effects in vitro. However, in vivo evidence of efficacy in hypoxic COVID-19 is sparse. This open label feasibility study was conducted at a single referral center in South India and evaluated the effectiveness of repurposed iNO in improving clinical outcomes in COVID-19 and its correlation with viral clearance. We recruited hypoxemic COVID-19 patients and allocated them into treatment (iNO) and control groups (1:1). Viral clearance on day 5 favored the treatment group (100% vs 72%, $P < 0.01$). The speed of viral clearance as adjudged by normalized longitudinal cycle threshold (Ct) values was positively impacted in the treatment group. The proportion of patients who attained clinical improvement, defined as a ≥2-point change on the World Health Organization ordinal scale, was higher in the iNO cohort (n = 11, 79%) as compared to the control group (n = 4, 36%) (odds ratio 6.42, 95% confidence interval 1.09–37.73, $P = 0.032$). The proportion of patients progressing to mechanical ventilation in the control group (4/11) was significantly higher than in the treatment group (0/14). The all-cause 28-day mortality was significantly different among the study arms, with 36% (4/11) of the patients dying in the control group while none died in the treatment group. The numbers needed to treat to prevent an additional poor outcome of death was estimated to be 2.8. Our study demonstrates the putative role of repurposed iNO in hypoxemic COVID-19 patients and calls for extended validation.

Keywords: nitric oxide; COVID-19; antiviral activity; WHO ordinal scale

Introduction

The coronavirus disease 2019 (COVID-19) pandemic has transcended borders and infected over 200 million people, resulting in more than 4 million deaths, which translates to a documented mortality of around 2%. Major efforts in development of therapeutics for moderate to severe COVID-19 have involved repurposing established drugs, vaccines and antibodies, although with limited success. Steroid therapy has by far been the most efficacious.[1]

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Author affiliations: [1] Department of Medicine, Division of Infectious Diseases, Amrita Institute of Medical Science, Amrita Vishwa Vidyapeetham, Kochi, Kerala, India, [2] Department of Cardiac Anaesthesiology, Amrita Institute of Medical Science, Amrita Vishwa Vidyapeetham, Kochi, Kerala, India, [3] Clinical Virology Laboratory, Amrita Institute of Medical Sciences, Amrita Vishwa Vidyapeetham, Kochi, Kerala, India, [4] Center for Research in Analytics and Technologies for Education (CREATE), Amrita Vishwa Vidyapeetham, Amritapuri, Kollam, Kerala, India, [5] Department of Infection Control and Epidemiology, Amrita Institute of Medical Science, Amrita Vishwa Vidyapeetham, Kochi, Kerala, India, [6] School of Medicine, Amrita Institute of Medical Science, Amrita Vishwa Vidyapeetham, Kochi, Kerala, India, [7] School of Biotechnology, Amrita Vishwa Vidyapeetham, Amritapuri, Kollam, Kerala, India, [8] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, California, USA, [9] Department of Pediatrics, University of California, San Diego, La Jolla, California, USA.

✉ Corresponding author: Aveek Jayant, Department of Anaesthesia, Critical Care and Pain, Homi Bhabha Cancer Hospital and Research Centre, Tata Memorial Centre, Visakhapatnam 530053, India. E-mail: jayant.aveek@gmail.com

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Nitric oxide (NO) is a biological molecule with pleotropic effects and wide presence across mammalian organ systems.[2] It performs a key role in maintaining vascular homeostasis.[2,3] NO or its donors have been used as anti-anginals[4] and in pulmonary hypertension.[5] NO possesses potent oxidant potential that is linked to putative harm,[6] but also makes it a potent broad spectrum antimicrobial.[7] NO donors have previously been shown to inhibit viral protein and ribonucleic acid synthesis in a coronavirus infection model of severe acute respiratory syndrome (SARS),[8] and more recently, in a SARS coronavirus 2 (SARS-CoV-2) infection model as well.[9] There has also been suggestive clinical evidence of its utility in SARS caused by Coronaviridae earlier.[10]

The proposed reasons for hypoxic respiratory failure resulting from lung injury in COVID-19 encompasses a spectrum, including viral replication, vasoconstriction, immune mediated injury, thrombotic damage, intrapulmonary shunting due to edema, and atelectasis. The viral loads are of clinical relevance as high viral loads and their persistence are linked to severity of the disease and the efficacy of an anti-viral is often best when initiated in the early phase of the disease.[11] This concept has been validated in earlier models of virus-induced SARS[10] and secondary analyses of the Adaptive COVID-19 Treatment Trial.[12–14] However, current evidence suggests that the maximum mortality benefit has actually been derived from dexamethasone, which modulates host immune responses to the virus.[1] Inhaled NO (iNO) has been reported as a simulator of immune function with proven beneficial effects on attenuating inflammatory mediated injury.

iNO has actually been the subject of investigation in several reports of therapies in COVID-19.[7,15] However, the overwhelming focus has been on the hemodynamic, gas exchange, or endothelial modulation effects of this drug,[16–21] in addition to its antiviral properties.[22] We hypothesized that the pleiotropic action of iNO could translate to a net clinical improvement as it targets both the virus and its downstream effects. Here, we report the results of a pilot study on the adjunctive effects of iNO administration in improving clinical outcomes and viral dynamics among patients with moderate to severe COVID-19.

Results

A total of 794 patients in the intensive care unit (ICU) diagnosed with COVID-19 between September to December 2020 were assessed as per the study inclusion criteria, among which 29 patients were recruited into the study. The Consolidated Standards of Reporting Trials diagram depicting the subject disposition is illustrated in Figure 1. Among the eligible patients post exclusion criteria assessment, 14 patients were randomly assigned to the treatment group, who were subsequently administered with iNO, and the remaining 15 patients were assigned to the control group. Later, four patients of the control group withdrew consent for the study, while all patients assigned to the treatment group completed the 3-day iNO therapy. The per protocol analysis of the study includes 25 patients (14 in the treatment group and 11 in the control group). An additional intention to treat (ITT) analysis was done for 29 patients (14 in the treatment group and 15 in the control group).

Baseline characteristics

Baseline characteristics of the study cohort are detailed in Table 1. The median age of the study cohort was 59 years [interquartile range (IQR) 48.5–66.5 years] with 72% male patients. Patients were randomly assigned to the treatment group at an average of 6.78 ± 3.84 days from symptom onset while the control group was enrolled at 8.27 ± 3.58 days from symptom onset. At the time of enrollment, 36% (n = 4) of the control group and 71% (n = 10) of the treatment group had severe COVID-19; however, none of the patients in the study cohort was in critical condition. Four patients in the treatment group and 2 patients in the control group required non-invasive ventilation (NIV) at enrollment. All patients received steroids and one or more antivirals, which included remdesivir and/or favipiravir. A total of 11 (79%) patients in the treatment group and all 11 (100%) patients in the control group received Azithromycin and other antibiotics. The presence of anti-SARS-CoV-2 spike IgG was reported in ten (71%) and six (55%) patients in the treatment and control groups, respectively.

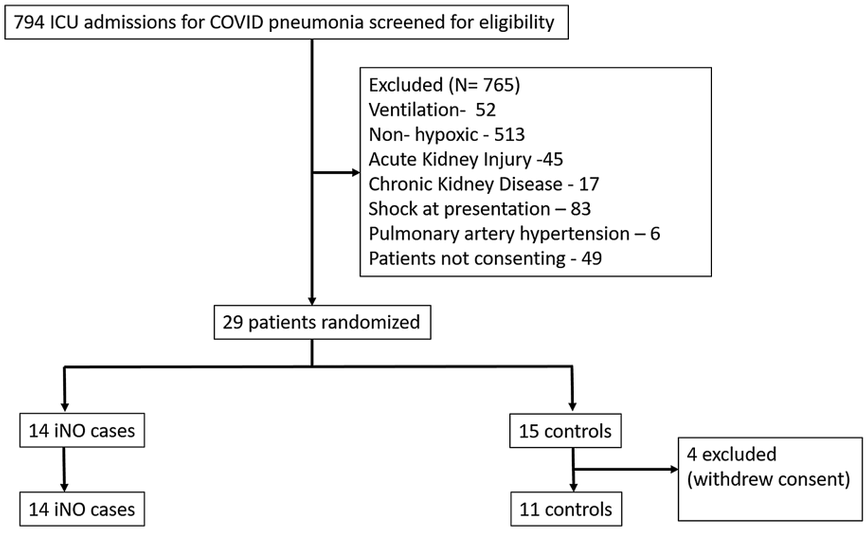

Figure 1. The Consolidated Standards of Reporting Trials diagram of patient disposition.

Table 1

Baseline characteristics of the study cohort

Characteristics	Treatment group (n = 14)	Control group (n = 11)	*P*-value
Age (years, mean ± SD)	53.87 ± 10.08	65.9 ± 10.78	<0.01
Males	12 (86%)	6 (55%)	0.08
Co-morbidities			
Diabetes mellitus	6 (43%)	8 (73%)	0.13
Hypertension	6 (43%)	5 (45%)	0.44
Coronary artery disease	2 (14%)	2 (18%)	0.79
Congestive heart failure	3 (21%)	3 (27%)	0.73
Chronic obstructive pulmonary disease	0	3 (27%)	0.03
Documented secondary infections	1 (7%)	2 (18%)	0.5
Therapy			
Remdisivir	11 (79%)	8 (73%)	0.7
Favipiravir	10 (71%)	9 (82%)	0.5
Steroids	14 (100%)	11 (100%)	–
Azithromycin	11 (79%)	11 (100%)	0.1
Plasma therapy	1 (7%)	1 (9%)	0.8
Antibiotics	11 (79%)	11 (100%)	0.7
Anticoagulation	13 (93%)	11 (100%)	0.3
Days of therapy prior to randomization			
Remdesivir therapy (days, mean ± SD)	1.00 ± 1.8	2.00 ± 2.23	0.22
Favipiravir therapy (days, mean ± SD)	0.36 ± 0.67	1.55 ± 2.0	0.04
Steroid therapy (days, mean ± SD)	1.09 ± 1.8	4.0 ± 2.44	0.002
Clinical status			
SOFA at enrollment (mean ± SD)	2.36 ± 1.33	2.73 ± 2.28	0.6
Resp-SOFA (mean ± SD)	1.96 ± 0.26	1.36 ± 0.80	0.01
NIV at enrollment	4 (29%)	2 (18%)	0.5
Time to randomization from symptom onset (mean ± SD)	6.78 ± 3.84	8.27 ± 3.58	0.33
Disease severity at enrollment			
Mild	0	0	
Moderate	4 (29%)	7 (64%)	0.07
Severe	10 (71%)	4 (36%)	
Critical	0	0	
Disease course phases			
Early (<10 days)	10 (71%)	7 (64%)	0.67
Immune (active phase) (>10 days)	4 (29%)	4 (36%)	
Viral markers at enrollment			
Presence of vRNA	8 (57%)	6 (55%)	0.8
Normalized Ct value *N* gene (mean ± SD)	8.058 ± 7.732	2.675 ± 4.796	<0.001
Normalized Ct value *ORF1ab* (mean ± SD)	8.243 ± 7.716	3.995 ± 5.909	0.002
Presence of Anti SARS-CoV-2 Spike IgG	10 (71%)	6 (55%)	0.3

Ct: cycle threshold; NIV: non-invasive ventilation; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SOFA: sequential organ failure assessment.

Primary outcomes

The proportion of patients who attained clinical improvement in terms of a ≥ 2-point change on the World Health Organization ordinal scale (WOS) at day 14 post randomization was significantly higher in the treatment group (n = 11, 79%) compared to the control group (n = 4, 36%) [odds ratio 6.42, 95% confidence interval (CI) 1.09–37.73, $P = 0.032$] (Figure 2). Table 2 details the outcomes of the study. Following baseline covariate adjustment with propensity scores, the effect of iNO administration on the ≥2-point WOS at day 14 remained significant (adjusted odds ratio 3.03, 95% CI 1.00–49.22, $P = 0.05$). However, the proportion of patients with ≥2-point change on WOS at day 7 was not significantly different (odds ratio 0.75, 95% CI 0.09–6.39, $P = 0.06$) between the treatment and control groups. The median time until a 2-point improvement on WOS was 14 days (IQR 15) in the control group and 11 days (IQR 3) in the treatment group. The cumulative event, when plotted, demonstrates a significant 2-point improvement on WOS in the iNO-treated patient cohort, as compared to the control cohort (Figure 3). However, the distribution of time to 2-point improvement on WOS did not significantly differ between the treatment and control groups after covariate adjustment (hazard ratio 1.45, 95% CI 0.74–2.84, $P = 0.55$).

Mixed effects model revealed iNO administration to have a significant effect on the viral load decline based on the normalized longitudinal cycle threshold (Ct) values until day 14 for both the nucleocapsid (*N*) gene ($P < 0.01$) and the O*rf1ab* gene ($P = 0.02$). The distribution of time to viral load reduction exhibited a significant difference between the treatment and control groups after covariate adjustment for both the *N* gene (adjusted hazard ratio 2.81, 95% CI 1.36–5.80, $P < 0.01$) and O*rf1ab* gene (adjusted hazard ratio 2.73, 95% CI 1.34–5.58, $P < 0.01$) (Figure 4A and 4B). *F* tests for the differences between the treatment and the control groups in the development of viral loads are shown in Table 3. Both time and grouping are seen to have a significant effect on viral loads. Within 5 days, 73% (8/11) and 64% (7/11) of patients in the control group cleared viral load as adjudicated by Ct values for the O*rf1ab* gene and *N* gene, respectively, as compared to all patients in the treatment group ($P = 0.04$) (Figure 5A and 5B).

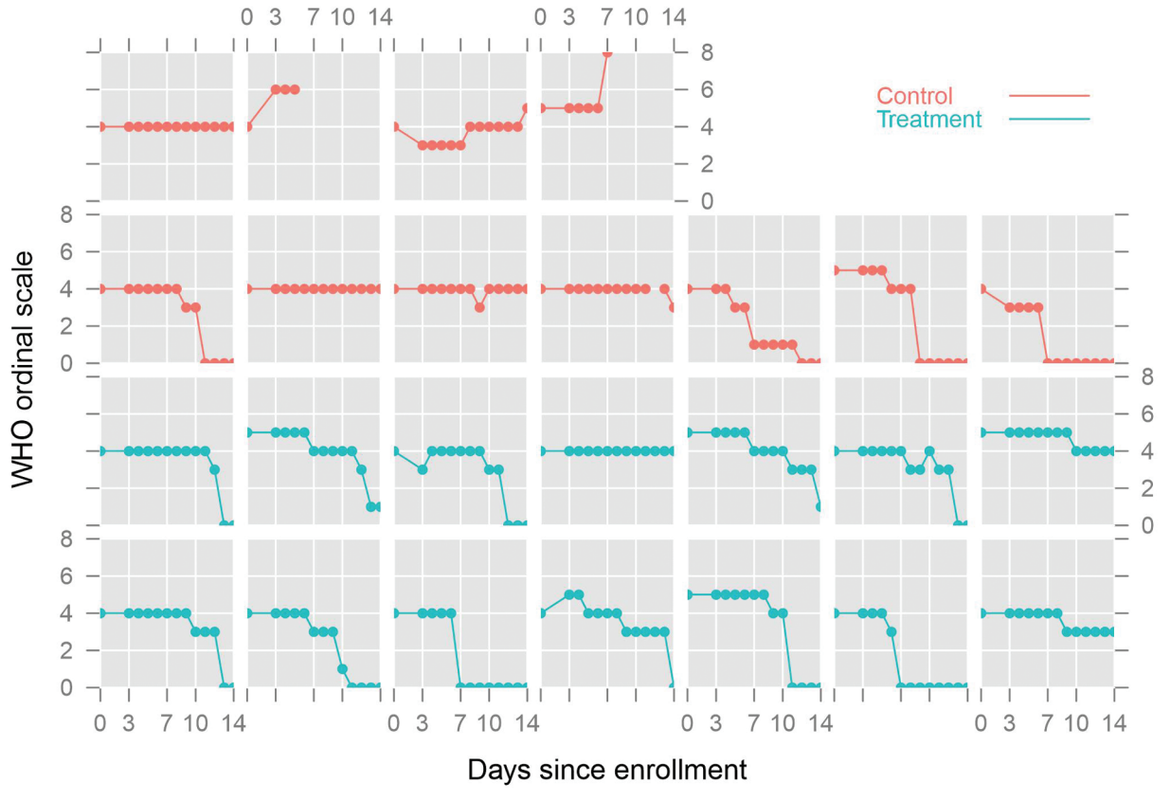

Figure 2. Individual values of the WHO ordinal scale (WOS) for patients in the treatment group and patients in the control group. WHO: World Health Organization.

Secondary outcomes

Ventilatory/supplemental oxygen requirements. The proportion of patients progressing to mechanical ventilation in the control group was significantly higher than in the treatment group (n=4, 36% vs none, $P=0.01$) (Table 2). In contrast, the treatment group cohort had a considerably higher mean value for the NIV hours over 14 days post enrollment (35.7±54.62) relative to the control group (10.3±21.8), but a significant difference was not observed ($P=0.06$). No significant difference was observed in sequential organ failure assessment (SOFA) scores after 72 hours between the treatment and control groups,

Table 2

Distribution of primary and secondary outcomes

Outcomes	Treatment group (n=14)	Control group (n=11)	*P*-value
WOS at enrollment	4.18±0.40	4.18±0.46	0.28
Primary outcomes			
≥2-point WOS improvement after 7 days	2 (14%)	2 (18%)	0.77
≥2-point WOS improvement after 14 days	11 (79%)	4 (36%)	0.032
Change in viral load by Day 3 (mean ± SD) – *N* gene (Difference in the normalized Ct values)	0.770±5.601	2.608±7.423	0.48
Change in viral load by Day 3 (Mean ±SD) – *ORF1ab* gene (Difference in the normalized Ct values)	1.180±4.984	0.863±7.797	0.9
Change in viral load by Day 5 (Mean ± SD) – *N* gene (Difference in the normalized Ct values)	2.805±8.239	0.704±4.354 (n=9)	0.45
Change in viral load by Day 5 (Mean ± SD) – *ORF1ab* gene (Difference in the normalized Ct values)	4.125±9.244 (n=11)	–0.103±4.935 (n=10)	0.2
Ct value ≤30 at end of 5 days	4 (33%)(n=12)	2 (29%)(n=7)	0.6
Secondary outcomes			
SOFA at 72 h post enrollment (mean ± SD)	1.93±0.73	4.18±5.19	0.06
ΔSOFA (mean ± SD)	0.42±1.55	–1.45±3.30	0.035
NIV hours (mean ± SD)	35.7±54.62	10.3±21.8	0.06
Need for invasive mechanical ventilation	0	4 (36%)	0.01
Incidence of methaemoglobin levels >3%	0	0	–
14-day mortality	0	2 (18%)	0.09
28-day mortality	0	4 (36%)	0.013
Average length of ICU stay (days, mean ± SD)	9.64±3.67	11.72±5.29	0.12
Average length of inpatient stay (days, mean ± SD)	16.21±7.26	18.5±9.34	0.26

Ct: cycle threshold; ICU: intensive care unit; NIV: non-invasive ventilation; SOFA: sequential organ failure assessment; WOS: WHO ordinal scale.

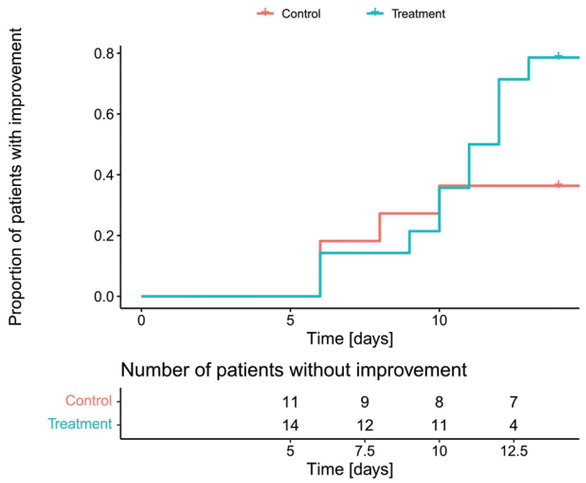

Figure 3. Proportion of patients with an improvement by at least 2 points on the WHO ordinal scale (WOS). During the 2-week study period, in the treatment group, 11 out of 14 (79%) of patients improved by at least 2 units on the WOS, while in the control group 4 out of 11 (36%) patients improved ($P=0.05$). WHO: World Health Organization.

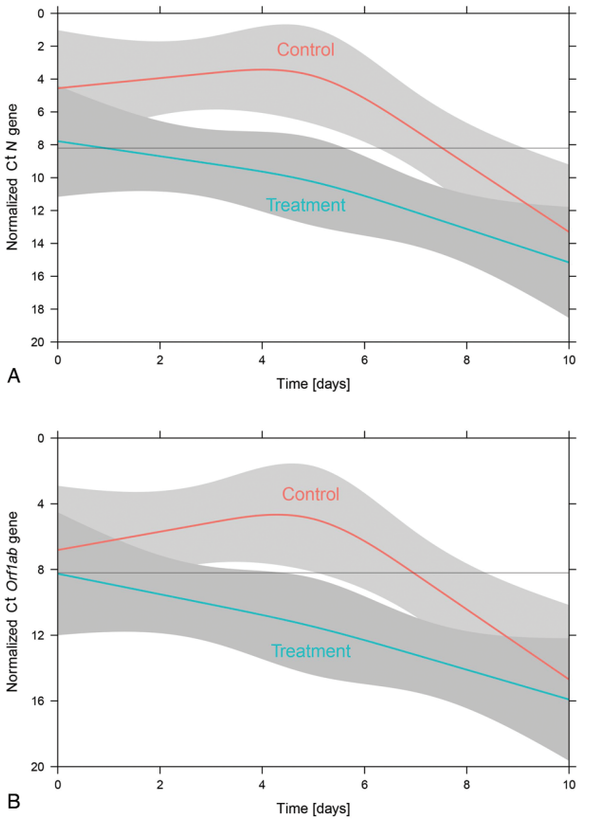

Figure 4. A: The average normalized Ct values for the *N* gene with 95% confidence intervals over time for the treatment and control groups. B: The average normalized Ct values for the *Orf1ab* gene with 95% confidence intervals over time for the treatment and control groups. The horizontal grey line indicates the Ct of 8.205. Ct: cycle threshold.

although the ΔSOFA depicting the difference of SOFA scores between enrollment and after 72 hours was significantly different between the treatment group (0.42 ± 1.55) and control group (-1.45 ± 3.30) ($P=0.035$).

All-cause mortality and length of stay. The all-cause 28-day mortality was significantly different between the study arms, with 36% (four of 11 patients) dying in the control group and none of the patients in the treatment group, resulting in a risk difference of −0.36 percentage points (95% CI −0.26 to −0.45, $P=0.013$). Kaplan-Meier survival plots were plotted to show the distribution between treatment and control groups (Figure 6). The hazard ratio for the need for mechanical ventilation or death within 28 days was calculated as 2.7. The absolute risk reduction was calculated to be 0.36 in terms of mortality and therefore the numbers needed to treat to prevent an additional poor outcome of death was estimated to be 2.8. However, a significant difference was not observed between the treatment and control groups for the length of inpatient stay and the length of ICU stay.

Adverse events

The incidence of methemoglobinemia was the adverse event monitored in the study and none of the patients in the treatment group developed methemoglobin levels >3% as per the adverse event definition during the treatment period.

ITT analysis

Serial assessment was carried out on nasopharyngeal specimens of patients that were enrolled throughout the trial except for the four patients that withdrew consent. An ITT analysis with all patients was carried out in addition to the per-protocol analysis with the patients that enrolled in the trial. The proportion of patients attaining a clinical improvement of ≥2 point change on WOS after 14 days remained significantly higher in the treatment group at 78% (11/14), as compared to 33% (5/15) in the control group. Similarly, the 28-day mortality ($P=0.03$) and the use of mechanical ventilation ($P=0.03$) were significantly different between the treatment and control groups.

Table 3

***F* tests for sets of parameters in the longitudinal models of viral loads**

	N gene				*Orf1ab* gene			
	df.	*F*	*P*-value (raw)	*P*-value (adjusted)	df.	*F*	*P*-value (raw)	*P*-value (adjusted)
Time	4	6.24	<0.01	<0.01	4	5.10	<0.01	<0.01
Group	3	2.80	0.06	0.08	3	3.52	0.02	0.05
Interaction	2	0.90	0.41	0.45	2	1.24	0.29	0.19

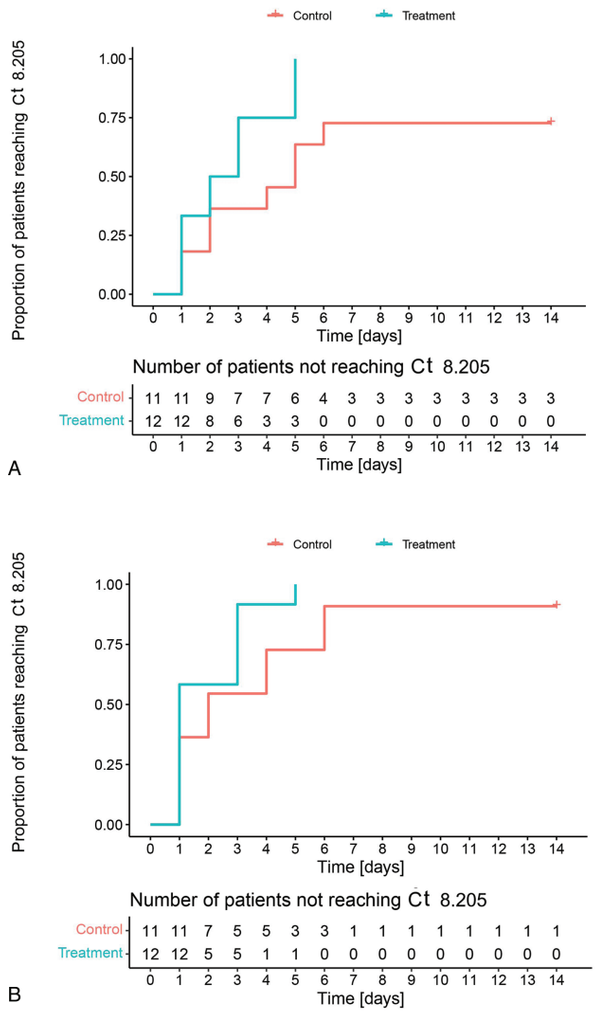

Figure 5. **Time until patients reached at least a normalized Ct value of at least 8.205, which corresponds to a Ct value of 35**. A: The proportion of patients that reached a normalized Ct value of at least 8.205 over time for the *N* gene ($P=0.04$). B: Proportion of patients that reached a normalized Ct value of at least 8.205 over time for the *Orf1ab* gene ($P=0.10$). Ct: cycle threshold.

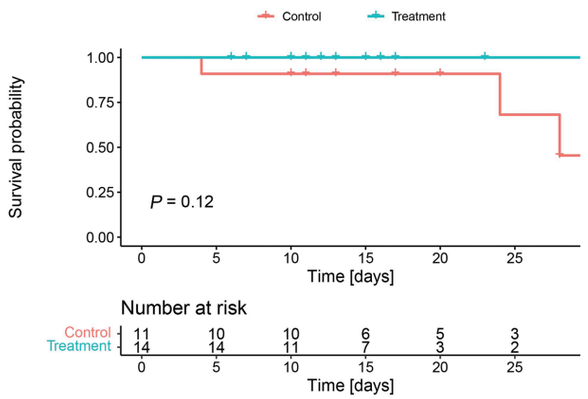

Figure 6. **Kaplan-Meier survival plot for iNO treatment and control groups**. The difference in survival times was not significant ($P=0.12$) There was 100% survival of patients in the treatment group during the first 28 days, as compared to 64% survival of patients in the control group ($P=0.03$ for difference between the two groups).

Discussion

Our proof-of-concept study demonstrating efficacy of iNO as adjunct therapy for the treatment of patients with moderate to severe COVID-19 presents pilot data that revealed clinical improvement of ≥2 points on the WOS in a significant proportion of patients by day 14 after commencement of iNO therapy, along with novel in vivo evidence of viral clearance.

NO has been shown to have a direct effect on inhibiting the replication of SARS-CoV-2. NO can cause a reduction in the palmitoylation of nascently expressed spike (S) protein, affecting the fusion between the S protein and its cognate receptor, which is the primary step mediating viral entry into host cells. In addition, NO also causes a reduction in viral RNA production in the early steps of viral replication, possibly due to an effect on one or both of the cysteine proteases encoded by *Orf1a* of SARS-CoV.[23] Besides the anti-viral properties, NO can also modulate the host immune response. NO exerts an inhibitory effect on helper T cell type 1 (Th1) lymphocytes and inhibits interleukin 2 (IL-2) and interferon-gamma, but NO does not affect IL-4 production by Th2 lymphocytes.[24] Thus NO can polarize the host immune response and modulate immunopathology. It is also interesting to study its anti-inflammatory effects in COVID-19 patients,[25] although in a mouse model of endotoxin-induced lung injury, NO did not modulate neutrophil activation.[26]

The majority of studies on iNO in COVID-19[20] have focused on its vascular effects. Yet, the use of iNO in improving gas exchange in other models of severe lung injury has been disappointing.[27] In our study, severe COVID-19 cases (71% vs 29%) and the respiratory SOFA score component (1.96 ± 0.26 vs 1.36 ± 0.80) were significantly higher in the iNO-treatment group at enrolment. Also, a relatively larger proportion of the treatment cohort (29% vs 18%) required NIV support at enrolment. Despite this evidently higher severity of disease in the treatment group, a significantly higher proportion of the control group progressed to invasive mechanical ventilation requirement and death. This resulted also in a higher number of NIV hours in the treatment group compared to the control group although it was statistically insignificant. This could potentially highlight the rescue role of iNO in COVID-19-related hypoxic respiratory failure. The treatment group also gained faster 2-point improvement on the WOS designed for SARS. Although both the treatment and the control groups did receive other antiviral medications, anticoagulants and dexamethasone, the outcome benefits persisted even with propensity score weighted adjustment. However, we believe that the observed benefits could represent an adjunct effect of NO. More definitive conclusions require a larger sample size.

All the patients in the treatment group achieved the normalized Ct value corresponding to a negative result for COVID-19 by day 5, indicating a significantly higher proportion and demonstrating a faster viral clearance compared with the control group. We acknowledge that Ct value is not a direct measurement of viral replication; however, this was used as the closest surrogate marker to understand viral dynamics during the hypoxic phase and the observed viral decline correlated well with the clinical outcomes. Barring the clinical benefits of steroids and a few immunomodulatory medications, an active medication with combined anti-viral properties and beneficial effects on pulmonary vasculature has not been developed. Given the urgent need to explore accessible and effective rescue therapies in severe COVID-19, our proof-of-

concept study offers a putative role towards repurposing of iNO for the therapy of patients with moderate to severe COVID-19.

Limitations

The small sample size limits the generalizability of our proof-of-concept study, requiring validation in a larger cohort. As very few of our patients had Ct values <25 at enrollment, the antiviral action of iNO may not be attributable to the observed change in viral dynamics alone. Additional mechanistic pathways such as on immune responses in the lung, modulation of pulmonary hemodynamics, intrinsic improvements in lung mechanics secondary to gas exchange, and effects of pulmonary improvement on the whole body inflammatory response were not studied and could also be contributory.

Materials and methods

Design

The study was conducted as an open-label, randomized controlled feasibility trial at a single tertiary referral center in South India. This study was approved by the Institutional Ethics Committee of the Amrita Institute of Medical Sciences and Research Center, Kochi and the Drug Controller General of India for permission to repurpose iNO (Approval Number: IEC AIMS 2020 CARDANES171; November 9, 2020). A description of the trial can be found in the ISRCTN registry (ISRCTN no 16806663).

Participants

All consenting adults (>18 years) who presented to our center with a confirmed diagnosis of SARS-CoV-2 infection and moderate to severe COVID-19 (pulse oximetry ≤94% and a respiratory rate >24 breaths/min) were enrolled after informed written consent. The diagnosis was carried out using one or more of the following tests: antigen testing (SD Biosensor, South Korea), polymerase chain reaction (PCR) for SARS-CoV-2 (details below) or the GeneXPert molecular platform (Xpert, Cepheid CA).[28,29] This was based on the guidelines from local and national health authorities as issued from time to time. Patients were excluded if they had mental obtundation and/or other contraindications to NIV or unwillingness to receive NIV; if they were deemed candidates for invasive mechanical ventilation at the time of screening; if they had Kidney Disease Improving Global Outcomes Stage II or higher renal failure,[30] or a pre-existing diagnosis of chronic renal failure; if they had known glucose-6-phosphate dehydrogenase deficiency, a baseline methemoglobin >3%, or hemoglobinopathies; if they had a mean arterial pressure <65 mm Hg, or presence of baseline pulmonary artery hypertension (as adjudged by a tricuspid regurgitation velocity of >2.8 m/s on resting trans thoracic echocardiography); if they were pregnant or lactating.

Randomization and masking

Randomization was performed by a computerized random number generator, and allocation was concealed in sealed opaque paper envelopes. While the attending medical team recruited patients, obtained consent and documented the clinical data, including daily assessments of WOS, a separate trial team opened the trial allocation envelopes and administered iNO. The laboratory personnel analyzing specimens was blinded to allocation. However, no placebo was used in the trial and allocation to treatment or control was visible both to the trial and attending teams.

Procedures

All enrolled patients received care in a designated isolation location, which comprised of ICU and non ICU beds. The treatment group received iNO delivered through a tight fitting face mask and the V60 respiratory assist system using continuous positive airway pressure values of 5–10 cm H_2O, in pulses as below for three consecutive days post enrolment. The iNO doses were administered in a crescendo-decrescendo fashion as follows:10 ppm, 0–5 minutes; 20 ppm, 5–7 minutes; 30 ppm, 7–9 minutes; 50 ppm, 9–11 minutes; 80 ppm, 11–23 minutes; 80 ppm-0 ppm, 23–30 minutes, decreased at 10 ppm/min.

Study patients also received oral Sildenafil at doses of 10 mg thrice daily (to primarily prevent iNO rebound) for 5 days from trial enrollment. iNO therapy was discontinued if the baseline or individual session methaemoglobin exceeded 3%, if serially obtained mean arterial blood pressures dropped below 60 mm Hg for >15 minutes (as this warranted discontinuing Sildenafil therapy), or if a subject was transitioned to invasive mechanical ventilation. Methaemoglobin values were measured at the end of each of the six sessions of therapy and 24 hours post cessation of therapy as a safety measure. During and after this trial period, standard of care COVID-19 therapies such as steroids (dexamethasone), antivirals (remdesivir and favipiravir), antibiotics, fluid restriction, NIV (and/or transition to invasive ventilation) and anticoagulation were also prescribed on an "as needed basis" by the immediate care team as per the local guidelines.[28,29] Patients were categorized depending on their clinical signs as per state government protocol into mild (respiratory rate <24/min, SpO_2 >94% in room air), moderate (respiratory rate 24–29/min, SpO_2 91%–94% in room air) and severe (respiratory rate >30/min, SpO_2 <90%).

Viral load assessments were performed as follows: nasopharyngeal swab samples collected on baseline (day 0) and thereafter on days 3, 5, 7, 10, and 14 post-treatment were measured for SARS-CoV-2 RNA. Briefly, viral RNA was extracted from the nasopharyngeal swabs using the QIAamp viral RNA kit (Qiagen, Hilden, Germany). The PerkinElmer SARS-CoV-2 RT-qPCR kit (CE/IVD, PerkinElmer, Turku, Finland), which targets two specific genomic regions of SARS-CoV-2, namely the *N* gene and *ORF1ab*, was used to assess qualitative detection of viral RNA in study samples. The limit of detection of this kit was 1 copy/μL (3 log10 copies/mL). According to the protocol of the RT-qPCR kit, a Ct value of ≤40 for either one of the two targets was defined as "positive," while a Ct >40 was considered "negative." The Ct value of the *N* gene or *ORF1ab* gene of SARS-CoV-2 is considered to inversely correlate with the viral load. In this study, viral load was calculated by the comparative Ct (ΔCt) method. The Ct value of the human RNAse *P* gene was used as reference gene (internal control), to normalize for sampling variations. For the analysis of "time to negativity" between the two groups, a cut off of Ct >35 was defined as "absence" of viral loads/virus particles.[31]

Outcomes

The defined primary outcomes were decline in viral load as defined by a surrogate change in Ct values and clinical

improvement of >2 points on the WOS.[32] A 2-point WOS improvement is considered as a major positive clinical outcome. The defined secondary outcomes were 28-day all-cause mortality, need for transition to invasive ventilation, duration of NIV (if applicable), duration of oxygen therapy, length of hospital and ICU stay, a change in the SOFA score from enrolment to 72 hours thereafter (ΔSOFA = SOFA at admission − SOFA after 72 hours), and incidence of methaemoglobin levels >3% during the trial. Viral loads were serially assessed on nasopharyngeal specimens obtained at enrolment, and on days 3, 5, 7, 10, and 14 thereafter, or until a Ct >35, whichever was earlier.

Statistical Methods

Baseline characteristics were summarized by means and standard deviations. QQ-plots were used to visually assess whether continuous variables were approximately normally distributed. Kaplan-Meier tests were used to estimate and visualize time to event and log rank tests were used to compare time-to-event data between groups. Cox regression, logistic regression and ANOVA were used to assess the differences in outcomes. Inverse probability weighted propensity scoring was done to adjust for gender, Charlson comorbidity index and respiratory-SOFA scores, days of symptoms prior to randomization, and days of steroid and antiviral therapy up to randomization between the treatment and the control groups at baseline. Longitudinal mixed-effects regression models with a random effect for the individual intercept were used to estimate the effect of the treatment group compared to the control group for WOS (treating the scale as a quantitative variable) and viral loads. Restricted splines with three interior knots were used for the dependency on time. *F*-tests were used to test for significance of difference groups of parameters in the longitudinal models. Multiple imputation was used to deal with missing data. A $P < 0.05$ was considered to be statistically significant. R language version 4.0.3 was used for the analyses.

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