MAJOR ARTICLE

Inhaled Nitric Oxide as an Adjunctive Treatment for Cerebral Malaria in Children: A Phase II Randomized Open-Label Clinical Trial

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Background. Children with cerebral malaria (CM) have high rates of mortality and neurologic sequelae. Nitric oxide (NO) metabolite levels in plasma and urine are reduced in CM.

Methods. This randomized trial assessed the efficacy of inhaled NO versus nitrogen (N_2) as an adjunctive treatment for CM patients receiving intravenous artesunate. We hypothesized that patients treated with NO would have a greater increase of the malaria biomarker, plasma angiopoietin-1 (Ang-1) after 48 hours of treatment.

Results. Ninety-two children with CM were randomized to receive either inhaled 80 part per million NO or N_2 for 48 or more hours. Plasma Ang-1 levels increased in both treatment groups, but there was no difference between the groups at 48 hours (P = not significant [NS]). Plasma Ang-2 and cytokine levels (tumor necrosis factor-α, interferon-γ, interleukin [IL]-1β, IL-6, IL-10, and monocyte chemoattractant protein-1) decreased between inclusion and 48 hours in both treatment groups, but there was no difference between the groups (P = NS). Nitric oxide metabolite levels—blood methemoglobin and plasma nitrate—increased in patients treated with NO (both $P < .05$). Seven patients in the N_2 group and 4 patients in the NO group died. Five patients in the N_2 group and 6 in the NO group had neurological sequelae at hospital discharge.

Conclusions. Breathing NO as an adjunctive treatment for CM for a minimum of 48 hours was safe, increased blood methemoglobin and plasma nitrate levels, but did not result in a greater increase of plasma Ang-1 levels at 48 hours.

Keywords. cerebral malaria; methemoglobin; nitric oxide; *Plasmodium falciparum*.

Malaria kills more than half a million people per year, and most are children in sub-Saharan Africa [1]. Cerebral malaria (CM) is a catastrophic sequel of *Plasmodium falciparum* malaria, with a mortality rate of approximately 20% [2], causing long-term neurological defects in 10% to 24% of survivors [3, 4]. The most effective antiparasitic therapy is intravenous (IV) artesunate, which reduced mortality from 21% to 18% in African children with CM [2]. Several adjunctive therapies have been tested, but none have proven to be effective [5].

Nitric oxide (NO) is an intercellular messenger molecule synthesized by various cell types, including vascular endothelial cells. Nitric oxide is a vasodilator and inhibits platelet aggregation, among numerous other effects [6]. Malaria severity was correlated with reduced levels of biochemical markers of NO production [7].

Received 2 June 2015; accepted 21 July 2015.

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DOI: 10.1093/ofid/ofv111

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Physiological markers of reduced NO bioavailability—reduced brachial artery postischemic vasodilation [8, 9] and increased pulmonary arterial pressures [10]—were correlated with increased cell-free hemoglobin levels in patients with severe malaria, reflecting the NO-scavenging property of cell-free hemoglobin.

Angiopoietin (Ang)-1 and its antagonist Ang-2 regulate endothelial activation, and serum, plasma, or whole blood levels have been studied as biomarkers of CM severity and disease progression. Angiopoietin-1 levels were reduced and Ang-2 levels were elevated in pediatric CM patients compared with healthy children [11] and uncomplicated malaria patients [12, 13]. Significantly elevated levels of Ang-2 were found in fatal cases of CM and severe malaria [13].

Breathing low concentrations of NO gas reduces pulmonary arterial pressures without producing systemic hypotension in animal models and children [14, 15], and inhaled NO has been safely used to treat persistent pulmonary hypertension and other pulmonary disorders [16, 17] in neonates and infants for over 20 years [18]. In *Plasmodium berghei* ANKA (PbA)-infected mice (a murine CM model), breathing 40 or 80 parts per million (ppm) of inhaled NO [19, 20] improved survival rates, reduced plasma inflammatory markers, and increased plasma Ang-1 levels. In other studies, inhaled NO reduced cerebral injury in murine models of stroke [21, 22] and traumatic brain injury [23].

Based on the well established clinical safety profile of inhaled NO and its efficacy in experimental models of CM, we conducted an open-label randomized trial of breathing 80 ppm NO versus nitrogen (N_2) as an adjunctive therapy to IV artesunate in pediatric CM patients in Uganda. The primary endpoint was to compare the increase of plasma Ang-1 levels over the first 48 hours of treatment in patients breathing either NO or N_2. Nitric oxide delivery and safety were monitored and assessed by measuring methemoglobin% (metHb%) and NO metabolites. Although the study was too small to detect significant changes, in-hospital mortality, time to recovery from coma, and neurologic sequelae for up to 6 months after discharge were also determined.

METHODS

Study Participants

We screened children aged 2 months to 12 years, weighing 5–20 kg, at the tertiary care Mbarara Regional Referral Hospital (MRRH) Pediatric Ward and the Holy Innocents Children's Hospital. We enrolled patients with a Blantyre coma score (BCS) [24] <3/5 (for children aged <4 years) or Glasgow coma score (GCS) <7/15 (for children aged 4–12 years), with a *Plasmodium* infection confirmed by thick blood smear (see Supplementary Box 1 for additional inclusion and exclusion criteria). Potential study patients were transferred to the research unit at MRRH, and Ugandan research staff obtained informed consent from first-degree relatives, using materials written in English and Runyankole (the local language). See the Supplement for the full description of the institutions and supporting companies involved.

Study Design and Randomization

This was a randomized open-label, phase II clinical trial comparing breathing air or oxygen supplemented with inhaled NO (80 ppm [by volume] in N_2) or N_2 in children with CM. Nitrogen has been used as an inert gas control in other trials of inhaled NO [25]. After consent was obtained, we randomized patients to either study arm using off-site computer-generated random allocation. Sealed envelopes contained the patient treatment designation and were opened after consent and enrollment.

Survival was defined as alive at the time of hospital discharge. Patients were examined for neurological sequelae at the time of discharge and at follow-up neurological assessments at 1, 3, and 6 months after discharge.

Study Oversight

This study was conducted in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association, and was approved by the institutional review boards of the following institutions: Massachusetts General Hospital, Mbarara University of Science and Technology, the Ugandan National Council for Science and Technology, the Comité de Protection des Personnes France, and Médecins Sans Frontières International. The US Food and Drug Administration was notified, but an Investigational New Drug approval was not required. The Ugandan National Drug Authority approved the study. A data and safety monitoring board was convened biannually, for a total of 3 meetings, to review the unblinded safety data based on serious adverse events and mortality. The trial was registered under the ClinicalTrials.gov: NCT01388842.

Study Procedures

The patient's vital signs and coma score were assessed and logged every 2 hours. Transcutaneous pulse oxygen saturation (SpO_2), methemoglobin (SpMET), hemoglobin levels, and pulse rate were continuously monitored noninvasively with a Radical 7 co-oximeter (Masimo, Irvine, CA). When elevated transcutaneous SpMET levels were detected, blood metHb% was measured with an Avox 4000 spectrophotometer (ITC, Cambridge, MA) for confirmation. Whole-blood hemoglobin (g/dL) levels were measured with a Hemo Control Hemoglobin Analyzer (EKF Diagnostics, Cardiff, UK). If the metHb% level was 8.5% or greater, the inhaled gas dose was reduced by 50% and then reduced by an additional 25% of the original dose if the metHb% did not decrease below 8.5% within 1–2 hours.

Blood was sampled by venipuncture at enrollment (15 minutes before initiating study gas treatment, time 0) and at 12, 24,

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48, 72, and 120 hours after commencing treatment with study gas. Blood-based measurements included the following: hemoglobin, complete blood count, parasite smears, and plasma levels of creatinine, lactate, Ang-1 and Ang-2, cytokines (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, interleukin [IL]-1β, IL-6, IL-10, and monocyte chemoattractant protein [MCP]-1), S100b, and NO metabolites (nitrate, nitrite, *N*-nitrosamines [RNNO], *S*-nitrosothiols [RSNO]). A total of 10 mL of blood was sampled for research analyses from each patient over the 5-day study period. See the Supplement for a description of specific laboratory procedures.

Gas Administration

Study gas was delivered to the patient via a nasal cannula with a metered dosing apparatus specifically developed for investigational use (INOPulse^{TM}, Ikaria, Inc., NJ) [25]. The gas volume, and thus the gas dose to be delivered, was determined with a nomogram, incorporating the child's weight (to estimate tidal volume) and respiratory rate (see the Supplement for further description). Because the INOPulse was limited to 10 mL of gas injection per breath, to avoid under-dosing NO gas we were restricted to enrolling children weighing less than 20 kg. Enrolled patients received study gas for a targeted minimum of 48 hours and a maximum of 120 hours. All patients with a $SpO_2 \leq 94\%$ were given supplemental oxygen by facemask.

If, during the course of treatment, a patient's respiratory rate (and therefore alveolar minute ventilation) changed, his/her INOPulse gas dose was adjusted using the nomogram. If the patient regained full consciousness (BCS 5/5 or GCS 15/15) and had received treatment gas for greater than 48 hours, or if the patient completed 120 hours of gas treatment regardless of clinical status, the treatment gas was reduced by 50% each hour until turned off.

Concomitant Treatment

Enrolled patients received IV artesunate per protocol for at least 48 hours and up to 5 days. Patients able to take oral medications were transitioned to a 3-day course of artemether/lumefantrine (Coartem; Novartis Pharma, Switzerland) after 48 hours of IV artesunate treatment (see the Supplement for additional treatments and interventions).

Study Endpoints

The primary study endpoint was the increase of plasma Ang-1 levels at 48 hours. The secondary laboratory endpoints included reduction in plasma Ang-2, the Ang-2/Ang-1 ratio, selected plasma cytokine levels and creatinine, and blood lactate. The Ang-2/Ang-1 ratio was calculated for each patient, and the mean of these ratios is reported. Additional secondary endpoints included mortality during hospitalization, time taken to recover from coma, and neurological sequelae occurring up to 6 months after discharge. We evaluated the safety of NO delivery, and we monitored metHb% and NO metabolites to confirm inhalation and uptake of NO.

Statistical Analysis

We compared proportions with a χ^2 or Fisher's exact test with 2-sided *P* values. We assessed data within groups using a 2-tailed paired *t* test or Wilcoxon matched-pairs signed-rank test, and we assessed data between groups using a Mann-Whitney *U* test. Data between the N_2 and NO treatment

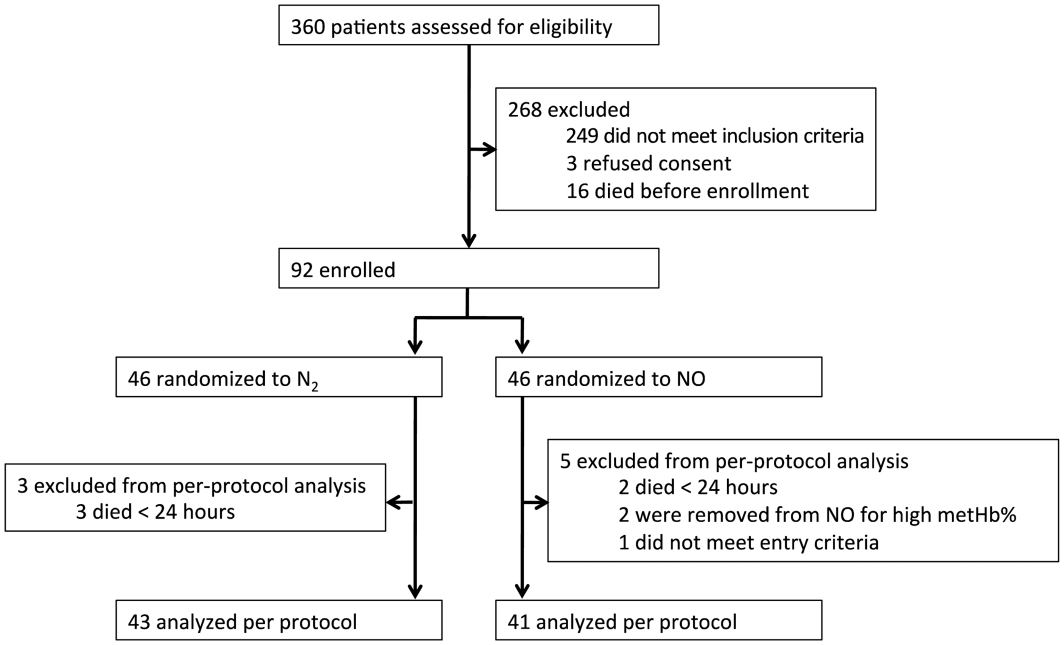


Figure 1. Screening, enrollment, and randomization flow diagram. Abbreviations: metHb%, percentage of methemoglobin; N_2, nitrogen; NO, nitric oxide.

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cohorts, and within treatment cohorts at hours 0 and 48, were compared from patients who received gas for at least 48 hours and those for whom we had paired data. We compared survival and time to coma recovery curves using Kaplan–Meier curves and log-rank test (or Wilcoxon Breslow when appropriate). Statistics and graphs were generated with Stata software (Release 13, StataCorp.) and GraphPad Prism 6 (GraphPad Software, Inc.). The level of statistical significance was set at 0.05. All results are displayed as mean ± standard deviation (SD). The target census for the study was 92 patients (see the Supplement for a description of how the study was powered).

RESULTS

Baseline Characteristics and Clinical Course

Between September 2011 and July 2013, 360 patients with a clinical diagnosis of CM were screened for eligibility. Ninety-two patients were included in the study, and their parent or guardian gave informed consent to enter the trial. Forty-six patients were randomly assigned to each treatment group. Eight patients enrolled in the study were not included in the per-protocol analysis: 3 patients in the N_2 group and 5 patients in the NO group (Figure 1). There was no difference in baseline characteristics or laboratory values between enrolled NO- and N_2-treated patients (Table 1).

There was no difference in the duration of treatment with study gas among patients treated ≥48 hours: 60.1 ± 23.3 hours and 63.9 ± 23.9 hours for N_2- and NO-treated patients, respectively (P = not significant [NS]). For patients receiving NO, the mean inhaled dose for the initial 48 hours of therapy was 0.55 ± 0.12 mg/kg per hour (N = 39) (Figure 2A).

Seventy-nine patients (38 N_2, 41 NO) returned for examination at 1 month after discharge, 76 patients (37 N_2, 39 NO) returned at 3 months, and 75 patients (36 N_2, 39 NO) returned at 6 months. The trial ended when the final enrolled patient completed the 6-month follow-up visit, in February 2014.

Primary and Secondary Endpoints and Outcomes

Table 2 shows the plasma Ang-1 and Ang-2 levels and the Ang-2/Ang-1 ratio at baseline (hour 0) and 48 hours later (hour 48). There was an increase of plasma Ang-1 levels in the NO group over 48 hours (P = .016), whereas there was no statistically significant increase in the N_2 group (P = .085). However, there was no difference in the change from baseline of plasma Ang-1 levels between the treatment groups. There was a significant decrease in plasma Ang-2 levels and the Ang-2/Ang-1 ratios over the 48 hours of treatment in both groups (P < .0001). There was no difference in (1) plasma Ang-2 or (2) the Ang-2/Ang-1 ratio between the treatment groups at either 0 or 48 hours.

In both groups, plasma TNF-α, IL-1β, and IL-6 levels were less at 48 hours than upon enrollment (Table 2), but there was no difference between the treatment groups at 0 or 48 hours (P = NS). Similar findings were obtained for plasma levels of IFN-γ, IL-10, and MCP-1 (Supplementary Table 1). Plasma levels of S100b, a biomarker of neural tissue injury [26], decreased after 48 hours in both treatment groups (Table 2), with no difference between treatment groups (P = NS).

Table 1. Baseline Clinical and Laboratory Data of Patients Enrolled in the Treatment Groups*

Characteristic	N_2 (N = 46)	NO (N = 46)
Female sex, no. (%)	24 (52.2%)	19 (41.3%)
Age, month	33.5 ± 22.4	42.4 ± 23.3
Age <48 month, no. (%)	36 (78.3%)	29 (63.0%)
Age ≥48 month, no. (%)	10 (21.7%)	17 (37.0%)
Weight, kg	11.6 ± 3.7	12.7 ± 3.3
Temperature, °C	38.1 ± 1.1	37.9 ± 1.3
SpO_2, %	97.8 ± 4.2	98.2 ± 2.0
Blood metHb, % (N)	0.6 ± 0.1 (37)	0.6 ± 0.1 (33)
Heart rate, BPM	147 ± 23	143 ± 25
Respiratory rate, BPM	37 ± 11	38 ± 13
Blood pressure, mmHg		
Systolic (N)	95 ± 11 (45)	98 ± 11 (44)
Diastolic (N)	53 ± 12 (45)	55 ± 10 (44)
Blantyre coma score (N)	1.3 ± 0.7 (36)	1.2 ± 0.8 (29)
Glasgow coma score (N)	5.6 ± 0.7 (10)	5.9 ± 0.5 (17)
History of seizures prior to admission, no. (%)	40 (87.0)	38 (82.6)
Malaria retinopathy (any finding), no. (%)	27 (58.7)	23 (50.0)
WBC, no. $\times 10^3/\mu L$	13.5 ± 7.1	16.8 ± 11.4
Hematocrit, %	23.3 ± 5.3	25.9 ± 6.7
Hemoglobin, g/dL	7.8 ± 2.1	8.7 ± 2.2
Platelets, no. $\times 10^3/\mu L$	93 ± 83	78 ± 68
Parasites, trophozoites, Parasite no. $\times 10^3/\mu L$ (N)	61 ± 100	56 ± 130

Abbreviations: BPM, beats per minute; metHb, methemoglobin; N_2, nitrogen; NO, nitric oxide; NS, not significant; SD, standard deviation; SpO_2, transcutaneous pulse oxygen saturation; WBC, white blood cells.

* All data are mean ± SD. P = NS comparing N_2 to NO.

There was no difference in the time to clear circulating parasites between the N_2 (N = 40) and NO (N = 43) treatment groups: 42.9 ± 26.2 and 40.7 ± 26.1 hours, respectively. Moreover, there was no significant difference in (1) the rate children became afebrile (<38°C) or (2) the change of blood creatinine or lactate levels over 48 hours between the gas treatment groups (see Supplementary Table 2 for creatinine and lactate levels).

There were no clinically significant differences in heart rate, systolic blood pressure, or SpO_2 after initiating treatment in patients receiving NO versus N_2. In addition, the number of patients affected by severe adverse events (SAEs), or any adverse events (AEs), including cardiopulmonary or respiratory complications, was similar in the 2 treatment groups.

Eleven (12.0%) of 92 enrolled CM patients died while in the hospital (Table 3), 7 of whom were treated with N_2 (15.2%) and

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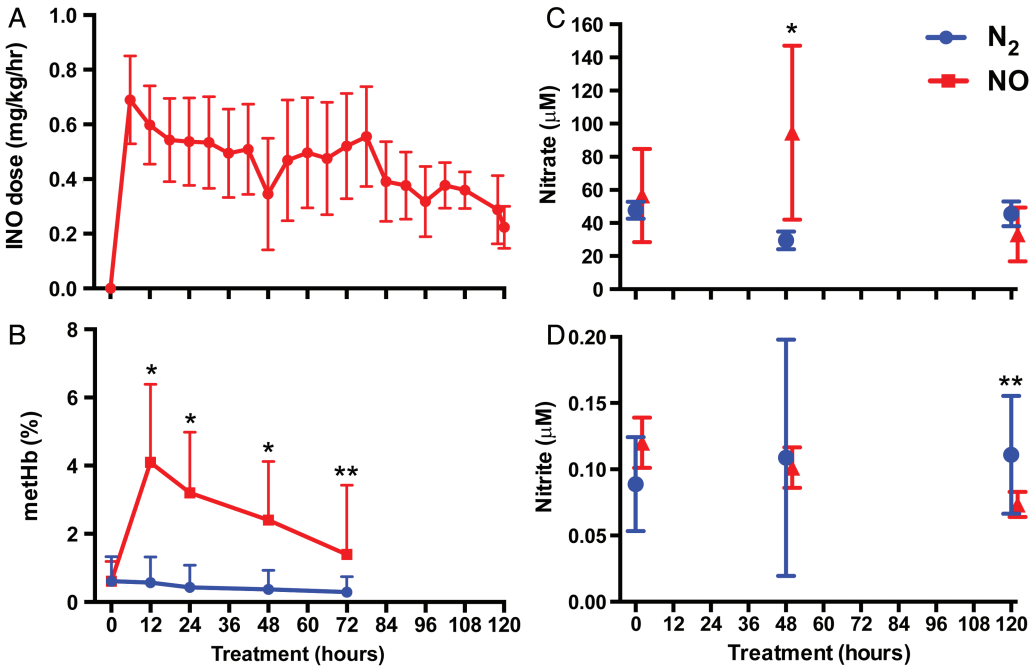


Figure 2. (A) Delivered dose of nitric oxide (NO) over 120 hours (mg/kg per hour); (B) blood methemoglobin (percentage of methemoglobin [metHb %]) in patients treated with nitrogen (N_2) and NO; (C) Plasma nitrate (μM) levels; and (D) plasma nitrite (μM) levels in patients treated with N_2 or NO. *P < .001 and **P < .05, NO versus N_2. All data mean ± standard deviation.

Table 2. Primary and Secondary Laboratory Outcomes*

Angiopoietins	Interval	N_2 (N = 42)	NO (N = 39)	Intragroup P Value	Intergroup P Value
Ang-1, pg/dL	Hour 0	2049 ± 2392	1733 ± 2485		.73
	Hour 48	3235 ± 3516	2837 ± 3152	N_2 = 0.085 NO = 0.016	.81
Ang-2, pg/dL	Hour 0	16235 ± 16216	14440 ± 12202		.41
	Hour 48	5901 ± 8424	4801 ± 4831	N_2 < 0.0001 NO < 0.0001	.78
Ang-2/Ang-1 Ratio	Hour 0	19.62 ± 24.29	35.35 ± 119.10		.94
	Hour 48	7.80 ± 20.58	5.71 ± 11.35	N_2 < 0.0001 NO < 0.0001	.96
Cytokines		N_2 (N = 40)	NO (N = 40)		
TNF-α, pg/mL	Hour 0	0.297 ± 0.495	0.311 ± 0.600		.90
	Hour 48	0.095 ± 0.410	0.008 ± 0.026	N_2 = 0.0005 NO < 0.0001	.99
IL-1β, pg/mL	Hour 0	0.359 ± 0.631	0.508 ± 0.910		.74
	Hour 48	0.151 ± 0.649	0.024 ± 0.074	N_2 = 0.007 NO < 0.0001	.93
IL-6, pg/mL	Hour 0	84.63 ± 108.60	99.19 ± 122.30		.88
	Hour 48	8.04 ± 27.56	13.46 ± 60.56	N_2 < 0.0001 NO < 0.0001	.70
S100b		N_2 (N = 37)	NO (N = 38)		
S100b, pg/mL	Hour 0	931.2 ± 813.7	922.7 ± 861.5		.80
	Hour 48	283.8 ± 400.4	318.8 ± 477.1	N_2 < 0.0001 NO = 0.0001	.85

Abbreviations: Ang, angiopoietin; IL, interleukin; N_2, nitrogen; NO, nitric oxide; SD, standard deviation; TNF, tumor necrosis factor.

* Plasma levels of Ang-1, Ang-2, cytokines, and S100b, as well as the ratio of plasma Ang-2 levels to plasma Ang-1 levels on enrollment and after 48 hours. All data are mean ± SD.

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Table 3. Secondary Clinical Outcomes*

	N_2	NO
Died before 7 days after enrollment	7 of 46 (15.2%)	4 of 46 (8.7%)
Survived 1 month after discharge	39 of 46 (84.8%)	42 of 46 (91.3%)
Neurological sequelae in 1-month survivors	5 of 39 (12.8%)	6 of 42 (14.3%)
Full recovery in 1-month survivors	34 of 39 (87.2%)	36 of 42 (85.7%)

Abbreviations: N_2, nitrogen; NO, nitric oxide; NS, not significant.

* All data are presented as number and percentage of patients in each treatment group. P = NS comparing N_2 to NO.

4 of whom were treated with NO (8.7%). Of the patients who were living at the time of hospital discharge (39 N_2, 42 NO), 5 of the patients treated with N_2 (12.8%) and 6 of the patients treated with NO (14.3%) exhibited neurological sequelae at the 1-month follow-up visit. Neurological sequelae included ataxia, hemiplegia, hypotonia, seizures, speech dysfunction, and/or behavioral disorders. Of the 11 patients with neurological sequelae at the time of discharge, 3 patients (1 N_2, 2 NO) resolved their neurological sequelae by the 6-month follow-up visit. Figure 3A shows the Kaplan-Meier survival curve for the 5 days after randomization into the study for patients treated with either N_2 or NO (P = NS). There was no difference in the rate of recovery from coma between the NO and N_2 treatment groups (P = NS; Figure 3B), with the duration of coma of 48.1 ± 58.2 hours and 55.8 ± 50.1 hours for the N_2 and NO treatment groups, respectively.

Focusing only on those patients with evidence of malarial retinopathy (N = 27, 58.7% N_2 group; N = 23, 50.0% NO group), we observed that there was no difference in the change in (1) Ang-1, Ang-2, Ang-2/Ang-1 ratio between 0 and 48 hours or (2) the survival rate between patients receiving NO or N_2.

Nitric Oxide Metabolic Products and Effects

Blood metHb, nitrate, nitrite, RSNO, and RNNO are NO reaction products. Before breathing NO, baseline blood metHb% levels in the N_2 and NO groups were 0.6 ± 0.1% (N = 37) and 0.6 ± 0.1% (N = 34), respectively (P = NS) (Figure 2B). In the NO group, blood metHb% levels increased to 4.1 ± 0.4% (N = 33) at 12 hours, 3.2 ± 0.3% (N = 33) at 24 hours, 2.4 ± 0.3% (N = 33) at 48 hours, and 1.4 ± 0.4% (N = 32) at 72 hours (P < .0001 at 12, 24, and 48 hours, and P = .03 at hour 72), when compared with patients breathing N_2, in whom levels of blood metHb% did not change. The decrease in metHb% levels between 24 and 72 hours reflects the reduction or cessation of NO administration, because coma was reversed during recovery. Of note, 6 NO patients transiently developed metHb% levels ≥10%, and a reduction of the inhaled NO dose per protocol led to a rapid decline in metHb% levels in each patient within 2 hours.

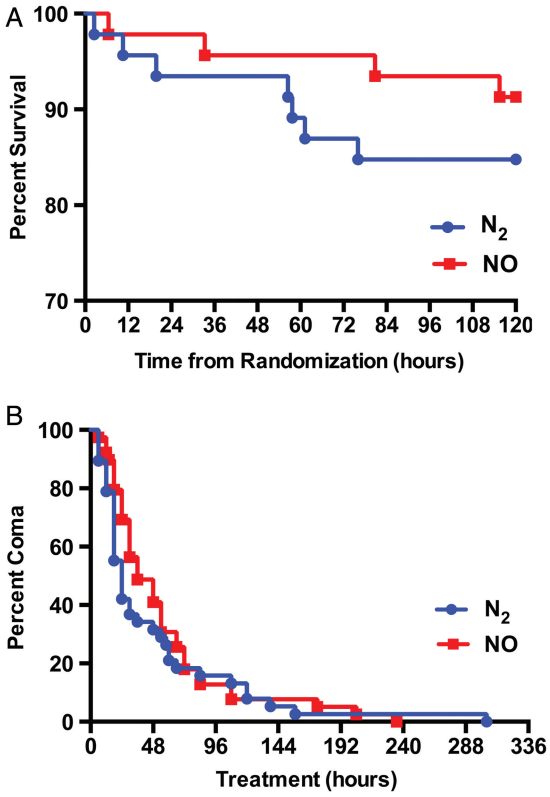


Figure 3. (A) Kaplan-Meier curves comparing survival in pediatric patients with cerebral malaria (CM) treated with nitrogen (N_2) or nitric oxide (NO). P = not significant (NS). (B) Kaplan-Meier curves for resolution of coma in pediatric patients with CM, N_2 vs NO; resolution of coma is defined as Blantyre coma score ≥ 3 or Glasgow coma score of ≥7; (N_2 N = 39, NO N = 41). P = NS. Abbreviations: INO, inhaled NO; metHb%, percentage of methemoglobin.

There was no difference of baseline nitrate levels in patients treated with N_2 or NO (P = NS) (Figure 2C). Nitrate levels decreased in the N_2-treated group over 48 hours, from 47.7 ± 21.1 to 29.5 ± 22.8 μM (P < .05). In contrast, nitrate levels increased over 48 hours with NO treatment from 56.7 ± 28.2 to 94.7 ± 52.5 μM (P < .05). Plasma nitrate levels at 48 hours were markedly greater in patients breathing NO versus N_2 (P < .01). After discontinuation of NO breathing, nitrate levels returned to baseline by 120 hours. Nitrite levels (Figure 2D) did not change significantly from hour 0 to 48 in either group but decreased from 48 hours to 120 hours in NO-treated patients (P < .05). The nitrite levels in the N_2- and NO-treated patients differed at 120 hours (P < .05). Plasma RSNO and RNNO levels were not significantly different between the treatment groups at hours 0, 48, and 120 of treatment (Supplementary Table 2).

DISCUSSION

This randomized trial compared breathing 80 ppm NO versus N_2 as an in-hospital adjunctive therapy for 92 comatose

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pediatric patients with CM. There was no significant difference of plasma Ang-1, Ang-2, or cytokine levels between the groups after 48 hours of treatment (Table 2).

Nitric oxide proved to be effective in murine CM models, wherein PbA-infected C57BL/6 mice treated with a systemic NO donor (dipropylenetriamine NONOate), or breathing 40 ppm NO, had improved survival [19]. Prophylactic treatment with 80 ppm NO in the same model, with and without antimalarial therapy, resulted in a reduced mortality rate, a greater increase of plasma Ang-1 levels, reduced brain mRNA copy numbers of the ratio of Ang-2/Ang-1, and reduced plasma levels of IFN-γ, TNF, and MCP-1 [20].

In this small clinical trial, we found an overall mortality rate of 12%, with fewer patients dying breathing NO; however, NO treatment did not significantly reduce the mortality rate. The overall mortality was less than the 18% reported in a recent multicenter study of 5425 children with severe malaria (of whom 1825 had CM) treated with IV artesunate [2]. The overall incidence rate of neurological sequelae in our study was 12%, which is similar to the 10%–25% rate reported in sub-Saharan Africa [3, 4]. We believe NO treatment to be safe because the mortality rate in our study was low, breathing NO did not increase the incidence of neurological sequelae, mean methemoglobin levels remained low (4.1% ± 0.4%), and the number of patients affected by SAEs and AEs were similar in both groups.

Plasma S100b, a biomarker of injury to astrocytes, oligodendrocytes, and other neural cell types, is increased in patients after traumatic brain injury and cardiac arrest [26–28]. Elevated S100b levels have been measured in adult and pediatric CM patients with seizures, but this was only investigated using cerebrospinal fluid [29, 30]. We measured plasma S100b levels in our CM patients, and, although we found no significant difference in the reduction of the levels between the treatment groups over 48 hours (Table 2), plasma S100b may be useful as a tool for assessing the level of central nervous system injury in future studies involving CM patients.

We delivered NO or N_2 with the INOPulse, a gas injector that was triggered on inspiration. Even though it was not possible to measure the precise NO concentration in the trachea of children receiving NO gas with the INOPulse, we confirmed that NO was inhaled by detecting elevated levels of blood metHb% and plasma NO metabolites (Figure 2B and C). Similar observations were reported by Gladwin et al [25], among adult sickle cell patients breathing 40 or 80 ppm NO using the INOPulse.

We measured plasma NO metabolites in an effort to better understand their changes in CM patients breathing NO, and we observed increased plasma nitrate levels in the NO arm and a reduction of nitrate levels in the N_2 arm after 48 hours of treatment (Figure 2C). After inhalation, NO has an extremely short half-life with 97% rapidly converted to nitrate and the remainder to stable adducts (*S*-nitrosylated proteins [eg, *S*-nitrosothiols or *N*-nitrosamines], nitrosyl-hemeproteins, and nitrite), which can release NO in systemic vascular beds by various mechanisms [31, 32]. Studies have shown beneficial extrapulmonary effects of inhaled NO in humans, including reduced platelet aggregation [33], reduced myocardial injury after cardiopulmonary bypass [34], increased renal blood flow [35], enhanced liver allograft function in liver transplant recipients [36], and reduced inflammation in lower extremity ischemia-reperfusion from tourniquet use [37].

Despite the possible vasodilatory and anti-inflammatory benefits of inhaled NO described above, including the positive outcomes of NO trials in murine models of CM [11, 12], we did not find a significant difference in the change of plasma Ang-1 or Ang-2 levels after 48 hours of inhaled NO treatment. Plasma angiopoietin levels have been correlated to the severity of illness in severe malaria and CM patients [11, 12]. In the future, it may be valuable to focus the administration of inhaled NO upon CM patients with laboratory evidence of NO scavenging and pulmonary or systemic vasoconstriction. We would suggest studying the subset of CM patients with elevated levels of plasma cell-free hemoglobin and therefore increased NO scavenging and consumption [38]. This subset has shown evidence of increased right heart/pulmonary pressures on echocardiogram [10]. It is also possible that this subset of patients suffer from increased cerebrovascular resistance with reduced cerebral blood flow. Breathing NO oxidizes cell-free hemoglobin to metHb, thereby reducing its ability to scavenge intrinsic NO [39]. Inhaling NO may have greater clinical efficacy in this subgroup of patients.

CONCLUSIONS

Treatment with NO did not significantly increase plasma Ang-1 levels at 48 hours compared with patients treated with N_2. This small trial could not demonstrate a reduction of mortality or neurological impairment in children treated with NO. Seven patients in the N_2 group and 4 patients in the NO group died. A larger trial, utilizing measurements of cell-free hemoglobin and right heart pressures to assess pulmonary vasoconstriction, would be required to learn whether breathing NO could lead to improved survival rates in this subgroup of malaria patients.

Supplementary Material

Supplementary material is available online at *Open Forum Infectious Diseases* (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

Acknowledgments

We sincerely thank the children and parents involved in the study. We also thank the following individuals: Sheilla Mbabazi, Jackline Tumuhairwe, James Kinyata, Amerias Arimpa, Gertrude Ngabirano, Allen Atukunda, and Addy Ahairwe for providing nursing care; Karin Hergarden and Susan Loosse for internal monitoring; Victoria Katawera, Davis Kaganzi, Arnold Ayebare, Harriet Adrama, and Daniel Omoding for the laboratory work; and Rinah Arinaite, Peace Magezi, and Ronie Mutabazi for data entry and management. We acknowledge Brian Bergmark and Regan Bergmark for

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contributing to the writing protocols while they were Harvard Medical School students working in Mbarara. Moreover, we thank the guards, cleaners, and drivers who facilitated the running of the study. We thank Dr. Emmanuel Buys for providing technical oversight of assays at Massachusetts General Hospital, Robert Tainsh for completing the cytokine assays, and Dr. Rajeev Malhotra for additional statistical assistance.

Author contributions. J. M.-A., R. W. C., E. B., Y. B., P. D. B., J. F.-E., K. D. B., M. F., B. O. F., and W. M. Z. developed the concept of the study. J. M.-A., R. W. C., E. B., K. D. B., and W. M. Z. were responsible for data analysis. J. M.-A., R. W. C., E. K., D. Na., K. M., D. S., D. Ny., D. I. N., and Y. B. are the trained physicians, nurses, and scientists who carried out patient care, gas administration, and laboratory processing. M. F. and B. O. F. carried out specialized laboratory work. All authors contributed to the development of the protocol, carrying out the protocol, and writing of the manuscript.

Disclaimer. No scientific writer was involved in the development of this manuscript. R. W. C. had full access to all of the data in the study, takes full responsibility for the integrity of the data and accuracy of the data analysis, and is the guarantor.

Financial support. This work was supported by financial contributions from the International Innovation Fund of Médecins Sans Frontières (MSF) and MSF France; the Anesthesia Center for Critical Care Research and the Department of Anesthesia, Critical Care, and Pain Medicine of Massachusetts General Hospital (Boston, MA); and the Mark and Lisa Schwartz Foundation. This study would not have been possible without generous gas and equipment support from Ikaria and Masimo, and funding for the NO metabolite assays from the University of Southampton School of Medicine, UK.

Potential conflicts of interest. A. B. was an employee of Ikaria, Inc. when she trained the clinical investigators on the use of the drug and the delivery devices provided by Ikaria Inc. W. M. Z. reports that the Massachusetts General Hospital has received royalties on the sales of nitric oxide for inhalation from Linde Corporation (Munich, Germany) and Ikaria (New Jersey), and he has received a portion of these royalties. Ikaria provided free gas and delivery devices for this study but played no other role. Massimo provided the oximeters but played no other role.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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