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MINIREVIEW

# Pathophysiological Mechanisms in Gaseous Therapies for Severe Malaria

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**Over 200 million people worldwide suffer from malaria every year, a disease that causes 584,000 deaths annually. In recent years, significant improvements have been achieved on the treatment of severe malaria, with intravenous artesunate proving superior to quinine. However, mortality remains high, at 8% in children and 15% in adults in clinical trials, and even worse in the case of cerebral malaria (18% and 30%, respectively). Moreover, some individuals who do not succumb to severe malaria present long-term cognitive deficits. These observations indicate that strategies focused only on parasite killing fail to prevent neurological complications and deaths associated with severe malaria, possibly because clinical complications are associated in part with a cerebrovascular dysfunction. Consequently, different adjunctive therapies aimed at modulating malaria pathophysiological processes are currently being tested. However, none of these therapies has shown unequivocal evidence in improving patient clinical status. Recently, key studies have shown that gaseous therapies based mainly on nitric oxide (NO), carbon monoxide (CO), and hyperbaric (pressurized) oxygen (HBO) alter vascular endothelium dysfunction and modulate the host immune response to infection. Considering gaseous administration as a promising adjunctive treatment against severe malaria cases, we review here the pathophysiological mechanisms and the immunological aspects of such therapies.**

Malaria exerts a heavy burden over human populations, with an estimated 124 to 283 million cases and 584,000 deaths in 2013 (1). Currently, intravenous (i.v.) artesunate is the treatment of choice in severe malaria cases in children and adults (2, 3). However, despite the efficacy of intravenous artesunate, mortality from severe malaria in general and from cerebral malaria (CM) in particular remains high, at 18% for African children and 30% for adults in Southeast Asia (2, 3). In addition, 11% of children who survive CM show severe neurological deficits, and up to 25% can maintain long-term cognitive deficits (4–8). Therefore, strategies focusing only on parasite killing may not be sufficient to prevent neurological complications and deaths related to severe malaria.

Accordingly, adjunctive therapies—defined as therapies administered in combination with antiparasitic drugs that modify pathophysiological processes caused by malaria—are being sought in order to mitigate complications caused by severe malaria (9). Considering the fact that currently administered antimalarial drugs often take 12 to 18 h to kill parasites, adjunctive therapies could reduce the risk of neurocognitive sequelae and mortality, particularly in patients with CM (10).

Different adjunctive therapies have been or are being tested, including treatments aimed at modulation of the immune response to infection (dexamethasone, intravenous immunoglobulin), reduction of iron burden, reduction of oxidative stress, modulation of the prothrombotic state, and reduction of parasitemia (blood transfusion), among others (reviewed in references 10 and 11). However, none of these adjunctive treatments has shown unequivocal evidence of improvement for patients in clinical trials, and therefore none of them can be definitely recommended as a treatment strategy (10, 11). Thus, pursuing new adjunctive therapies for malaria remains a research priority.

It is in this scenario that the gas-based therapies for malaria arise. The study of administration of gas therapies has advanced in some areas, such as hyperbaric (pressurized) oxygen (HBO) for complicated wound healing (12–14) and nitric oxide (NO) for acute respiratory distress syndrome (15), although not without controversy (16, 17). Nevertheless, the use of gaseous therapy for malaria is incipient. At the moment, only two phase II clinical trials have been completed, both examining the effect of NO administration for children with severe malaria (18, 19). Nevertheless, some *in vitro* and *in vivo* studies—using the experimental cerebral malaria (ECM) murine model—have shed light on the topic and opened perspectives for adjunctive therapies in malaria. ECM is the result of the infection of susceptible mouse strains, such as C57BL/6 and CBA, with *Plasmodium berghei* strain ANKA (20). The relevance of this model is a matter of heated debate and has been discussed in depth elsewhere (21–24). Of critical importance is the fact that in both human and murine severe malaria, ischemia and hypoxia resulting from hypoperfusion play a key role in pathogenesis, and in both cases hypoperfusion results from vascular occlusion and dysfunction. Human severe malaria findings, such as retinal hypoperfusion (25), impaired reactive hyperemia-peripheral arterial tonometry index (RH-PAT index; a measurement of reactive vasodilation) (26), low NO bioavailability (26), increased levels of plasma cell-free hemoglobin (27), elevated asymmetric dimethylarginine-to-arginine plasma ratios (28, 29), and low levels of plasma angiopoietin-1 (30), are closely

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mimicked in *P. berghei* ANKA-infected mice displaying severe malaria (31–34). Since ischemia and vascular dysfunction are the prime targets of gaseous therapies, the murine model of severe malaria may work as a reliable surrogate to address these issues. However, the limitations of any experimental model need to be considered, with findings requiring subsequent confirmation in human studies. Having these considerations in mind, and due to the obvious restrictions imposed on studies in humans, experimental models may represent valuable sources of insights and establishing proof of concepts for the discovery of mechanisms of pathogenesis and novel therapeutic targets. Herein, we review the state of the art of the study of carbon monoxide (CO), NO, and HBO as adjunctive therapies for malaria.

## CARBON MONOXIDE

CO is physiologically produced as a by-product of the degradation of heme, in a reaction catalyzed by heme oxygenase 1 (HO-1) and which also produces $Fe^{2+}$ and biliverdin (35). Although widely known for its toxicity due to its high-affinity binding to hemoglobin, CO has drawn scientific attention for its role as a signaling molecule in the gastrointestinal tract, a paracrine mediator of smooth muscle hyperpolarization, and an immunomodulatory effector (35–37). The immune actions of CO take part in the "immunological web" of HO-1, the inducible form of heme oxygenase, whose expression is upregulated in situations of cellular exposure to oxidant agents, pathogens, and other stressors (35). The colocalization of HO-1 expression and vascular lesions in brains of patients that died from CM provides evidence of HO-1 induction (38), albeit not necessarily indicating increased or sufficient enzymatic activity (39). In ECM, *P. berghei* ANKA-infected BALB/c mice exhibited a higher expression of the heme oxygenase-1 gene (*Hmox-1*) and HO-1 than did C57BL/6 mice and were less likely to die of ECM (40). Furthermore, deletion of *Hmox-1* rendered BALB/c mice susceptible to death by ECM (40). The protective action of the augmented expression of HO-1 is believed to take place through CO production and its binding of cell-free hemoglobin (40–42). In malaria, cell-free hemoglobin is produced due to hemolysis, and its degradation leads to the formation of free heme, a highly oxidant molecule proposed to be a key mediator of blood-brain barrier (BBB) dysfunction, a hallmark of CM (43, 44). In this regard, Pena et al. (41) developed a CO-releasing molecule (CO-RM) that fully protected mice from death due to ECM when administered before the onset of symptoms, preventing inflammation and BBB disruption. The use of the CO-RM in combination with artesunate improved survival for 83%, compared to artesunate alone, indicating the potential of this molecule as an adjunctive therapy.

Nevertheless, the effect of CO and HO-1 in CM is a matter of debate. Studies from Myanmar, Angola, and Gambia have found an association between shorter $(GT)_n$ dinucleotide repeat polymorphisms in the *Hmox-1* promoter region—correlated with higher expression of the gene and higher levels of HO-1 in peripheral blood—and the incidence of severe malaria (45–47). The authors of the Gambian study argued that while this observation may simply reflect an adequate but insufficient response, the higher induction of HO-1 in patients with shorter $(GT)_n$ repeat alleles indicates that levels of HO-1 above a certain threshold might directly participate in the disease pathogenesis (48). Such deleterious effects might involve oxidative pathways via activation of the neutrophil oxidative burst (46) and release of iron (48). These findings highlight a problem in experimental models dealing with CO and HO-1 in malaria, as inbred mice lack the variability of HO-1 $(GT)_n$ repeat polymorphisms (49).

Considering that in ECM the liver phase of malarial infection is skipped (40, 41, 50), discrepancies in HO-1 levels between the mouse model and human infections might occur (51). For example, Epiphanio et al. found that when *Hmox-1*$^{-/-}$ mice were infected with sporozoites, instead of being directly inoculated with blood-stage parasites, infection failed to develop, and inhalation of CO by *Hmox-1*$^{+/+}$ mice in this setting led to a 4-fold increase in *P. berghei* liver infection (51). Given that malaria is diagnosed during the blood phase of infection, HO-1/CO-based therapeutic approaches possibly would not face the dilemma of increasing parasite load in *Plasmodium falciparum* infection, but the same is not warranted for species that produce hypnozoites, such as *Plasmodium vivax*.

A major concern, however, for the application of CO-based therapies is the gas intrinsic toxicity. CO poisoning is a leading cause of unintentional poisoning (52), and carboxyhemoglobin (COHb) levels as low as 3% are indicative of exposition in nonsmokers. Toxicity from free hemoglobin released during malaria may be prevented by CO's ability to bind hemoglobin, which is the exact same mechanism that drives its toxicity. Yeo et al. (53) described an association between COHb levels and severity of malaria disease in Indonesian adults; however, no such association was found for Kenyan children (39). While in the former study COHb might have been generally overestimated, there was a significant increase in COHb levels from healthy controls with moderately severe and severe malaria. While the smaller but significant increase in COHb in patients with moderately severe malaria might indeed reflect a protective effect from an adequate increase in HO-1 activity, further increases in COHb seem insufficient or harmful (46, 53). Besides a possible harmful effect of HO-1 superactivation, induction of COHb leads to a decrease in blood oxygen-carrying capacity. In severe malaria patients, hemoglobin levels are already reduced, imposing serious risks and limitations for the use of CO as an adjunctive therapy (53).

## NITRIC OXIDE

NO plays physiological roles in neuronal and vascular cells, regulating vasodilation and blood pressure, among other biological effects. It is produced by the activity of enzymes known as NO synthases (NOSs), whose substrates are the amino acid L-arginine and $O_2$. Three NOS isoforms have been identified: neuronal (NOS1), inducible (NOS2), and endothelial (NOS3). Both NOS1 and NOS3 are calcium-dependent enzymes expressed constitutively, whereas NOS2 is expressed in response to acute inflammatory stimuli (54). NO has been related to numerous pathological conditions, including artery disease (55), cerebrovascular stroke (56), sepsis (57), and ischemic injury (58).

Reduced NO bioavailability has been reported in human malaria (59) and ECM (33), and this phenomenon could contribute to the development of disease by impairment of endothelial function and vascular perfusion, as reviewed elsewhere (60). NO decreases the expression of endothelium activation markers and reduces the expression of adhesion molecules, such as ICAM-1 and P-selectin, resulting in decreased vascular permeability (61) and leukocyte and platelet adhesion (62).

Autopsy of CM patients revealed the sequestration of infected red blood cells (iRBC) in the capillaries and postcapillary venules

of multiple organs, suggesting a role for iRBC cytoadherence in the pathogenesis of severe malaria (61, 63, 64). NO exposure led to reduced iRBC adherence to endothelium under flow conditions *in vitro* (65) as well as a decreased biomass of infected erythrocytes on cerebral tissue in ECM (66). Thus, NO may play a role against CM via antiadhesive effects.

Mice with ECM show widespread cerebrovascular constriction, leading to marked ischemic hypoxia (67) and decreased blood flow (31). In addition, pial vessels of mice with ECM show impaired NOS1- and NOS3-mediated vasodilatory responses to pharmacological stimulation (32). Evidence of vascular dysfunction has been documented also in human CM, with the observations of retinal vascular occlusion, hypoperfusion, and hemorrhage (25) and impaired vasodilation, along with low exhaled NO levels (26). Several factors are thought to contribute to low NO bioavailability, such as hypoargininemia (low plasma L-arginine concentration) (68), increased concentration of NOS inhibitor, and reduced expression of NOS (28, 59, 69).

Therefore, adjunctive therapies aimed at restoring NO levels were developed. In *P. berghei* ANKA-infected mice, treatment with the NO donor dipropylenetriamine NONOate (DPTA-NO) prevented the neurological syndrome, with increased endothelial barrier integrity and protection of the brain tissue from extravasation and petechial hemorrhaging, but it led to hypotension in mice (70). Treatment with *S*-nitrosylated glutathione (GSNO), an endogenous, physiological NO donor, prevented ECM development while having milder effects on blood pressure (71). Glyceryl trinitrate (nitroglycerin; GTN) not only prevented ECM but also worked as adjunctive therapy with artemether, markedly increasing survival of mice with late-stage ECM compared to artemether alone (72). The benefit in survival was associated with reversal of cerebrovascular constriction, suggesting that the effect was due to improved brain perfusion. Finally, novel hybrid drugs combining dihydroartemisinin with NO donors were shown to be more effective than artemether in rescuing mice with ECM (73). The benefits of NO donors, such as the ones described above, have not yet been shown in human CM.

An alternative form of NO treatment is the inhalation of NO (iNO), which is approved by the FDA for the treatment of respiratory failure, hypoxia, and pulmonary hypertension (74). During ECM, iNO treatment reduced the activation of endothelial cells, decreased the number of parasites in the brain, and maintained BBB integrity, and when combined with artesunate improved mouse survival rates compared to artesunate alone (66). However, it must be emphasized that mice were treated before the neurological syndrome was established. Given that iNO is used in the treatment of other diseases, with a well-established safety profile and low cost, along with positive results in animal models, it is an attractive option for clinical tests in malaria patients. Based on these advantages, two randomized phase II clinical trials in patients with severe malaria have been recently reported in Uganda (18, 19, 75). The first study compared 88 children who received iNO at 80 ppm with 92 children who received placebo (all subjects received artesunate i.v.) and showed that iNO failed to reduce angiopoietin-2 (Ang-2; a marker of endothelial dysfunction) levels and had no effect on mortality (18). Methemoglobinemia did develop in 25% of children in the treated group, but without sequelae. The second study compared 46 children receiving iNO at 80 ppm with 46 children in the placebo group, with similar results. Plasma levels of Ang-2 and inflammatory cytokines remained similar between groups, and there was no difference in mortality (19). Treatment with iNO resulted in increased levels of plasma nitrate, and methemoglobinemia developed, but without sequelae. The fact that iNO combined with artesunate did not result in a greater reduction of Ang-2 levels compared to artesunate alone in these trials indicates that a measurable biological effect on the endothelium was not achieved with this NO dose and route of administration (69, 70). A major potential limitation with iNO is that NO may not exert its expected effects systemically, rather being restricted to the lung endothelium. In such a scenario, rapid conversion of iNO to nitrate and other stable adducts may result in decreased levels of bioavailable NO, although pharmacological effects beyond the pulmonary vasculature have been reported in other studies in humans (76). The use of better, more reliable readouts of NO action in the systemic vasculature in these trials is imperative to ensure that it is being properly delivered.

Infusion of L-arginine is another candidate for adjunctive treatment based on increased NO levels. Patients with severe falciparum malaria treated with antimalarial drugs showed a correlation between increased levels of L-arginine and the improvement of endothelial function (77). Infusion of L-arginine improved NO bioavailability without significant adverse effects on vital signs (26). Despite these encouraging results, in patients with severe falciparum malaria infusion of L-arginine at low doses over 8 h failed to change lactate clearance time and RH-PAT (78). However, this was a small pilot study, and as such lacked sufficient power to show beneficial effects.

Despite advances reported with NO therapy studies, the molecular mechanisms involved in induction of protection have not been completely elucidated. Data from animal studies suggest its main effect takes place by restoring vascular tonus and hence reversing cerebral ischemia/hypoxia (32, 70). Recent research demonstrated in ECM that NO regulates *Hmox*-1 expression by a mechanism involving the transcription factor Nfr-2 and consequently CO production. The proposed mechanism is that CO prevents Hb oxidation and heme release, while NO exerts a prooxidant effect, preventing activation, proliferation, and expansion of T cells and thus inhibiting a deleterious response to malaria infection (50) (Fig. 1a and b). However, this remains to be further confirmed for human disease.

## HYPERBARIC OXYGEN

The inhalation of oxygen (95%) under normobaric (1 atmosphere) conditions was found to be ineffective for the treatment of malaria (79); therefore, an alternative form of $O_2$ delivery, as hyperbaric (pressurized) oxygen (HBO), has been developed. HBO is defined as a treatment of exposure to oxygen (100%) at a pressure greater than 1 atmosphere absolute (ATA) (80). It is the only treatment for decompression sickness (80) and is recommended for complicated wound healing (14). In addition, HBO is widely used as an adjunctive therapy for many conditions, such as diabetic ulcer healing, traumatic brain injury, and ischemic stroke. However, a recent meta-analysis of clinical trials for the latter three conditions found no conclusive evidence for benefit to the patient after HBO therapy (13, 81, 82).

HBO treatment is relatively safe (83, 84), and some studies have shown it has anti-inflammatory activity (85–87). These features support further research into HBO treatment as an adjunctive therapy candidate for a wide range of diseases (88). Observations drawn from human studies suggest that HBO might be

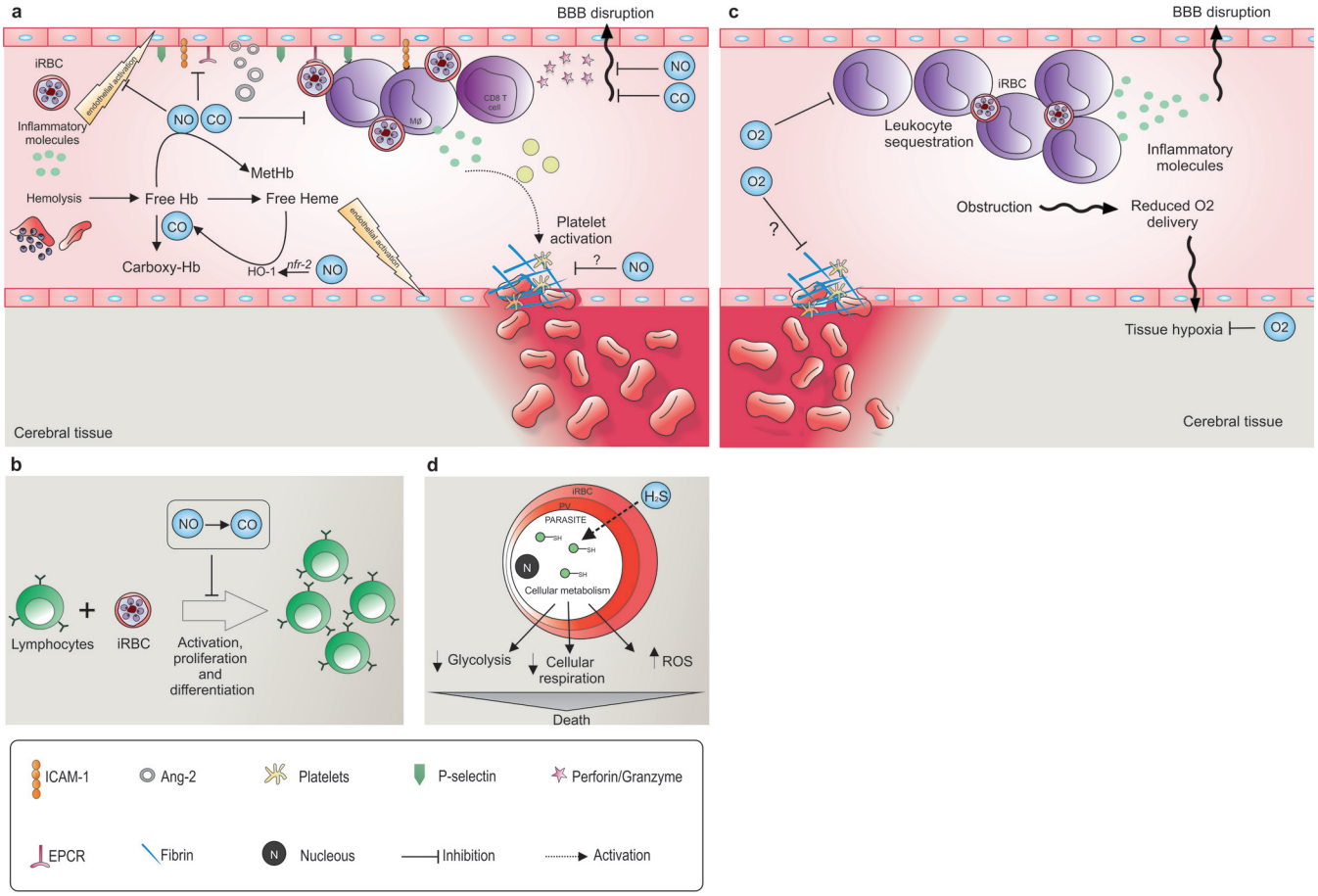


**FIG 1** Effects of NO, CO, HBO, and $H_2S$ on cerebral malaria. (a) NO and CO inhibit endothelial activation, decreasing expression of adhesive receptors and secretion of proinflammatory molecules. Consequently, sequestration of leukocytes and mature iRBC is blocked, as well as BBB disruption triggered by perforin and granzyme released by $CD8^+$ T cells. Proinflammatory molecules secreted by leukocytes may promote activation of platelets, which adhere to the endothelium and initiate the coagulation process, a relevant event in cerebral malaria. Free heme, a toxic agent generated from free Hb oxidation during hemolysis, reacts with NO and CO, producing metha- and carboxy-Hb, respectively. Besides promoting vasodilation, NO also induces HO-1 expression with the involvement of the transcription factor nrf-2, and HO-1 breaks free heme, forming endogenous CO. (b) Some experimental results indicate that NO and CO lead to an immunological tolerance to parasites, inhibiting activation, proliferation, and differentiation of T cells. (c) Hyperbaric oxygen protects mice from CM by decreasing proinflammatory cytokine levels and leukocyte sequestration in the brains of infected mice, which in turn could increase microvascular blood flow, decreasing tissue hypoxia and thus preventing BBB disruption. (d) $H_2S$ is harmful to parasites. Thiolation of parasite proteins alters cellular metabolism, resulting in generation of reactive oxygen species (ROS), which impair glycolysis and parasite respiration. EPCR, endothelial protein C receptor.

useful in the treatment of some bacterial and fungal infections, like purpura fulminans (89) and necrotizing fasciitis (90). However, only a few studies have investigated the application of HBO to protozoan infections (91, 92), including ECM (93, 94).

Blanco and colleagues (93) demonstrated that HBO therapy was neuroprotective in ECM. In that study, HBO treatment prevented clinical signs and improved mortality for up to half of treated mice. HBO treatment decreased mRNA levels of gamma interferon, tumor necrosis factor alpha, and interleukin-10 and reduced sequestration of γδ and αβ $CD4^+$ and $CD8^+$ T lymphocytes in the brain; these findings support its neuroprotective effect. In addition, HBO therapy prevented BBB dysfunction and hypothermia and significantly decreased parasite burden of *P. berghei* ANKA-infected mice as well as in mice infected with *P. berghei* NK65 (a non-ECM strain) (93). These data pointed to the possibility for HBO as an adjunctive therapy for CM. However, a better understanding of the mechanisms involved in the protection of ECM by HBO is needed. Figure 1c summarizes current knowledge of mechanisms of pressurized $O_2$ treatment.

## HYDROGEN SULFIDE

$H_2S$ is a gas produced endogenously as a by-product of the metabolism of the amino acid L-cysteine, which occurs via at least three enzymes: cystathionine β-synthase, cystathione γ-lyase, and 3-mercaptopyruvate sulfurtransferase. Considered a toxic gas, $H_2S$ has emerged as an important signaling molecule, a gas transmittor, influencing physiological and pathological processes (95–97). Its pleiotropic effect has been reported in inflammation, neuromodulation, and apoptosis (98). Protective effects of $H_2S$ were observed in animal models of atherosclerosis (99), shock (100), cardiac arrest (101), and cerebral ischemia (102). Fast and slow donors of $H_2S$ (NaHS and GYY4137, respectively) were tested *in vitro* against *P. falciparum* (strains 3D7, PA and HB3) and were shown to inhibit parasitemia in a dose-dependent manner (103). $H_2S$ acted against the parasite by directly altering its cellular metabolism. However, *in vivo* treatment did not prevent development of ECM or death of infected mice. This study indicated that $H_2S$ could contribute to protein thiolation and interfere with cel-

**TABLE 1** Gaseous treatments for cerebral malaria

| Gaseous molecule | Therapeutic delivery method | ECM outcome | Clinical trial outcome |
|---|---|---|---|
| CO | Inhaled CO | Increased parasite load in liver (51); prevented ECM syndrome and BBB disruption (40) | —[a] |
| | Molecules releasing CO (CO-RM) | Prevented ECM syndrome and death; protected mice from acute lung injury (41) | — |
| NO | Donor of NO: dipropylenetriamine NONOate | Increased survival and protected BBB disruption in ECM (70) | — |
| | Donor of NO: GSNO | Prevented ECM syndrome and death; decreased inflammation and edema in ECM (71) | — |
| | Donor of NO: GTN | Protected mice from ECM; when combined with artemether increased survival (72) | — |
| | Artemisinin-NO donor hybrid | Antiplasmodial activity; rescued mice from ECM (73) | — |
| | Inhaled NO | Increased survival and reduced systemic inflammation in mice infected with *P. berghei* ANKA (66) | Safe but with no effect on mortality in children with severe malaria (iNO at 80 ppm) (18, 19, 75) |
| | L-Arginine infusion | —— | In severe falciparum malaria, it showed no increase of NO endothelial production (78) |
| $O_2$ | Hyperbaric oxygen | Protected mice from CM, decreasing clinical symptoms and mortality rate (93) | — |
| $H_2S$ | $H_2S$ donors | Antiplasmodial activity; no effect on ECM at dose tested (103) | — |

[a] —, no clinical trial data available.

lular redox balance, but the mechanisms were not elucidated (Fig. 1d). Although preliminary results with $H_2S$ have not shown exciting results against malaria *in vivo*, a reformulation of the $H_2S$ delivery system that allows a prolonged half-life may generate promising results, opening perspectives for its use as an antimalarial therapy.

Table 1 provides a summary of findings from both ECM and clinical trials.

## CONCLUSION

In spite of advances in malaria therapeutics, the morbidity and mortality rates attributable to CM are still high. Therefore, an adjunctive therapy preventing the complications, sequelae, and deaths of CM patients is urgent. Gas-based therapies are an attractive complement for CM treatment, although the emphasis on the toxic properties of some of the gases discussed in this review may have limited their study. However, as more information about the physiological roles of these gases emerges, greater scientific interest builds on their research. NO is the most investigated among the gas-based therapies; nevertheless, its beneficial effect is yet to be validated in human CM. The investigation of the pleiotropic activities of these molecules, which regulate a large number of biologic processes, is needed, considering that cerebral malaria is a multifactorial process. More intense research with these and other molecules with therapeutic potential is necessary to open perspectives to combat a disease that costs hundreds of thousands of lives every year.

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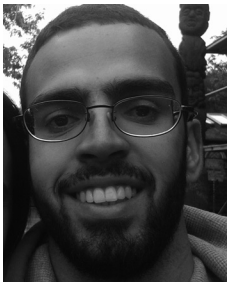

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**Dr. Júlio Aliberti**'s work as a postdoctoral fellow at the Immunobiology Section, Laboratory of Parasitic Diseases, NIAID, NIH, gave him the basis for the formation of his research program that is focused on understanding the cellular and molecular bases for the development and regulation of protective immune responses against infectious agents. Dr. Aliberti's strongest expertise focuses on the cellular and molecular bases for immunopathogenesis and microbial recognition. His position in this field of research gives him a perspective to approach the questions involved in induction and modulation of innate mechanisms in the development of disease in mouse models. Dr. Aliberti has over 22 years of experience, with studies focusing on the role of cytokines, chemokines, and lipid mediators in the induction and control of the immune response to infection.

**Dr. Fabio T. M. Costa** is Associate Professor of Parasitology at the University of Campinas (UNICAMP) located in Campinas (São Paulo State, Brazil). As a malaria researcher at UNICAMP, Dr. Costa is an expert in basic research focusing on the immunopathological aspects of *Plasmodium* species infections and on the discovery and development of experimental drugs and vaccines. Dr. Costa also works as an academic editor for the journals *PLoS One* and *Frontiers in Immunology*. Dr. Costa graduated in the Biological Sciences at the University of Brasilia in 1994. He obtained his Masters' and Ph.D. degrees at Federal University of São Paulo in 1998 and 2001, respectively. From 2001 to 2003, he attended the Université de La Méditerranée/Institut Pasteur (France) as a postdoctoral fellow, working on human and experimental malaria. Dr. Costa has worked for almost 20 years on malaria research, and he has published more than 60 articles, which have been cited more than 1,000 times.