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 modulate 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 (ARDS) (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
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 inflammation, and CO has been considered as an adjunctive therapy. (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).

**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).
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 dipropylentriamine 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). Glycerol 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 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 pro-oxidant 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 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 O2 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
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 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 O2 treatment.

HYDROGEN SULFIDE
H2S 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-mercaptopropionate sulfoximine lyase. Considered a toxic gas, H2S 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 H2S were observed in animal models of atherosclerosis (99), shock (100), cardiac arrest (101), and cerebral ischemia (102). Fast and slow donors of H2S (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). H2S 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 H2S could contribute to protein thiolation and interfere with cel-
lular redox balance, but the mechanisms were not elucidated (Fig. 1d). Although preliminary results with H₂S have not shown exciting results against malaria in vivo, a reformulation of the H₂S 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 gaseous treatments; 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.

ACKNOWLEDGMENTS
This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grant 2012/16525-2), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and NIH (AI118302-02). A.C.A.V.K., J.C.K.S., and M.F.B. were sponsored by FAPESP fellowships. F.T.M.C. and L.J.C. are CNPq research fellows. A.C.A.V.K., J.C.K.S., and M.F.B. were sponsored by FAPESP fellowships. Conrado Khouri Dos-Santos, Marcele F. Bastos, and Fabio Trindade Maranhão Costa under grant number 2012/16525-2. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES


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