Transdermal glyceryl trinitrate as an effective adjunctive treatment with artemether for late stage experimental cerebral malaria

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ABSTRACT

Cerebral malaria (CM) is associated with low nitric oxide (NO) bioavailability, cerebrovascular constriction, occlusion and hypoperfusion. Administration of exogenous NO partially prevents the neurological syndrome and associated vascular pathology in an experimental CM mouse model (ECM). In this study, we evaluated the effects of transdermal glyceryl trinitrate in preventing ECM and, in combination with artemether, rescuing late-stage ECM mice from mortality. The glyceryl trinitrate and/or artemether effect on survival and clinical recovery was evaluated in C57BL/6 mice infected with *P. berghei* ANKA. NO synthase (NOS) expression in mouse brain was determined by western blots. Mean arterial pressure (MAP) and pial arteriolar diameter were monitored using a tail-cuff blood pressure system and a cranial window preparation, respectively. Preventative administration of glyceryl trinitrate 0.025 mg/hour decreased ECM mortality from 67% to 11% and downregulated inducible NOS expression in the brain. When administered as adjunctive rescue therapy with artemether, glyceryl trinitrate increased survival from 47% to 79%. The adjunctive therapy caused a sustained reversal of pial arteriolar vasoconstriction in ECM mice, an effect not observed with artemether alone. Glyceryl trinitrate induced a 13% decrease in MAP in uninfected mice, but did not further affect MAP pressure in hypotensive ECM mice. Glyceryl trinitrate, when combined with artemether, was an effective adjunctive rescue treatment for ECM. This treatment ameliorated pial arteriolar vasospasm and did not significantly affect MAP. These results indicate that transdermal glyceryl trinitrate has potential to be considered as a candidate for adjunctive therapy for CM.
INTRODUCTION

Cerebral malaria (CM) is a lethal complication of *Plasmodium falciparum* infection and largely responsible for the estimated 1 million-plus malaria deaths every year (1). CM has high mortality rates of 20% even upon administration of prompt antimalarial treatment, which is based on parenteral administration of quinine or artemisinin derivatives. In an attempt to reduce mortality, various adjunctive treatments for CM have been evaluated in clinical trials, however mostly with unfavorable outcomes (2). Human CM is a severe vasculopathy (3) and is commonly associated with acidosis and other complications (4). Post-mortem studies show diffuse microhemorrhages and cerebrovascular obstruction by parasitized RBCs (pRBCs) and often leukocytes sequestered in inflamed endothelium via receptors such as intercellular adhesion molecule 1 (5-7). *In vivo* studies of the retinal microcirculation of CM patients revealed vascular obstruction, hypoperfusion and intravascular filling defects (8). Endothelial dysfunction in CM has been demonstrated, with low nitric oxide (NO) bioavailability (9), elevated plasma levels of cell-free hemoglobin (10), asymmetric dimethylarginine (11), endothelin 1 (12) and angiopoietins (13), and spastic constriction of cerebral arterioles (14).

*Plasmodium berghei* ANKA (PbA) infection in susceptible mice induces a neurological syndrome known as experimental cerebral malaria (ECM) whose pathogenesis shares similarities with human CM (15). The relevance of this model has recently been debated (16-21). Similarly to human severe malaria, low NO bioavailability has been linked to the genesis of experimental cerebral malaria (ECM) (9, 22, 23). We have shown that exogenous NO administration in the form of NO-donors such as...
dipropylentetramine NONOate (DPTA-NO) and S-nitrosoglutathione (GSNO) decreases ECM incidence as well as cerebral edema, leukocyte accumulation and hemorrhages (22, 24, 25). Similar findings were obtained with inhaled NO (26). Using intravital microscopy of the pial microcirculation through a closed cranial window, we demonstrated that ECM is associated with cerebrovascular constriction, hypoperfusion, vessel blockage, marked decreases in cerebral blood flow and eventually vascular collapse (27), features similar to those in human CM (8). More importantly, co-administration of the calcium channel blocker nimodipine, a potent cerebral vasodilator, with artemether markedly increased survival and recovery of mice with late-stage ECM (27), indicating that interventions to counteract cerebral vasoconstriction and improve cerebral blood flow are logical and potentially powerful approaches for CM adjunctive therapies.

Glyceryl trinitrate is used for the treatment of angina and heart failure due to its dilator activity in large veins and arteries (28). Glyceryl trinitrate induced vasodilatation occurs via a biotransformation process through denitrification to yield NO, which activates soluble guanylate cyclase and thus relaxes vascular smooth muscle (29). Besides vasodilatation, glyceryl trinitrate has also been proven to decrease inflammation (30). Since orally administered glyceryl trinitrate has very short elimination half-life, and undergoes extensive gastrointestinal and hepatic first-pass metabolism, the use of transdermal administration of glyceryl trinitrate represents an interesting approach to obtain good bioavailability and to prolong the duration of action (31). Moreover, glyceryl trinitrate patches are inexpensive and generic formulations can be found for less than 1 USD per unit. We hypothesized that since transdermal glyceryl trinitrate can be used as a
source of sustained and slow NO release, glyceryl trinitrate patches may be promising for
ECM adjunctive therapy. In the present study, we report the efficacy of transdermal
glyceryl trinitrate in preventing ECM and, in combination with artemether, rescuing mice
from late-stage ECM. We also describe the effects of this adjunctive therapy in mouse blood
pressure and cerebrovascular dilatation.

**MATERIAL AND METHODS**

**Mice, *P. berghei* ANKA infection and parasitemia follow-up**

All protocols for animal handling and care were approved by the La Jolla
Bioengineering Institute’s Animal Care and Use Committee. Eight to ten week old female
C57BL/6 mice (The Jackson Laboratory, Sacramento, CA) were infected intraperitoneally
with $10^6$ PbA parasites expressing the green fluorescent protein (obtained from the MR4,
Manassas, VA, reference MRA−865, deposited by CJ Janse, AP Waters). Parasitemia levels
were monitored by flow cytometry or by microscopy in mice under artemether treatment.

**Clinical evaluation and ECM definition**

ECM was defined by the occurrence of at least one of the following clinical signs:
ataxia, limb paralysis, roll-over, seizures, convulsions, poor righting reflex, hypothermia
and/or coma. Body temperature was monitored using an Acorn Series Thermocouple with
a mouse rectal probe (Oakton Instruments, Vernon Hills, IL, USA). In addition, a set of six
motor behavior tests, with scores ranging from 0 (complete impairment) to 23 (maximum performance), was performed as described (27, 32).

**Treatments**

Two different types of experimental treatments were evaluated: (i) preventative treatment to assess whether glyceryl trinitrate protects against ECM; (ii) rescue treatment to evaluate whether glyceryl trinitrate was able to increase the efficacy of artemether in rescuing mice presenting late-stage ECM.

**(i) ECM preventative treatment**

Three days before infection, mice were mildly anesthetized with isoflurane and part of the back fur was removed with hair removal cream (Nair lotion, Princeton, NJ, USA). After PBA inoculation, a quarter of a glyceryl trinitrate patch (Nitroglycerin Transdermal System 0.1 mg/hour, Mylan Pharmaceuticals, Inc.; Morgantown, WV, USA) delivering 0.025 mg/hour was applied to the back of the animal in cycles of 12 hours to avoid the development of glyceryl trinitrate tolerance until day 8 of infection. The control group consisted of infected mice which were subjected to back fur removal under light anesthesia three days after infection but had no patch implanted. The lack of a placebo patch was a limitation in the experimental procedure. Parasitemia, rectal temperature and motor behavior scores were recorded daily (32). On day 6 and 12 of infection, hematocrit levels were measured (33). After the cessation of glyceryl trinitrate treatment on day 8, survivor mice were followed up to day 12 of infection. Mortality rates were recorded and at the end
of the experimental protocol (day 12) mice were euthanized with sodium pentobarbital (390 mg) plus sodium phenytoin (50 mg/ml) (Euthasol, 100 mg/kg, intraperitoneally).

(ii) ECM rescue treatments

On day 3 of infection, mice were shaved. Beginning on day 4 and until the end of the experiment, parasitemia, rectal temperature and motor behavior were monitored daily. Mice with at least one of the ECM clinical signs and showing body temperatures between 32−34°C (used as an objective, quantitative criterion for treatment and for group comparison) on days 5−7 were randomly assigned to the different treatment groups: i) artemether (Artesiane, Dafra Pharma, Belgium) 25 mg/kg, intraperitoneally without patches; ii) artemether 25 mg/kg, intraperitoneally plus glyceryl trinitrate patches delivering 0.025 mg/hour; iii) artemether 25 mg/kg, intraperitoneally plus glyceryl trinitrate patches delivering 0.1 mg/hour. Glyceryl trinitrate patches were left on the mouse skin for 24 hours, after which they were removed. Therefore, after 24 hours all mice in all groups received only artemether 25 mg/kg intraperitoneally daily for a total course of 5 days (32). Mortality rates were recorded for all treatments. One week after the cessation of artemether, mice were euthanized with Euthasol (100 mg/kg, intraperitoneally).

Brain sample preparation and NO synthase (NOS) expression

Uninfected mice and PbA-infected mice undergoing or not glyceryl trinitrate preventative treatment were euthanized (Euthasol 100 mg/kg, intraperitoneally) on day 6 of infection; brains were immediately harvested, flash frozen in liquid nitrogen and stored
at −80°C. Brains were homogenized, lysates subjected to SDS–PAGE, transferred to polyvinylidene fluoride membranes and incubated against different antibodies (see supplementary data for details). The following primary antibodies were used: β-tubulin, total iNOS and nNOS (Santa Cruz, CA, USA); total eNOS (Stressgen Bioregents, Victoria, BC, Canada) and P-eNOS (S1176) (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were used for detection. Band intensity was quantified on unsaturated X-ray films and quantified with ImageJ software (NIH, Bethesda, MD, USA). Total NOS expression levels are presented as a ratio over β-tubulin content.

Determination of plasma nitrite and nitrate content

Uninfected and PbA-infected mice with ECM were treated with either artemether monotherapy, artemether plus glyceryl trinitrate patch 0.025 mg/hour or artemether plus glyceryl trinitrate patch 0.1 mg/hour. During the course of the experiment, blood (30 µl) samples were collected from saphenous vein before (0 hours) and at different time points after the treatment (3, 6, 24 hours). Plasma was recovered by centrifugation (800g, 10 min, 4°C) and mixed with equal volume of methanol. After protein precipitation (5,000g, 10 min, 4°C), plasma nitrite (NO₂⁻) and nitrate (NO₃⁻) concentrations were quantified by ion chromatography (ENO20 Analyzer; Eicom, Kyoto, Japan). Concentrations of nitrite and nitrate were estimated by assessing the peak height of the absorption compared to sodium nitrite and sodium nitrate standard solutions.
Blood pressure measurements

Mean arterial blood pressure (MAP) was recorded in conscious mice using the CODA non-invasive tail-cuff system (Kent Instruments, Torrington, CT, USA) (34). Mice were allowed to acclimate to the restrainer for 5 minutes prior to initiating the blood pressure measurement. At least 10 readings were taken from each animal at each time point. MAP was measured on day 0 and the animals were subsequently infected with PbA. Mice showing signs of ECM on day 6 were treated with either artemether or artemether plus glyceryl trinitrate patch 0.1 mg/hour and had MAP recorded just before and at four time points after glyceryl trinitrate patch implantation (0, 3, 6, 24 and 48 hours). All MAP measurements were normalized to day 0 baseline.

Cranial window preparation and intravital microscopy

The chronic closed cranial window preparation, as previously described (35, 36), was utilized for monitoring changes in pial arteriolar diameters (see supplementary data for details). Briefly, two weeks after surgery, the mouse was mildly anesthetized with isofluorane and transferred onto an intravital microscope stage (customized Leica-McBain, San Diego, CA, USA). Pial arterioles (N: 2-6, baseline vessel diameters: 35-115 µm) were visualized by epi-illumination using a 20X water immersion objective and their diameters measured using an image shear device (0.213 µm/pixel; Image Shear, Vista Electronics, San Diego, CA, USA). After microscopy, the mice were infected with PbA (10^6) and intravital procedures repeated on day 6 of infection before and at multiple time points (0, 3, 6, and 24 hours) after the rescue treatment (artemether or artemether plus glyceryl trinitrate 0.1
Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM), unless otherwise indicated. Significant differences in survival rates were determined using Log-rank (Mantel-Cox) tests. One-way or two-way ANOVA followed by Bonferroni test comparisons were used to test the significance of differences in western blot analysis, during the preventative and rescue treatments follow-ups with regards to temperature, motor behavior score, parasitemia, hematocrit, plasma nitrite/nitrate levels, MAP and vessel diameters. Evaluated group sizes (N) are reported in graphs and/or figure legends. Differences were considered statistically significant at $P \leq 0.05$. Statistical analyses were performed with GraphPad 5.0 (Graph Pad Software, San Diego, CA, USA).

RESULTS

Glyceryl trinitrate treatment protects against ECM

PbA-infection led to the development of ECM and 67% mortality in untreated mice (Fig. 1A), with ECM manifestation starting on day 6 and most deaths occurring on day 7. Application of glyceryl trinitrate delivering patches to the skin of PbA-infected mice daily from days 0-8 led to a marked reduction in mortality by ECM (11%; $P < 0.0001$). Glyceryl trinitrate treatment positively influenced the animals' body temperature (Fig. 1B). Two mice in each group showed low body temperatures (< 33°C) on day 6 or 7 but recovered. At
least part of the protection could be attributed to glyceryl trinitrate effect in inhibiting parasite growth, as glyceryl trinitrate treated animals better controlled parasitemia at a critical period for ECM development (days 6–7) (Fig. 1C). Despite the effect on parasitemia, hematocrit values were not different between glyceryl trinitrate treated and untreated PbA-infected mice on day 6 or 12 (Fig. 1D).

**Glyceryl trinitrate treatment prevents iNOS and eNOS upregulation in PbA-infected mice**

Nitric oxide is synthesized by NO synthases (NOS) whose dysfunction was recently shown to contribute to impaired cerebroarteriolar reactivity in ECM (37). Brains of PbA-infected mice on day 6 of infection showed increased expression of both the inducible NOS (iNOS) and the endothelial specific NOS (eNOS) isoforms, but not of the neuronal NOS (nNOS) (Fig. 2A–C). eNOS activation can be induced by phosphorylation of Serine 1176 (S1176) by mechanical forces of fluid shear stress on the endothelium of the blood vessel wall. As we previously reported eNOS S1176 phosphorylation was downregulated during ECM (Fig. 2D). Glyceryl trinitrate treatment prevented iNOS and eNOS upregulation during ECM (Fig. 2A and C, P < 0.05), although the effect was stronger and more uniform in preventing iNOS than eNOS upregulation. Glyceryl trinitrate treatment did not affect the levels of phosphorylation of eNOS during infection (Fig. 2D).

**Adjunctive therapy with glyceryl trinitrate markedly increases the efficacy of artemether in rescuing mice from late-stage ECM**
Mice with ECM treated with artemether plus glyceryl trinitrate at 0.1 mg/hour showed significantly increased survival rates as compared to artemether monotherapy (79% versus 47%, respectively; \( P = 0.01 \)) (Fig. 3A). This beneficial effect of glyceryl trinitrate on survival was not observed with the 0.025mg/hour dose. There were no significant differences in rectal temperature and motor behavior scores (Fig. 3B–C) among the different groups at the time of the first dose (time zero on day 6 of infection). There was no difference as well in parasitemia between each glyceryl trinitrate treated group and the artemether only-treated group at the time of treatment, although the group of mice treated with artemether plus glyceryl trinitrate 0.025 mg/hour showed higher parasitemias compared to the artemether plus glyceryl trinitrate 0.1 mg/hour group (Fig. 3D). Glyceryl trinitrate treatment did not affect the recovery in body temperature and motor behavior scores, or the rate of parasite clearance after treatment, when compared with the artemether-treated group (Fig. 3B–D).

**Plasma nitrite and nitrate levels are significantly increased after glyceryl trinitrate treatment**

Treatment with glyceryl trinitrate at 0.1mg/hour or 0.025mg/hour resulted in significant increases of both plasma nitrite and nitrate levels in both uninfected and ECM mice (Fig. 4A and B). Nitrite is the first oxidation product of NO and because it is rapidly oxidized to nitrate, its levels represent a reliable and real-time measure of NO production. Nitrate is the end product of NO oxidation and provides an estimate of its cumulative production. In uninfected mice, plasma nitrite levels peaked at 3 hours and were still high at 6 hours, but it was no longer detected at 24 hours (Fig. 4A). Plasma nitrate levels followed a similar trend, but the peak was observed at 6 hours and, despite a marked drop
it was still detected after 24 hours (Fig. 4B), which is consistent with its cumulative nature. As expected, glyceryl trinitrate at 0.1mg/hour dose induced higher levels of both nitrite and nitrate than did the 0.025mg/hour dose (about 4-fold higher nitrite levels overall). Interestingly, plasma nitrite levels in mice with ECM were much lower than in uninfected mice at 3 and 6 hour time points after glyceryl trinitrate patch administration, but remained stable even at the 24 hour time point with the 0.1mg/hour dose (Fig. 4A). Plasma nitrate levels followed a similar trend, except for an increase at the 24 hour time point with the 0.1mg/hour dose (Fig. 4B). Again, in mice with ECM glyceryl trinitrate at 0.1mg/hour dose induced higher and longer-lasting levels of both nitrite and nitrate than did the 0.025mg/hour dose. These findings indicate that release of glyceryl trinitrate is slower and longer-lasting in mice with ECM.

Glyceryl trinitrate lowers blood pressure of healthy mice but does not affect blood pressure of ECM mice

Glyceryl trinitrate is recognized as a potent vasodilator affecting systemic blood pressure. The potential hypotensive effect of glyceryl trinitrate, especially at the high doses used in this study, could be a major concern when considering its use in the clinical setting. Glyceryl trinitrate 0.1 mg/hour induced a 13% decrease in the MAP values 3 hours after the patch implantation in control, uninfected mice (baseline: 116 ± 1.6 mmHg); this relatively mild hypotension persisted during the 24 hours of patch treatment and MAP returned to baseline after its removal (Fig. 4C). No changes in MAP were observed in uninfected mice receiving only artemether. PbA-infected mice with ECM signs were
hypotensive, with MAP values already decreased by around 30%. Glyceryl trinitrate treatment caused no further deterioration in MAP, which remained relatively stable up to the 6 hour mark examined and started to recover by 24 hours, returning to pre-infection baseline levels by 48 hours (Fig. 4C). Conversely, PbA-infected but untreated mice remained hypotensive throughout the course of investigation and died within 24 hours of ECM development.

**Glyceryl trinitrate dilates brain arterioles of mice with late-stage ECM**

Using intravital microscopy through a closed cranial window, the effects of artemether and artemether plus glyceryl trinitrate treatments on pial arteriolar diameters in day 6 PbA-infected mice were examined. All PbA-infected mice with ECM signs presented marked vasoconstriction of pial arterioles from baseline pre-infection values (Fig. 5). Vessels in untreated mice remained constricted at 3 and 6 hours after treatment (these mice succumbed to the disease before 24 hours). Similarly, vessels from artemether-treated mice remained constricted throughout the course of investigation (time 0: −16%, 3 hours: −24%, 6 hours: −26%, and 24 hours: −29%). There were no significant differences between the extent of vasoconstriction in untreated and artemether-treated mice. In contrast, mice receiving artemether plus glyceryl trinitrate 0.1 mg/hour showed reversal of vasoconstriction with vessel diameters returning to their pre-infection levels after 3 hours, and were maintained in their recovered states at subsequent time points (Fig. 5).

Discussion
Effective adjunctive therapies capable of increasing survival and decreasing incidence of sequelae in patients with cerebral malaria treated with artemisinin derivatives remain elusive (2). In the present study we describe that glyceryl trinitrate increases survival of mice with late-stage ECM when given in combination with artemether. The relevance of this experimental model for human CM has been heavily debated in the past years (16-21). The main criticism conveyed is that pRBC sequestration in brain vessels is the histopathological hallmark of human CM, accompanied by little inflammatory response, whereas ECM is associated with inflammation and leukocyte (rather than pRBC) sequestration in the brain (16). However, several aspects of inflammation have been demonstrated in human CM, and an inflamed endothelium has even been described as a condition for pRBC attachment and accumulation in the brain, with increased expression of MHC Class II and ICAM-1 (the latter incriminated as the receptor for PfEMP1, and hence for pRBC, in the brain) (38, 39), frequent presence though in low numbers of leukocytes (5), high cytokine (40) and chemokine (41) levels, and loss of endothelial protein C receptors (42), among other findings. Conversely, *P. berghei* pRBC accumulation in the brain has been shown in a number of studies (43-46). As we have previously discussed (17), although the two syndromes show quantitative differences in the blood cell type sequestered (more pRBCs than leukocytes in human CM and the opposite in ECM), the cause of sequestration (endothelial inflammation with ICAM-1 upregulation) and its most obvious consequence (vascular obstruction of blood flow) are similar, resulting in hypoperfusion, ischemia and hypoxia. The difference between the pathologies makes it evident that not every intervention would work in human CM compared to ECM. For instance, pRBCs bind to
ICAM-1 through PfEMP-1, whereas leukocytes do it through LFA-1, and specific binding inhibitors aimed to detach adherent cells might not work in both cases. However, in both cases restoration of vascular perfusion is expected to have a beneficial effect and in such cases the ECM model can work as a viable and relevant surrogate for human CM. This is also the case for interventions that address mechanisms of pathogenesis and damage that have been shown to be shared by the two pathological entities (15, 40). This includes endothelial dysfunction, low NO bioavailability and hypoperfusion-hypoxia (37). We emphasize, nevertheless, that findings in this model obviously cannot be directly translated to the human situation, this being dependent upon the performance of properly designed clinical studies.

The objective of the present study was to address whether interventions to reverse cerebral vasoconstriction and to improve cerebral blood flow would promote sustenance of life in moribund animals displaying ECM, allowing time for the full action of artemether and therefore increasing survival. We have previously shown that NO-donors such as DPTA-NO and GSNO partially prevent ECM development and decrease vascular pathology, improving pial blood flow and attenuating vasoconstriction, inflammation and hemorrhages (24, 25, 33). Glyceryl trinitrate showed similar effects and as a drug for potential use in the clinical setting it has a number of advantages over DPTA-NO and GSNO: (i) clinical data on glyceryl trinitrate is abundant, with a long history of efficacy and safety; (ii) several formulations are available, including transdermal patches that allow continuous delivery of sustained levels for prolonged periods of time; (iii) available formulations are stable for long periods at room temperature, and (iv) glyceryl trinitrate is inexpensive and easily accessible.
Similarly to DPTA-NO and GSNO, glycercyl trinitrate delivering patches largely protected against ECM development. Since glycercyl trinitrate showed some inhibitory effect on parasitemia at a critical time for ECM development (days 6–7 of infection), it is unclear whether the glycercyl trinitrate protective effect was solely secondary to this anti-parasite effect or resulted from other beneficial actions on the host itself. The latter seems to be the case as glycercyl trinitrate treatment downregulated iNOS expression in the brain of PbA-infected mice, suggesting that it also displayed anti-inflammatory activity. iNOS upregulation is expected to occur in a highly inflammatory condition such as ECM, and eNOS upregulation might occur as a tentative response to reverse the state of low NO bioavailability. Indeed, iNOS and eNOS upregulation has been shown to be triggered by an increase in oxidative stress under conditions of low NO bioavailability (47), similar to the conditions observed in ECM (37). Therefore, providing exogenous NO during PbA infection may prevent the very causes of iNOS and eNOS upregulation, decreasing inflammation and ameliorating endothelial function in ECM (22, 24, 25). Glycercyl trinitrate had a more uniform effect in preventing iNOS than eNOS upregulation, resulting in more variable expression of the latter. In addition, glycercyl trinitrate was unable to prevent S1176-P-eNOS downregulation. S1176-P-eNOS activation occurs mainly as a result of mechanical stimulation of endothelial cells by shear stress (48, 49), and decreased shear rates on the contrary promotes P-eNOS downregulation. Indeed, we have recently shown that PbA infection leads to decreased microvascular wall shear rates due to decreased hematocrit and decreased RBC velocities (48). The fact that glycercyl trinitrate treatment did not prevent S1176-P-eNOS downregulation indicate that it did not prevent the PbA-induced
decrease in shear rates, which is consistent with the fact that glyceryl trinitrate treatment did not prevent the PbA-induced decrease in hematocrit.

The most striking observation in this study, however, was the demonstration of the adjunctive effect of glyceryl trinitrate in association with artemether in mice with ECM. A number of studies have recently been published describing adjunctive therapies in ECM (50-53). One disadvantage is that more often than not intervention is given early, before full development of the neurological syndrome occurs. We have previously developed a model system for testing the effect of antimalarial drugs in late-stage ECM (32). The advantage of this model is that treatment is given only after mice display clear signs of neurological derangement, therefore mimicking more closely the situations under which a CM patient will receive treatment. In mice, hypothermia precedes death by ECM (54, 55) and therefore body temperature is an objective, quantifiable parameter allowing precise staging of the disease. The relatively tight pre-set range for treatment (32–34°C) allowed better intergroup homogenization to improve comparison, reduce variability and decrease the number of animals needed to achieve significance. At this stage, mice also generally show other signs of neurological involvement, low motor scores and hypotension, denoting the advanced stage of the disease. Using this system, we show that glyceryl trinitrate 0.1 mg/hour, but not 0.025 mg/hour, increased survival of mice with ECM from 47% to 79%. We have previously shown that ECM is associated with a vasospasm-like brain microcirculatory dysfunction (27). The vasodilatation effect in pial arterioles after glyceryl trinitrate administration has been well characterized in vivo mainly in the study of migraine (56-58). Our data show that indeed the success of glyceryl trinitrate therapy in
improving ECM survival was associated with a marked reversal of cerebral vasospasm. Conversely, artemether alone was unable to reverse vasospasm even 24 hours after treatment, despite its dramatic effect in decreasing parasitemia (32). Although artemether did not decrease the number of vessels containing leukocytes after 24 hours, it did decrease the number of leukocytes per vessel (32) indicating that vascular constriction is more resilient and harder to reverse than inflammation and, perhaps, vascular occlusion. The positive effects of glyceryl trinitrate in reversing vasospasm are in line with our previous findings that nimodipine, a calcium channel blocker used to prevent vasospasm in patients with subarachnoid hemorrhages, is also beneficial in late-stage ECM (27). Overall, these data support the concept that tackling vasospasm can be of great benefit in CM.

Most of the potentially severe side effects of glyceryl trinitrate overdose are related to its systemic vasodilator effect, which can cause hypotension and headaches (59, 60). We therefore used blood pressure as the indicator for glyceryl trinitrate in vivo activity and to assess severe side effects. The effective glyceryl trinitrate dose used was high, as 0.1 mg/hour in a 20 g mouse is roughly equivalent to 80 µg/kg/minute. Doses given to humans using glyceryl trinitrate patches usually do not exceed 0.8 mg/hour in adults (28, 61) but trials with much higher doses (160 µg/minute – 10 mg/hour) have been performed (62). These doses are still 30–40 times lower than those used in the present study, yet the side effects associated with this high dose in the mouse seemed to be relatively mild. Indeed, the hypotensive effect of glyceryl trinitrate 0.1 mg/hour caused a drop of 13% in MAP during the 24 hours of glyceryl trinitrate treatment in uninfected mice. This decrease in MAP was much lower than that observed with bolus intraperitoneally injection of DPTA-NO and
GSNO in mice (22, 33), or with similar doses of glyceryl trinitrate intravenously infused in rabbits (63), which can cause acute drops in MAP of over 40%. On the other hand, glyceryl trinitrate administration to mice with ECM, which already show hypotension, did not cause further decreases in blood pressure. There is a number of considerations to be made. First, ECM mice were hypothermic (32−34°C) and it has been shown that skin temperature changes can cause major short-term modifications in glyceryl trinitrate bioavailability (64). Indeed, the plasma levels of nitrite and nitrate, the surrogate markers for NO production, were at least 2 to 4-fold lower in mice with ECM compared to uninfected mice 3−6 hours after patch implantation. The plasma nitrite levels achieved with the 0.025mg/hour dose were even lower and therefore in this case NO generation may have failed to reach the threshold needed to be effective in mice with ECM. Second, mice with ECM show high plasma levels of NO-scavenging cell-free hemoglobin (22). It means that a large fraction of the NO derived from the glyceryl trinitrate patches is diverted to react and be scavenged by cell-free hemoglobin instead of playing its expected effect on blood vessels. Third, it has been shown that the vasodilatory action of low-dose glyceryl trinitrate is partly due to NOS-dependent mechanisms (65). NOS (both eNOS and nNOS isoforms) are dysfunctional in mice with ECM (37), therefore it is conceivable that the action of glyceryl trinitrate through this mechanism is impaired in mice with ECM, also explaining the need for higher doses. Therefore, decreased glyceryl trinitrate delivery due to hypothermia, increased NO consumption due to scavenging by plasma cell-free hemoglobin and decreased action due to NOS dysfunction may explain the need for higher doses of glyceryl trinitrate to rescue mice with ECM. Finally, the baseline vascular diameter upon which the glyceryl trinitrate-
derived NO will exert its actions is markedly lower in mice with ECM than in uninfected animals due to vasoconstriction (27). This means that NO will be basically used to reverse vasoconstriction rather than to cause deleterious vasodilation in sick animals. Indeed, this conclusion is supported by Fig. 5 as well as by the absence of deleterious effects on blood pressure shown in Fig 4C. In human CM, the required glyceryl trinitrate dose would likely be much lower than that needed in mice as hyperthermia rather than hypothermia is usually observed (4). On the other hand, high plasma levels of cell-free hemoglobin and endothelial dysfunction are also a feature of human severe malaria (11) and therefore doses higher than usually prescribed for angina would probably be necessary.

In conclusion, glyceryl trinitrate has been shown in this study to be an effective adjunctive therapy for ECM in association with artemether, and this effect was associated with reversal of pial arteriolar vasospasm and also with limited side effects as measured by changes in systemic blood pressure. Further studies to back the potential of glyceryl trinitrate as an adjunctive therapy for cerebral malaria are warranted.

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**Figure legends**

**FIG 1** Glyceryl trinitrate protects from ECM. Effect of glyceryl trinitrate 0.025 mg/hour on cumulative survival (A), temperature (B), parasitaemia (C) and haematocrit (D). In panel (A), the number of mice (N) is shown. In panel (D), hematocrit values were recorded at day 6 (PbA N = 15; PbA-GTN N = 18) and day 12 (PbA N = 4; PbA-GTN N = 8) of infection; data from panel (B) to (D) are derived from the same animals of the survival experiment (A) and are expressed as the mean of two separate experiments ± SEM (except D12 hematocrit data, which are data from one experiment). *P < 0.05, **P < 0.001, ***P < 0.0001. GTN: glyceryl trinitrate; PbA: *P. berghei* ANKA.

**FIG 2** Glyceryl trinitrate preventative treatment limits NOS expression. Panels (A) to (C), bar graphs from western blot analyses of NOS isoforms expression as a ratio over total β-tubulin. A representative blot is shown above the graph. Panel (D) immunoblot and bar graph showing the ratio of phosphorylation at eNOS (S1176) specific site to total amount of respective NOS. Results are derived from one experiment evaluating 8 mice treated with
FIG 3 Efficacy of artemether plus glyceryl trinitrate rescuing mice from late stage ECM.
Cumulative survival of rescue treatments (A) and follow-up of survivors: restoration of
temperature (B) and behavior motor score (C), and parasitemia clearance (D). All data is
derived from eight individual experiments. The number of mice evaluated in each group
(N) is shown in panel (A). Data from panels (B) to (D) are expressed as mean ± SEM. *P <
0.001. ARM: artemether; GTN: glyceryl trinitrate; PbA: P. berghei ANKA.

FIG 4 Effect of artemether plus glyceryl trinitrate treatment in plasma nitrite/nitrate levels
and systemic blood pressure from uninfected and ECM mice. Uninfected mice: open
symbols, dashed lines; PbA-infected mice with ECM: solid symbols, solid lines; Artemether
monotherapy: triangles; Artemether plus glyceryl trinitrate 0.025 mg/hour: squares (grey
color); Artemether plus glyceryl trinitrate 0.1 mg/hour: circles. The number of mice (N)
evaluated in each experiment is shown. Kinetics changes of nitrite (A) and nitrate (B) in
plasma samples collected before (0 hours) and at 3, 6, and 24 hours after treatment. In case
of PbA-infected mice, treatment was performed like in previous experiments (mice shaved
on day 4 of infection, artemether administration and patch implantation when mice
showed at least one clinical sign of ECM and rectal temperatures between 32-34°C); this
time point is shown as “0 hr” in the figures. Values represent mean ± SEM. Asterisks
(PbA-infected) and crosses (uninfected) denote a significant increase in nitrite/nitrate levels at individual time points. (C) MAP was measured before infection (day 0), on day 6 of infection at the moment of the ECM manifestation as described above (“0 hr”) and then at 3, 6, 24 hours and 48 hours after artemether with or without glyceryl trinitrate treatment (day 8). Asterisks represent a significant drop in MAP values from uninfected mice treated with artemether plus glyceryl trinitrate patch in relation to day 0 baseline. Crosses represent a significant drop in MAP values from PbA-infected mice with respect to day 0 baseline on day 6 of infection. Data is expressed as the mean of at least 10 MAP readings per mouse ± SEM. Horizontal dotted line represents baseline. +P < 0.05; ++P < 0.01; +++ or ***P < 0.001. ARM: artemether; GTN: glyceryl trinitrate; ECM: experimental cerebral malaria.

**FIG 5** Beneficial effect of artemether plus glyceryl trinitrate treatment in pial arterioles from late-stage ECM mice. Changes in arteriolar diameter were recorded at day 0 and day 6 of infection before (time 0) and after dosing (3, 6 and 24 hours). In panel (A) groups are displayed as follow: Untreated PbA-infected mice (white bars), PbA-infected mice treated with artemether (slash-lined bars), and PbA-infected mice treated with artemether plus glyceryl trinitrate 0.1 mg/hour (black bars). In panel (B), five sections of the same arteriole showing the effect of ECM and artemether plus glyceryl trinitrate treatment in arteriole diameters. The number of mice (N) and arterioles evaluated in each group are shown. Data are expressed as the mean ± SEM. *P < 0.001. ARM: artemether; GTN: glyceryl trinitrate; ECM: experimental cerebral malaria; hr: hour.
FIG 1 Glyceryl trinitrate protects from ECM. Effect of glyceryl trinitrate 0.025 mg/hour on cumulative survival (A), temperature (B), parasitemia (C) and hematocrit (D). In panel (A), the number of mice (N) is shown. In panel (D), hematocrit values were recorded at day 6 (PbA N = 15; PbA+GTN N = 18) and day 12 (PbA N = 4; PbA+GTN N = 8) of infection; data from panel (B) to (D) are derived from the same animals of the survival experiment (A) and are expressed as the mean of two separate experiments ± SEM (except D12 hematocrit data, which are data from a single experiment). *P < 0.05, **P < 0.001, ***P < 0.0001. GTN: glyceryl trinitrate; PbA: P. berghei ANKA.
FIG 2 Glyceryl trinitrate preventative treatment limits NOS expression. Panels (A) to (C), bar graphs from western blot analyses of NOS isoforms expression as a ratio over total β-tubulin. A representative blot is shown above the graph. Panel (D) immunoblot and bar graph showing the ratio of phosphorylation at eNOS (S1176) specific site to total amount of respective NOS. Results are derived from one experiment evaluating 8 mice treated with glyceryl trinitrate, 10 naïve mice and 10 mice infected and untreated. *P < 0.05, **P < 0.001.

GTN glyceryl trinitrate; PbA P. berghei ANKA.
FIG 3 Efficacy of artemether plus glycyril trinitrate rescuing mice from late stage ECM. Cumulative survival of rescue treatments (A) and follow-up of survivors: restoration of temperature (B) and behavior motor score (C), and parasitemia clearance (D). All data is derived from eight individual experiments. The number of mice evaluated in each group (N) is shown in panel (A). Data from panels (B) to (D) are expressed as mean ± SEM. *P < 0.001. ARM artemether; GTN glycyril trinitrate; PbA P. berghei ANKA.
FIG 4 Effect of artemether plus glyceryl trinitrate treatment in plasma nitrite/nitrate levels and systemic blood pressure from uninfected and ECM mice. Uninfected mice: open symbols, dashed lines; PbA-infected mice with ECM: solid symbols, solid lines; Artemether monotherapy: triangles; Artemether plus glyceryl trinitrate 0.025 mg/hour: squares and grey color; Artemether plus glyceryl trinitrate 0.1 mg/hour: circles. The number of mice (N) evaluated in each experiment is shown. Kinetics changes of nitrite (A) and nitrate (B) in plasma samples collected before (0 hours) and at 3, 6, and 24 hours after treatment. In case of PbA-infected mice, treatment was performed like in previous experiments (mice shaved on day 4 of infection, artemether administration and patch implantation when mice showed...
crosses (uninfected) denote a significant increase in nitrite/nitrate levels at individual time points. (C) MAP was measured before infection (day 0), on day 6 of infection at the moment of the ECM manifestation as described above (0 hours) and then at 3, 6, 24 hours and 48 hours after artemether with or without glyceryl trinitrate treatment (day 8). Asterisks represent a significant drop in MAP values from uninfected mice treated with artemether plus glyceryl trinitrate patch in relation to day 0 baseline. Crosses represent a significant drop in MAP values from PbA-infected mice with respect to day 0 baseline on day 6 of infection. Data is expressed as the mean of at least 10 MAP readings per mouse ± SEM. Horizontal dotted line represents baseline. + or * P < 0.05; ** P < 0.01; +++ or ***P < 0.001. In panel (A) and (B) the significant increase in plasma nitrite/nitrate levels of mice treated with artemether plus glyceryl trinitrate 0.025 mg/hour or glyceryl trinitrate 0.1 mg/hour versus artemether monotherapy is shown with crosses and asterisks respectively. ARM: artemether; GTN: glyceryl trinitrate; ECM: experimental cerebral malaria.
**FIG 5** Beneficial effect of artemether plus glyceryl trinitrate treatment in pial arterioles from late-stage ECM mice. Changes in arteriolar diameter were recorded at day 0 and day 6 of infection before (time 0) and after dosing (3, 6 and 24 hours). In panel (A) groups are displayed as follow: Untreated PbA-infected mice (white bars), PbA-infected mice treated with artemether (slash-lined bars), and PbA-infected mice treated with artemether plus glyceryl trinitrate 0.1 mg/hour (black bars). In panel (B), five sections of the same arteriole showing the effect of ECM and artemether plus glyceryl trinitrate treatment in arteriole diameters. The number of mice (N) and arterioles evaluated in each group are shown. Data are expressed as the mean ± SEM. *P < 0.001. ARM artemether; GTN glyceryl trinitrate; ECM experimental cerebral malaria; hr hour.