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Inhibition of nitric oxide synthesis promotes increased mortality despite the reduction of parasitemia in *Plasmodium berghei*-infected mice

Inibição da síntese de óxido nítrico promove aumento da mortalidade, apesar da redução da parasitemia em camundongos infectados pelo *Plasmodium berghei*

La inhibición de la síntesis de óxido nítrico promueve el aumento de la mortalidad a pesar de la reducción de la parasitemia en ratones infectados por *Plasmodium berghei*

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Aline da Silva Barbosa
ORCID: https://orcid.org/0000-0003-4125-4392
Federal University of Pará, Brazil
E-mail: alinesb100@gmail.com
Mayani Costa Ribeiro Temple
ORCID: https://orcid.org/0000-0001-5482-7861
Federal University of Pará, Brazil
E-mail: mayaniribeiro@gmail.com
Everton Luiz Pompeu Varela
ORCID: https://orcid.org/0000-0001-9710-3791
Federal University of Pará, Brazil
E-mail: evertonlpvarela@gmail.com
Antonio Rafael Quadros Gomes
ORCID: https://orcid.org/0000-0002-4700-7942
Federal University of Pará, Brazil
E-mail: rafaelquadros13@hotmail.com
Edvaldo Lima Silveira
ORCID: https://orcid.org/0000-0002-0961-635X
Federal University of Pará, Brazil
E-mail: edvaldolsilveira@gmail.com
Eliete Pereira de Carvalho
ORCID: https://orcid.org/0000-0003-1454-9638
Federal University of Pará, Brazil
E-mail: elicar28@uolcom.br
Maria Fani Dolabela
ORCID: https://orcid.org/0000-0002-3073-3518
Federal University of Pará, Brazil
E-mail: fanidolabela20@gmail.com
Sandro Percario
ORCID: https://orcid.org/0000-0002-9528-0361
Federal University of Pará, Brazil
E-mail: percario@ufpa.br

Abstract
Nitric oxide (NO) is an important mediator molecule in inflammatory processes, but its role in the pathophysiology of malaria is still uncertain. To investigate the NO synthesis inhibition on the oxidative changes induced by *Plasmodium berghei* infection in mice, malaria was induced in 150 animals, of which 75 animals were treated with the NO inhibitor L-NAME; the remaining animals were sham controls. All animals underwent euthanasia 1, 5, 10, 15, or 20 days after infection for the collection of lungs, brain, and blood. Parasitemia was determined, and the survival of the animals was evaluated. Tissue samples were assayed for nitrites and nitrates (NN), thiobarbituric acid reactive substances (TBARS), and total Trolox equivalent antioxidant capacity (TEAC). A histopathological study was performed. Mortality rates in the L-NAME group were always higher than those in the controls. In the brain, NN was lower in the L-NAME group. Parasitemia and its progression rate were greater in the control group. By the 5th day of infection, mice treated with L-NAME showed cerebral edema and interstitial pneumonia of greater intensity than controls. In conclusion, the anti-inflammatory and hemodynamic effects of NO surpass its pro-oxidant role in murine malaria.

Keywords: Nitric oxide; Malaria; Nitric oxide synthase; Oxidative stress.

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Resumo

O óxido nítrico (NO) é uma importante molécula mediadora em processos inflamatórios, mas seu papel na fisiopatologia da malária ainda é incerto. Para investigar a inibição da síntese NO sobre as alterações oxidativas induzidas pela infecção por *Plasmodium berghei* em camundongos, a malária foi induzida em 150 animais, dos quais 75 animais foram tratados com o inibidor de NO L-NAME; os demais animais eram controles sham. Todos os animais foram submetidos à eutanásia 1, 5, 10, 15 ou 20 dias após a infecção para coleta de pulmões, cérebro e sangue. A parasitemia foi determinada, e a sobrevivência dos animais foi avaliada. As amostras de tecido foram avaliadas para nitritos e nitratos (NN), substâncias reativas de ácido tiobarbitúrico (TBARS) e capacidade antioxidante equivalente trolox total (TEAC). Foi realizado um estudo histopatológico. As taxas de mortalidade no grupo L-NAME sempre foram maiores do que as dos controles. No cérebro, NN era menor no grupo L-NAME. A parasitemia e sua taxa de progressão foram maiores no grupo controle. No 5th dia de infecção, os camundongos tratados com L-NAME apresentaram edema cerebral e pneumonia intersticial de maior intensidade do que os controles. Em conclusão, os efeitos anti-inflamatórios e hemodinâmicos de NO superam seu papel pró-oxidante na malária murina.

Palavras-chave: Óxido nítrico; Malária; Óxido nítrico sintase; Estresse oxidativo.

Resumen

El óxido nítrico (NO) es una molécula mediadora importante en los procesos inflamatorios, pero su papel en la fisiopatología de la malaria sigue siendo incierto. Para investigar la inhibición de la síntesis de NO en los cambios oxidativos inducidos por la infección por *Plasmodium berghei* en ratones, la malaria se indujo en 150 animales, de los cuales 75 animales fueron tratados con el inhibidor de NO L-NAME; los animales restantes eran controles sham. Todos los animales se sometieron a eutanasia 1, 5, 10, 15 o 20 días después de la infección para la recolección de pulmones, cerebro y sangre. Se determinó la parasitemia, y se evaluó la supervivencia de los animales. Se ensayaron muestras de tejido para nitritos y nitratos (NN), sustancias reactivas de ácido tiobarbitúrico (TBARS) y capacidad antioxidante total equivalente a Trolox (TEAC). Se realizó un estudio histopatológico. Las tasas de mortalidad en el grupo L-NAME siempre fueron más altas que las de los controles. En el cerebro, NN era más baja en el grupo L-NAME. Parasitemia y su tasa de progresión fueron mayores en el grupo de control. Para el 5o día de infección, los ratones tratados con L-NAME mostraron edema cerebral y neumonía intersticial de mayor intensidad que los controles. En conclusión, los efectos antiinflamatorios y hemodinámicos de NO superan su papel pro-oxidante en la malaria murina.

Palabras clave: Óxido nítrico; Malaria; Óxido nítrico sintasa; Estrés oxidativo.

1. Introduction

Malaria is an infectious disease caused by several species of protozoa of the genus *Plasmodium*, with five species of human importance (*P. vivax, P. falciparum, P. ovale, P. malariae and P. knowlesi*). Malaria caused by *P. falciparum* has the greater potential for morbidity and mortality, but *P. vivax* is the most widespread throughout the world. In 2015, the World Health Organization (WHO) envisaged that 3.4 billion people were at risk of acquiring malaria, with 212 million new cases of the disease and 429,000 deaths (WHO, 2016).

Between 2010 and 2015, the WHO estimated a 29% reduction in malaria global mortality. However, despite this important accomplishment, resistance to antimalarials, with emphasis on artemisinin combination treatments - wrongly employed as *P. falciparum* monotherapy - has concerned WHO; therefore, new therapeutic drugs are still needed, as well as a better knowledge of the physiopathology of the disease (WHO, 2016).

Current evidence has indicated that oxidative stress is a fundamental factor in its pathophysiology (Erel et al., 1997; Pablón et al., 2002; Percário et al., 2012; Gomes., et al, 2015), mainly because *Plasmodium* is highly sensitive to changes in the redox balance, and host defenses rely on free radical production to fight the infection. The parasite itself produces oxidizing agents and has its own system of antioxidant enzymes to defend itself (Becker et al., 2004; Percário et al., 2012). This excess oxidative aggression causes apoptosis of erythrocytes and injuries host organs, such as the brain and lungs (Pino et al., 2003; Becker et al., 2004).

Cerebral and pulmonary complications in malaria, more frequent in infections caused by *P. falciparum*, seem to occur due to the activation of the inflammatory cascade, with consequent generation of free radicals and causing lipid peroxidation and endothelial changes that generate ischemia or tissue hemorrhage (Van Der Heyde et al., 2006).

Among the free radicals involved in malaria physiopathology lies nitric oxide (NO), which presents a controversial yet fundamental role. NO is an important mediator of inflammatory processes and, in addition to being one of the reactive oxygen and nitrogen species (RONS), is a vasodilator produced by the endothelium and a neurotransmitter (Dusse et al., 2003).

Among the multitude of roles played by NO, NO produced by inflammatory cells reacts with other RONS, yielding other even more powerful oxidizing agents, such as peroxynitrite (Xia et al., 1996; Xia & Zweier, 1997; Dusse et al., 2003; Barreto et al., 2005; Percário et al., 2012) which can attack both *Plasmodium* and host cells (Anstey et al., 1999; Becker et al., 2004; Percário et al., 2012). On the other hand, it is a very important cellular signaling molecule that regulates arterial pressure at the microcirculation level and displays antioxidant actions when stimulating the expression of the enzyme superoxide dismutase, in addition to inducing the synthesis of ferritin, which bounds free iron and prevents the generation of superoxide radicals in the Haber-Weiss and Fenton reactions (Dusse et al., 2003; Barreto et al., 2005, Halliwell & Gutteridge, 2007).

This multiplicity of effects attributed to NO makes quite difficult to interpret the results from scientific experiments, with several controversial reports existing in the scientific literature so far. In this sense, although there is evidence of increasing NO production during the evolution of parasitemia (Nussler et al., 1994**;** Anstey et al., 1999), there are other reports that have found an inverse relationship between NO levels and the severity of the disease (Anstey et al., 1996).

Thus, the objective of this study was to investigate the effects of NO synthesis inhibition on parasitemia, survival rate, and oxidative changes induced by plasmodial infection in an experimental model of mouse malaria.

2. Methodology

For the present study, 150 young adult male Swiss mice (*Mus musculus*) were used from the vivarium of the Evandro Chagas Institute (Belém, PA, Brazil). Animals were randomly divided into two groups with five subgroups each, as follows:

Groups I-V: (N=15 each), in which animals were inoculated with *Plasmodium berghei* ANKA. Animals simultaneously received physiological saline solution (1.5 µL/g body weight; intraperitoneal) before (48 h and 24 h) and during (every 24 h) the study period and were submitted to euthanasia after 24 h, 5, 10, 15 or 20 days after infection.

Groups VI-X: (N=15 each), in which animals were pre-treated (48 h and 24 h before infection) and post-treated (every 24 h) with N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of constitutive and inducible forms of nitric oxide synthase (NOS; aqueous solution 10%; 1.5 µL/g body weight; intraperitoneal). Mice were inoculated with *P. berghei* and submitted to euthanasia in the same manner and periods of animals in Groups I to V.

Animals were maintained in the vivarium of the Institute of Biological Sciences of the Federal University of Pará (UFPA, Brazil) in polystyrene cages of five animals each, with a light/dark cycle of 12 h, controlled temperature and humidity, as well as food and water *ad libitum*. The project was approved by the Ethics Committee for Research with Experimentation Animals of the Federal University of Pará (CEPAE/UFPA; report No. MED014/2008).

2.1 Induction of malaria

Mice were induced to malaria by intraperitoneal inoculation of 10^6 erythrocytes infected by *P. berghei* ANKA diluted in 0.2 mL of sterile saline solution. The strain of *P. berghei* was supplied by the Evandro Chagas Institute (Belém, PA, Brazil).

Features of the animal model

The use of Swiss mice as a model for malaria infection is widely used and presents the same pattern of infection progression and basic features of lung malaria as other mouse species. As described by Sadavongvivad & Aviado (1969), the main histopathological lung findings in P. berghei-infected mice are the presence of inflammatory reactions around the alveoli and intra-alveolar hemorrhages. Moreover, the presence of large alveolar edema is a common finding, often causing an over

40% increase in the lung-to-body weight ratio (Aviado & Cambar, 1969; Weiss & Kubat, 1983). Additionally, the infiltration of macrophages and lymphocytes is observed as the infection progresses and is responsible for septal thickening (Moore et al., 2008; Martins et al., 2009), as well as the presence of cytoadherence of mononuclear cells in pulmonary vessels (Martins et al., 2009). Taken together, the histopathological features described are similar to those displayed in severe malaria human cases.

2.2 Sample collection

At the end of each period (1, 5, 10, 15, or 20 days after infection), animals were inoculated with heparin sulfate (100 IU: i.p.), anesthetized (50 µL of 5% ketamine + 2% xylazine solution), and then underwent euthanasia. The thoracic cavity was exposed, and the skull was opened for extraction of the lungs and brain, followed by perfusion of both lungs and brain with saline solution (9% NaCl) for the removal of an excess of blood, with the organs subsequently processed.

2.3 Sample processing

Homogenization of tissues: After the collection of a small fragment of the tissue for the anatomopathological exam, the remaining pulmonary or cerebral tissue was weighed and diluted in saline phosphate buffer solution (PBS) in proportions of 1:10 (m:m) for the brain and 1:20 (m:m) for the lung. In sequence, in the same beaker in which they were weighed, the lungs and brains were shredded with scissors to produce smaller fragments with the aim of facilitating homogenization. The homogenization process was performed in an ultrasonic cell disrupter (Thornton, DCel, Indaiatuba, SP, Brazil) at 4 minutes after the tuning of the homogenization needle at 10 W. During the homogenization process, the beaker containing the material was kept in crushed ice to prevent sample denaturation. Subsequently, samples were centrifuged (175 x *g* x 15 min), and the supernatant was collected and stored (-20°C) until analysis.

Tissue preparation for the anatomopathological exam: the material was fixed in buffered neutral formalin solution (10%), where it remained for three months, being hereinafter subjected to the usual processes of dehydration, clarification and inclusion in paraffin. Subsequently, the blocks containing the fragments of the material were sectioned into serial slices 4 to 7 µm thick, mounted on glass slides and transferred to an incubator (56°C) until staining by the methods of Giemsa and May-Grunwald.

2.4 Technical procedure

Laboratory measurements of Trolox equivalent antioxidant capacity (TEAC), thiobarbituric acid reactive substances (TBARS), and nitrites and nitrates (NN) were performed in duplicate. Internal controls and standards were inserted into each batch for the quality assurance of determinations.

2.4.1 Trolox equivalent antioxidant capacity (TEAC)

The potential antioxidant was determined according to their equivalence to Trolox (6-hydroxy-2,5,7,8-tetrametilcromono-2-carboxylic acid; Aldrich Chemical Co; 23881-3), a synthetic soluble analogue of vitamin E. The technique is based on the colorimetric reaction between ABTS (2,2'-azinobis-3--ethylbenz-thiazoline-6-acid-6-sulfonic-diammonium; Sigma St Louis; 1888) with potassium persulphate ($K_2S_2O_8$), yielding the radical cation $ABTS^{\bullet+}$, chromophore of green/blue staining, with maximum absorbance at 734 nm (Fento, Sao Paulo, Brazil; 800XI; Miller et al., 1993; Re et al., 1999). The final results were expressed in micromoles per liter (µM/L) corresponding to the concentration of Trolox equivalent to the antioxidant capacity of the tested sample. Finally, the total antioxidant activity of the sample is calculated as its

relationship with the reactivity of the Trolox standard through the implementation of a standard curve under the same conditions.

2.4.2 Thiobarbituric acid reactive substances (TBARS)

For the assessment of oxidative stress in the samples, we opted to assay TBARS, a marker of lipid peroxidation. The dosage is based on the reaction of two molecules of thiobarbituric acid (TBA) with a molecule of malondialdehyde (MDA), forming a pinkish complex TBA-MDA-TBA, with absorbance at 535 nm (Fento, Sao Paulo, Brazil; 800XI; Percario et al., 1994). 1,1,3,3, tetraethoxypropane (Sigma-Aldrich; T9889) was used for the implementation of the standard curve.

2.4.3 Nitrites and nitrates (NN)

The evaluation of this parameter was performed by means of spectrophotometry (Kit Total Nitrite/Nitrate, R & D Systems, KGE001). This technique is based on the quantitative determination of NO, involving the enzyme nitrate reductase, which converts nitrate to nitrite, followed by colorimetric detection of nitrite as a product of pink color, produced by the Griess reaction and that absorbs visible light at 540 nm (PerkinElmer, Victor X3). The nitrite concentration was calculated based on the nitrite standard curve.

2.5 Determination of parasitemia

Parasitemia assessment was conducted on blood smears, stained by the methods of Giemsa and May-Grunwald, counting, in a total of 300 erythrocytes, the number of parasitized erythrocytes. The ratio between the number of parasitized red blood cells and the number of blood cells counted (300) was considered the degree of parasitemia.

2.6 Histopathological analysis

Slides of pulmonary tissue and brain were examined under light microscopy, and the alterations found were classified as absent, mild, moderate, or severe by comparison between the groups, scored as 1, 2, 3, and 4 points, respectively.

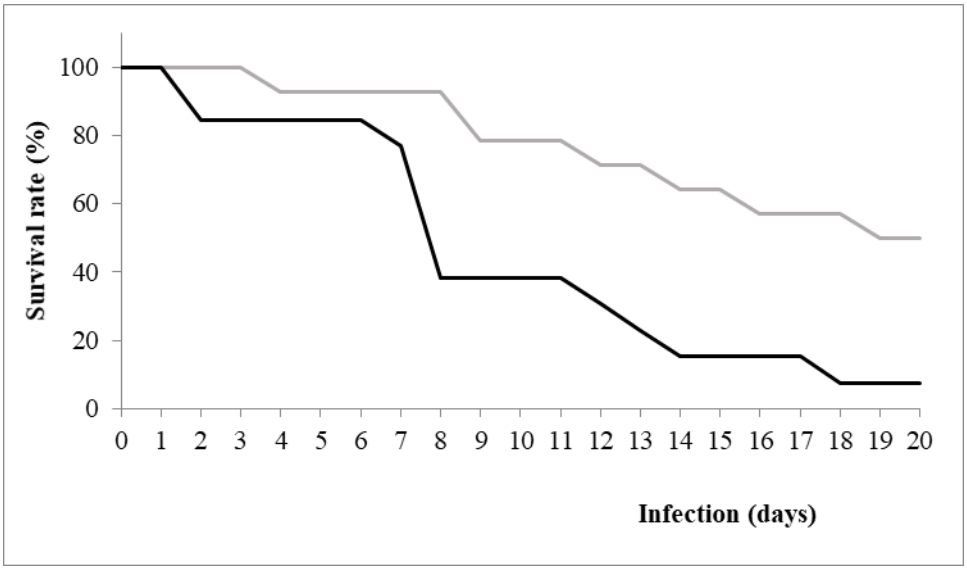
2.7 Statistical analysis

The differences were explored by paired comparison between groups through a multiple variance test followed by *post hoc* analysis through the Tukey test. To check for possible correlation between parameters, Pearson's correlation test was performed, considering the paired values of two parameters obtained from the same animal, with the data obtained from all animals simultaneously, regardless of the group to which they belonged. In the histopathological analysis, the Kruskal-Wallis test (test H) was used, followed by Dunn's test. In all tests, a significance level of 5% ($p \leq 0.05$) was considered.

3. Results

Figure 1 shows the survival rate of animals in each group in relation to the time of infection. A progressive decrease in the survival rate over time was noted for both groups, with the groups treated with L-NAME (Groups V to X) having a lower survival rate than the control groups (Groups I to V).

Figure 1. Survival rate of mice infected with *Plasmodium berghei* and treated with L-NAME (black line) or controls (gray line).

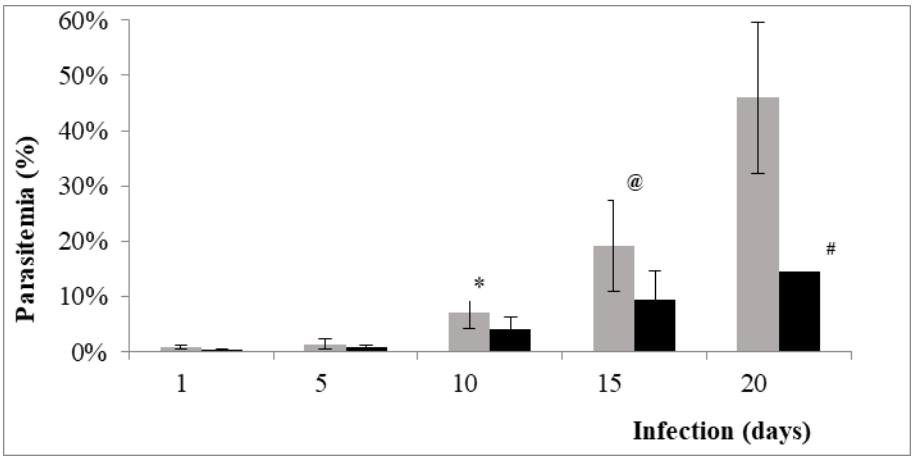


Source: Authors.

3.1 Parasitemia

Figure 2 shows the evolution of parasitemia in the control groups and in those treated with L-NAME. A significant reduction was found in the groups treated with L-NAME ($p<0.001$), especially at 10 days ($p=0.012$) and 15 days ($p<0.001$) postinfection.

Figure 2. Evolution of parasitemia in *Plasmodium berghei*-infected mice treated with L-NAME (black bars) and in controls (gray bars).



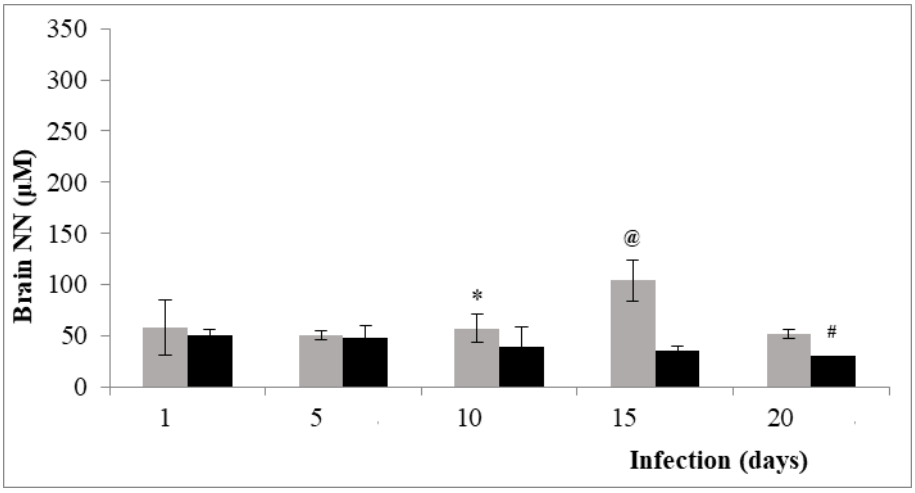
Error bars represent the standard deviation of measurements (n=15). *= $p=0.012$ *versus* L-NAME; [@]= $p<0.001$ *versus* L-NAME; [#]= impossible to run statistics due to the reduced number of samples in group L-NAME.
Source: Authors.

3.2 Nitrites and nitrates

Figure 3 shows the levels of cerebral NN found in groups treated with L-NAME or controls. A significant reduction was found in the groups treated with L-NAME in relation to the controls at 10 days ($p=0.029$) and 15 days ($p<0.001$) after infection. For the lung samples, groups treated with L-NAME presented similar values to the control group, except for the 10th

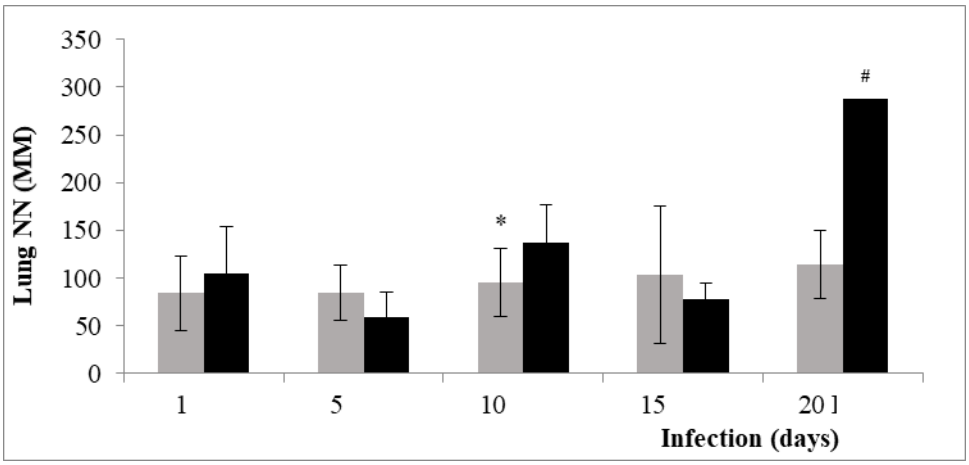
day after infection, where the value displayed by the L-NAME group was statistically higher than that of the control group (p=0.019; Figure 4).

Figure 3. Levels of nitrites and nitrates (NN) in brains of mice treated with L-NAME (black bars) and in controls (gray bars), according to the duration of *Plasmodium berghei* infection.



Error bars represent the standard deviation of measurements (n=15). *= p=0.029 *versus* L-NAME; @= p<0.001 *versus* L-NAME; #= impossible to run statistics due to the reduced number of samples in group L-NAME.
Source: Authors.

Figure 4. Levels of nitrites and nitrates (NN) in the lungs of mice treated with L-NAME (black bars) and in the controls (gray bars), according to the duration of *Plasmodium berghei* infection.

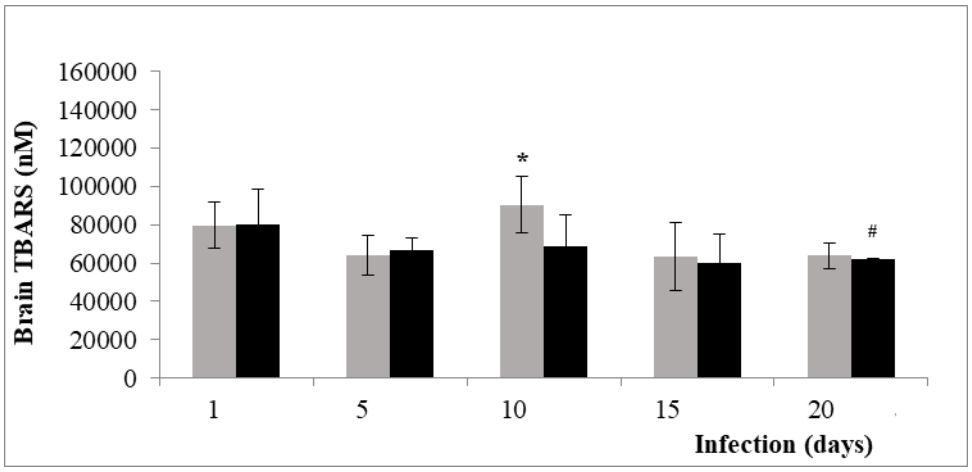


Error bars represent the standard deviation of measurements (n=15). *= p=0.019 *versus* L-NAME; #= impossible to run statistics due to the reduced number of samples in group L-NAME.
Source: Authors.

3.3 TBARS

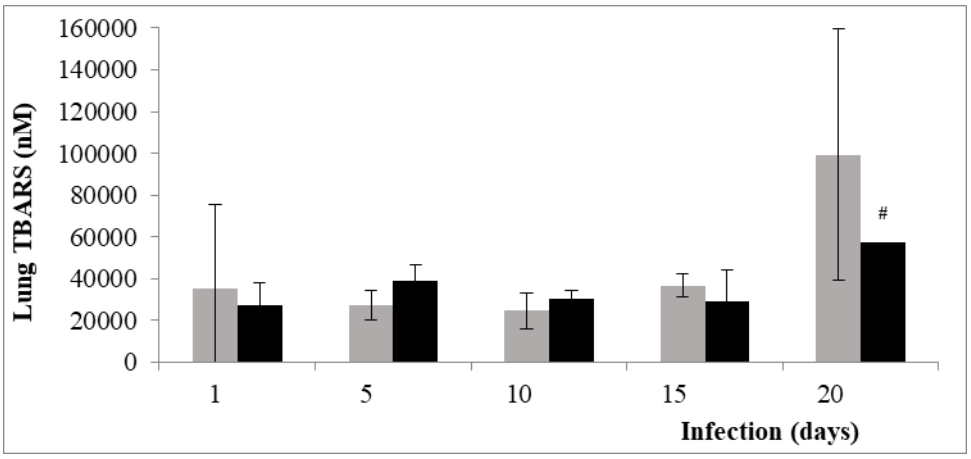
Figure 5 shows the levels of cerebral TBARS found in groups treated with L-NAME or controls. In the comparison between these groups, a statistically significant reduction was observed only in the L-NAME group after 10 days of infection (p=0.002). There were no differences between the groups for the values of pulmonary TBARS (Figure 6).

Figure 5. Levels of thiobarbituric acid reactive substances (TBARS) in the brains of mice treated with L-NAME (black bars) and controls (gray bars), according to the duration of *Plasmodium berghei* infection.



Error bars represent the standard deviation of measurements (n=15). *= p=0.002 *versus* L-NAME; #= impossible to run statistics due to the reduced number of samples in group L-NAME.
Source: Authors.

Figure 6. Levels of thiobarbituric acid reactive substances (TBARS) in the lungs of mice treated with L-NAME (black bars)



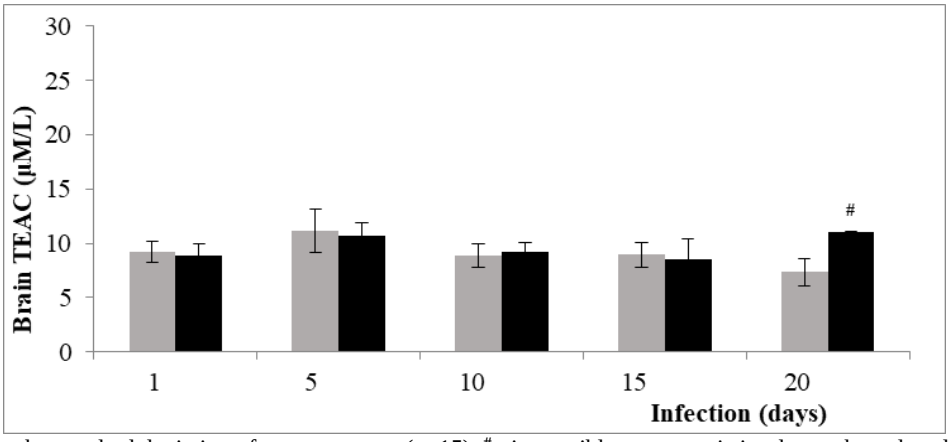
and controls (gray bars), according to the duration of *Plasmodium berghei* infection.

Error bars represent the standard deviation of measurements (n=15). #= impossible to run statistics due to the reduced number of samples in the group.
Source: Authors.

3.4 TEAC

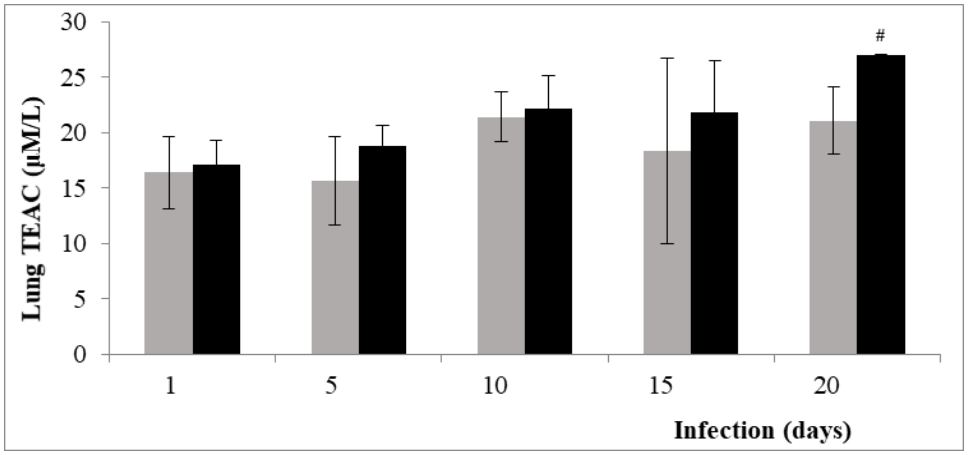
Figures 7 and 8 present TEAC values found in groups treated with L-NAME and in controls. No differences were found between the groups for brain (Figure 7) or pulmonary samples (Figure 8).

Figure 7. Levels of Trolox equivalent antioxidant capacity (TEAC) in brains of mice treated with L-NAME (black bars) and controls (gray bars), according to the duration of *Plasmodium berghei* infection.



Error bars represent the standard deviation of measurements (n=15). #= impossible to run statistics due to the reduced number of samples in group L-NAME.
Source: Authors.

Figure 8. Levels of Trolox equivalent antioxidant capacity (TEAC) in the lungs of mice treated with L-NAME (black bars) and controls (gray bars) according to the duration of *Plasmodium berghei* infection.



Error bars represent the standard deviation of measurements (n=15). #= impossible to run statistics due to the reduced number of samples in group L-NAME.
Source: Authors.

3.5 Correlation studies

Although animals treated with L-NAME displayed lower parasitemia levels than controls, no significant correlation between NN and parasitemia was found. The same occurred for NN and TBARS. However, a positive correlation between NN and TEAC was found for both lung ($p=0.019$ and $r=0.434$) and brain samples ($p=0.004$ and $r=0.419$).

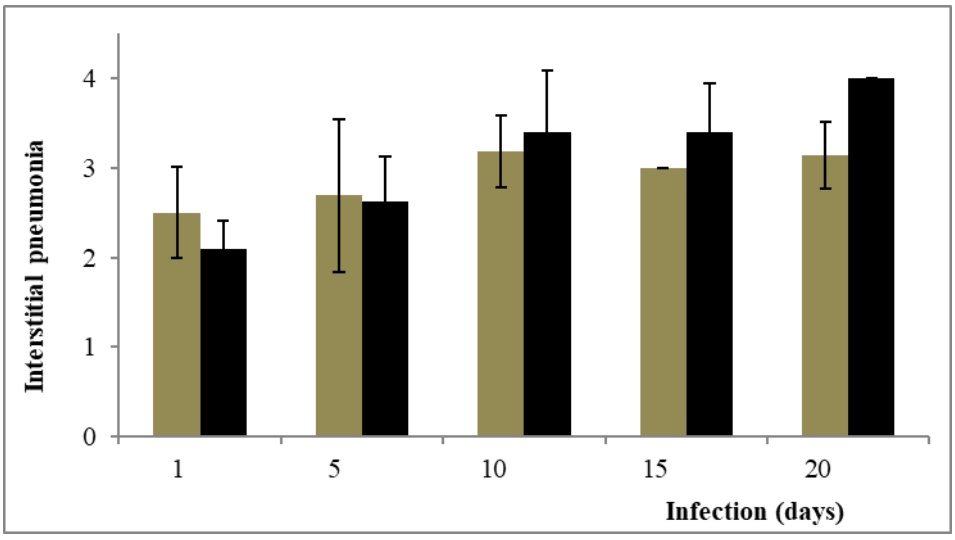
3.6 Histopathological exam

In all groups, intense vascular congestion was observed with intense leukocyte margination in brains and lungs, as well as mild alveolar hemorrhage in lung samples. No group developed cerebral petechial hemorrhage or pulmonary edema. Histological changes were characterized as cerebral edema and interstitial pneumonia and classified as absent, mild, moderate, and severe, with values from 0 to 4 depending on the intensity of the alterations, 0 for absence of alterations and 4 for intense alterations. All animals presented some level of cerebral edema and interstitial pneumonia.

Interstitial pneumonia

The degree of interstitial pneumonia progressively increased in mice treated with L-NAME (p=0.0317; 15 days *versus* 1 day) but remained without significant differences when compared to controls (Figure 9).

Figure 9: Interstitial pneumonia according to the duration of the disease in *Plasmodium berghei*-infected mice treated with L-NAME (black bars) and controls (gray bars)

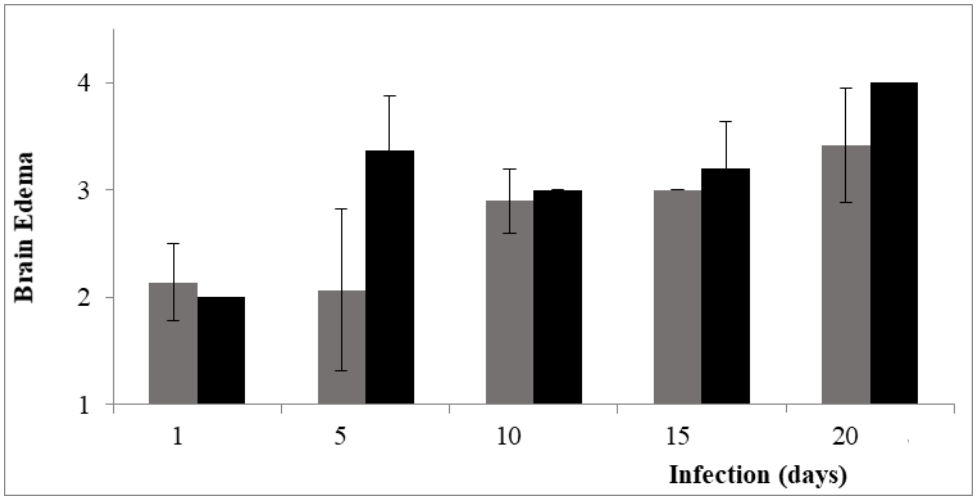


Error bars represent the standard deviation of measurements (n=15).
Source: Authors.

Cerebral edema

The degree of cerebral edema was higher in mice treated with L-NAME than in controls on days 5 and 15 postinfection (p<0.001; Kruskal-Wallis test; Figure 10).

Figure 10: Brain edema in *Plasmodium berghei*-infected mice treated with L-NAME (black bars) and controls (gray bars) according to the duration of infection

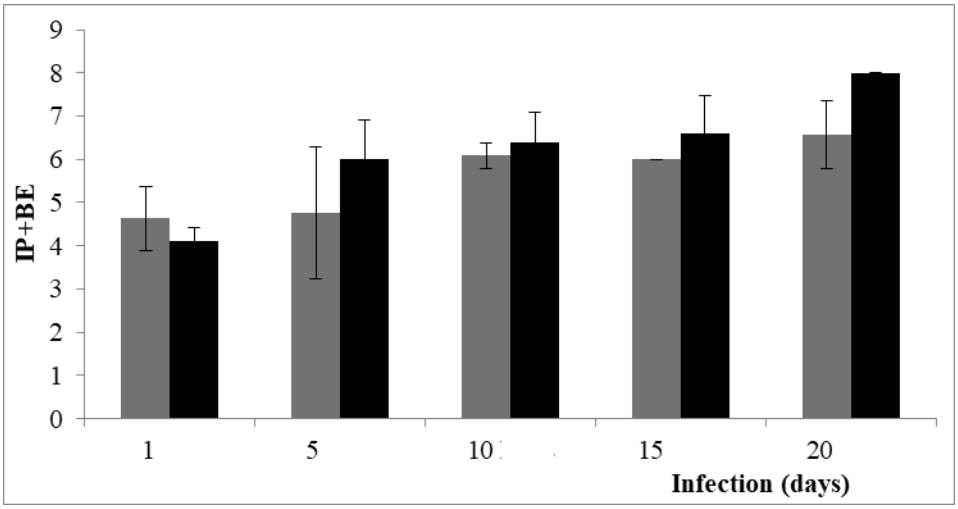


Error bars represent the standard deviation of measurements (n=15)
Source: Authors.

Interstitial pneumonia + brain edema

When the scores of interstitial pneumonia and cerebral edema in each group were summed, a greater degree of histopathological changes was observed in groups treated with L-NAME on days 5, 10, 15, and 20 post-infection in relation to controls; however, this difference was not statistically significant (Figure 11).

Figure 11: The sum of interstitial pneumonia and cerebral edema scores (IP+BE) in mice treated with L-NAME (black bars) and controls (gray bars), according to the duration of infection by *Plasmodium berghei*



Error bars represent the standard deviation.
Source: Authors.

4. Discussion

Often used to assess the effects of NO synthesis inhibition in the most diverse pathologies, L-NAME is an L-arginine analog that competitively inhibits the production of NO from all isoforms of NOS. NO plays a role in many essential functions of the body, including neuronal plasticity, blood pressure regulation, vasodilation, inflammation, and nonspecific immunity (Förstermann & Sessa, 2012).

In brain samples of mice treated with L-NAME, NN levels remained significantly lower than in the control group ($p<0.001$), showing that L-NAME was effective in inhibiting NO synthesis in this tissue. However, it was not evident in L-NAME-treated lung samples because they presented equal or higher NN levels than the control group for the entire period of the study. At the pulmonary level, to compensate for the reduction in NO production, it is possible that L-arginine was mobilized to compete with L-NAME for its binding site in the NOS molecule, which would explain the findings of this study. Another possibility is that the dose of L-NAME inoculated daily was insufficient to inhibit NOS in this tissue.

Additionally, this fact can result from compensatory physiological effects, such as the vasoconstriction caused by NO inhibition, which seems to stimulate the release of mediators that cause vasodilation, such as acetylcholine and bradykinin, which, however, are bronchoconstrictors. On the other hand, it is possible that the occurrence of pulmonary hypertension in malaria, a side effect of NO inhibition as reported by Lacerda et al. (2009) combined with the need for oxygen as a result of hemolysis, stimulates the synthesis of endothelial nitric oxide synthase (eNOS) by erythropoietin, which increases the expression of eNOS receptors in the lung (Beleslin-Čokić et al., 2011). Another important factor to be considered is the

existence of a complex system of non-adrenergic non-cholinergic (NANC) neural fibres in mammalian lungs capable of producing significant quantities of NO (Gaston et al., 1994**)** that overcome malaria-derived NO synthesis in this tissue.

In the scientific literature, the antiparasitic effect of NO against all species of *Plasmodium* stands out in several surveys conducted both *in vitro* and *in vivo* (Green et al., 1994; Balmer et al., 2000; Fritsche et al., 2001; Becker et al., 2004). Nevertheless, this study demonstrated that mice treated with L-NAME presented significantly lower parasitemia than controls, along with a lower rate of parasitemia progression, suggesting that NO might be a factor required by the parasites for its growth or that it can contribute somehow to the invasion of erythrocytes by the parasites. Similar findings were found in another recent study conducted in our laboratory (Moreira, in press). Sobolewski et al. (2005) have also questioned the role of NO in the progression of malarial parasitemia, affirming that *Plasmodium* is protected by hemoglobin from oxidative aggression within erythrocytes, requiring very high levels of ROS to surpass the protective effects of hemoglobin. In their experiments, NO could destroy the parasites only when at levels close to saturation. Likewise, studies with inducible nitric oxide synthase (iNOS)-/- mice found parasitemia similar to that of controls (Favre et al., 1999; Van Der Heyde et al., 2000).

NO produced by eNOS in the endothelium of all tissues acts as a blood flow regulator, causing vasodilation, inhibiting platelet aggregation, and preventing lymphocytes and monocytes from adhering to the endothelium (Dusse et al., 2003; Lacerda et al., 2009), which prevents local ischemia from arising. These effects are essential in the prevention of cerebral malaria, and the administration of exogenous NO, or NO-releasing substances, has been investigated as an adjuvant treatment to malaria, with great results in improving microcirculation, reducing cerebral inflammation, and protecting the integrity of the blood brain barrier (Cabrales et al., 2011; Hawkes et al., 2011; Serghides et al., 2011; Zanini et al., 2011; Martins et al., 2012).

On the other hand, in the present study, treatment with L-NAME did not significantly reduce lipid peroxidation in the brain, except on the 10th day postinfection ($p=0.002$). The reductions found at 15 and 20 days after infection were mild and not significant. It is possible that in this experimental model, other oxidizing agents play more important roles than NO in the course of the disease, as well as that the eventual benefits generated by the reduction of the deleterious effects of this molecule in the cerebral tissue had been surpassed by the damage caused as a consequence of the decrease of its protective effects. Among other oxidizing agents of importance in this model lies superoxide ($O_2^{\bullet-}$), produced by NOS when in monomers, and peroxynitrite ($ONOO^-$), produced by the reaction between $O_2^{\bullet-}$ and NO (Lacerda et al., 2009).

Not only would cerebral malaria be avoided with increasing NO levels but also other forms of severe malaria, such as respiratory distress accompanied by metabolic acidosis (Lovegrove et al., 2008). To support this suggestion, there are reports in the literature on the use of exogenous NO as a prophylaxis for lung damage in malaria (Hawkes et al., 2011). In parallel, eNOS seems to have a protective role, and there is also evidence that iNOS may act as an anti-inflammatory molecule at the pulmonary level (Speyer et al., 2003; Zeidler et al., 2004).

Nevertheless, in the present study, no significant differences in pulmonary lipid peroxidation levels between groups were found, which can be explained by hypoxemia or by ischemia and inflammation caused by NO inhibition. These cerebral and pulmonary effects of decreased NO synthesis may explain the higher mortality observed in this study for the groups treated with L-NAME (Figure 1).

In this work, the potential role of NO in inhibiting leukocyte adhesion was not evident, since there was intense vascular congestion with leukocyte margination in the brains and lungs of mice of both groups. However, from the 5th day postinfection, control mice showed cerebral edema and interstitial pneumonia of lesser intensity than the ones treated with L-NAME, findings that indicate the anti-inflammatory role of NO, as well as its important regulatory function over cerebral

hemodynamics (Speyer et al., 2003; Zeidler et al., 2004; Lovegrove et al., 2008; Cabrales et al., 2011; Hawkes et al., 2011; Serghides et al., 2011; Zanini et al., 2011; Martins et al., 2012).

Pulmonary malaria presents clinical and histopathological findings similar to adult respiratory distress syndrome (ARDS) that occurs in sepsis (Hawkes et al., 2011), with a histological pattern of interstitial pneumonia. In the lungs of mice with severe malaria, inflammation of the alveolar septa was found without cellular transmigration for the interior of the alveoli (Lovegrove et al., 2008), similar findings to the present study.

In the brains of control animals, a progressive decrease in cerebral TEAC during the course of infection was observed, and the same effect was observed in L-NAME-treated animals, a phenomenon compatible with an alteration of redox balance (Erel et al., 1997; Pablón et al., 2002; Pino et al., 2003; Becker et al., 2004). In fact, experimental models with administration of antioxidants showed a reduced incidence of cerebral malaria (Reis et al., 2010). In the present study, as there was no significant difference between the groups, it is inferred that higher or lower levels of NO were not a determinant to interfere with the concentration of antioxidants in that tissue.

Unlike the brain, in lung samples, an increased level of antioxidant molecules in the course of malarial infection was noted, both in groups treated with L-NAME as in controls, perhaps by the readiness of these molecules to reach the pulmonary circulation, while in brain, the blood-brain barrier and cerebral vascular changes imposed by malaria could create a greater difficulty in the intake of antioxidant molecules to this tissue. The positive correlation found between NN and TEAC for both samples suggests the importance of NO as a vasodilator, which enables the inflow of antioxidant molecules from other tissues, as well as the potential anti-inflammatory effects of iNOS (Martins, et al., 2012).

In view of the present results, further investigation of hemodynamic and anti-inflammatory actions of nitric oxide may lead to the development of new strategies for the treatment of severe forms of malaria.

5. Conclusion

From this murine *P. berghei*-induced experimental model of malaria, it is concluded that the inhibition of NOS by L-NAME increased mouse mortality, even with lower values of final parasitemia and decreased speed of parasitemia progression and is associated with more intense interstitial pneumonia and cerebral edema. In the face of these results, it is suggested that the hemodynamic and anti-inflammatory actions of NO overcome its pro-oxidant effects.

References

Anstey, N. M., Granger, D. L., Hassanali, M. Y., Mwaikambo, E. D., Duffy, P. E., & Weinberg, J. B. (1999). Nitric oxide, malaria, and anemia: inverse relationship between nitric oxide production and hemoglobin concentration in asymptomatic, malaria-exposed children. *The American journal of tropical medicine and hygiene*, 61(2), 249-252. https://doi.org/10.4269/ajtmh.1999.61.249

Anstey, N. M., Weinberg, J. B., Hassanali, M. Y., Mwaikambo, E. D., Manyenga, D., Misukonis, M. A., Arnelle, D. R., Hollis, D., McDonald, M. I., & Granger, D. L. *(1996).* Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *Journal of Experimental Medicine, 184(2), 557-567.* https://doi.org/10.1084/jem.184.2.557

Aviado, D. M., & Cambar, P. J. (1969). Pathologic physiology and chemotherapy of *Plasmodium berghei*: X. Pulmonary edema and naphthoquinones. *Experimental Parasitology*, 26(3), 354-368. https://doi.org/10.1016/0014-4894(69)90129-5

Balmer, P., Phillips, H. M., Maestre, A. E., McMonagle, F. A., & Phillips, R. S. (2000). The effect of nitric oxide on the growth of *Plasmodium falciparum*, *P. chabaudi* and *P. berghei* in vitro. *Parasite Immunology*, 22(2), 97-106. https://doi.org/10.1046/j.1365-3024.2000.00281.x

Barreto, R. L., Correia, C. R. D., & Muscará, M. N. (2005). Óxido nítrico: propriedades e potenciais usos terapêuticos. *Química Nova*, 28(6), 1046-1054. https://doi.org/10.1590/S0100-40422005000600020

Becker, K., Tilley, L., Vennerstrom, J. L., Roberts, D., Rogerson, S., & Ginsburg, H. (2004). Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *International Journal for Parasitology*, 34(2), 163-189. https://doi.org/10.1016/j.ijpara.2003.09.011

Beleslin-Čokić, B. B., Čokić, V. P., Wang, L., Piknova, B., Teng, R., Schechter, A. N., & Noguchi, C. T. (2011) Erythropoietin and hypoxia increase erythropoietin receptor and nitric oxide levels in lung microvascular endothelial cells. *Cytokine*, 54(2), 129-135. https://doi.org/10.1016/j.cyto.2011.01.015

Cabrales, P., Zanini, G. M., Meays, D., Frangos, J. A., & Carvalho, L. J. M. (2011). Nitric Oxide protection against murine cerebral malaria is associated with improved cerebral microcirculatory physiology. *The Journal of Infectious Diseases*, 203(10), 1454-1463. https://doi.org/10.1093/infdis/jir058

Dusse, L. M. S., Vieira, L. M., & Carvalho, M. G. (2003). Revisão sobre óxido nítrico. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 39(4), 343-350. https://doi.org/10.1590/S1676-24442003000400012

Erel, O., Kocyigit, A., Avci, S., Aktepe, N., & Bulut, V. (1997). Oxidative stress and antioxidative status of plasma and erythrocytes in patients with *vivax* malaria. *Clinical Biochemistry*, 30(8), 631-639. https://doi.org/10.1016/s0009-9120(97)00119-7

Favre, N., Ryffel, B., & Rudin, W. (1999). Parasite killing in murine malaria does not require nitric oxide production. *Parasitology*, 118(2), 139-143. https://doi.org/10.1017/S0031182098003618

Förstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *European Heart Journal*, 33(7), 829-837. https://doi.org/10.1093/eurheartj/ehr304

Fritsche, G., Larcher, C., Schennach, H., & Weiss, G. (2001). Regulatory interactions between iron and nitric oxide metabolism for immune defense against *Plasmodium falciparum* infection. *The Journal of Infectious Diseases*, 183(9), 1388-1394. https://doi.org/10.1086/319860

Gaston, B., Drazen, J. M., Loscalzo, J., & Stamler, J. S. (1994). The biology of nitrogen oxides in the airways. *American Journal of Respiratory and Critical Care Medicine*, 149(2), 538-551. https://doi.org/10.1164/ajrccm.149.2.7508323

Gomes, B. A. Q., Silva, L. F. D., Gomes, A. R. Q., Moreira, D. R., Dolabela, M. F., Santos, R. S., Green, M. D., Carvalho, E. P., & Percário, S. (2015). N-acetyl cysteine and mushroom *Agaricus sylvaticus* supplementation decreased parasitemia and pulmonary oxidative stress in a mice model of malaria. *Malaria Journal*, 14(202), 1-12. https://doi.org/10.1186/s12936-015-0717-0

Green, S. J., Scheller, L. F., Marletta, M. A., Seguin, M. C., Klotz, F. W., Slayter, M., Nelson, B. J., & Nacy, C. A. (1994). Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. *Immunology Letters*, 43(1-2), 87-94. https://doi.org/10.1016/0165-2478(94)00158-8

Halliwell, B. & Gutteridge, J. M. C. (2007). Free Radicals in Biology and Medicine. (4th ed.) Oxford University Press.

Hawkes, M., Opoka, R. O., Namasopo, S., Miller, C., Conroy, A. L., Serghides, L., Kim, H., Thampi, N., Liles, W. C., John, C. C., & Kain, K. C. (2011). Nitric oxide for the adjunctive treatment of severe malaria: Hypothesis and rationale. *Medical Hypotheses*, 77(3), 437-444. https://doi.org/10.1016/j.mehy.2011.06.003

Lacerda, M. V. G., Mourão, M. P. G., Santos, P. J. T., & Alecrim, M. G. C. (2009). Malária álgida: um diagnóstico sindrômico. *Revista da Sociedade Brasileira de Medicina Tropical*, 42(1), 79-81. https://doi.org/10.1590/S0037-86822009000100017

Lovegrove, F. E., Gharib, S. A., Peña-Castillo, L., Patel, S. N., Ruzinski, J. T., Hughes, T. R., Liles, W. C., & Kain, K. C. (2008). Parasite burden and CD36-mediated sequestration are determinants of acute lung injury in an experimental malaria model. *PLoS Pathogens*, 4(5), e1000068. https://doi.org/10.1371/journal.ppat.1000068

Martins, Y. C., Smith, M. J., Pelajo-Machado, M., Werneck, G. L., Lenzi, H. L., Daniel-Ribeiro, C. T., & Carvalho, L. J. M. (2009). Characterization of cerebral malaria in the outbred Swiss Webster mouse infected by *Plasmodium berghei* ANKA. *International Journal of Experimental Pathology*, 90(2), 119-130. https://doi.org/10.1111/j.1365-2613.2008.00622.x

Martins, Y. C., Zanini, G. M., Frangos, J. A., & Carvalho, L. J. M. (2012). Efficacy of different nitric oxide-based strategies in preventing experimental cerebral malaria by *Plasmodium berghei* ANKA. *PLoS ONE*, 7(2), e32048. https://doi.org/10.1371/journal.pone.0032048

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Sciense*, 84(4), 407-12. https://doi.org/10.1042/cs0840407

Moore, B. R., Jago, J. D., & Batty, K. T. (2008). *Plasmodium berghei*: Parasite clearance after treatment with dihydroartemisinin in an asplenic murine malaria model. *Experimental Parasitology* ,118(4), 458-467. https://doi.org/10.1016/j.exppara.2007.10.011

Moreira, D. R, Uberti, A. C. M. G., Gomes, A. R. Q., Ferreira. M. E. S., Ferrari, C. K. B., Santos, R. S., et al. Inhibition of nitric oxide synthesis by dexamethasone increases survival rate in *Plasmodium berghei*-infected mice. In press.

Nussler, A. K., Eling, W., & Kremsher, P. G. *(1994).* Patients with Plasmodium falciparum malaria and Plasmodium vivax malaria show increased nitrite and nitrate plasma levels. *The Journal of Infectious Diseases, 169(6), 1418-1419.* https://doi.org/10.1093/infdis/169.6.1418

Pablón, A., Carmona, J., Burgos, L. C., & Blair, S. (2002). Oxidative stress in patients with non-complicated malaria. *Clinical Biochemistry*, 36(1), 71-78. https://doi.org/10.1016/S0009-9120(02)00423-X

Percário, S., Moreira, D. R., Gomes, B. A. Q., Ferreira, M. E. S., Gonçalves, A. C. M., Laurindo, P. S. O. C., Vilhena, T. C., Dolabela, M. F., & Green, M. D. (2012). Oxidative stress in malaria. *International Journal of Molecular Sciences*, 13(12), 16346-16372. https://doi.org/10.3390/ijms131216346

Percario, S., Vital, A. C. C., & Jablonka, F. (1994). Dosagem do malondialdeido. *Newslab*, 2(6), 46-50.

Pino, P., Vouldoukis, I., Dugas, N., Hassani-Loppion, G., Dugas, B., & Mazier, D. (2003). Redox-dependent apoptosis in human endothelial cells after adhesion of *Plasmodium falciparum*-infected erythrocytes. *Annals of the New York Academy of Sciences*, 1010 (1), 582-586. https://doi.org/10.1196/annals.1299.109

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26(9-10), 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Reis, P. A., Comim, C. M., Hermani, F., Silva, B., Barichello. T., Portella, A. C., Gomes, F. C. A., Sab, I. M., Frutuoso, V. S., Oliveira, M. F., Bozza, P. T., Bozza, F. A., Dal-Pizzol, F., Zimmerman, G. A., Quevedo, J., & Castro-Faria-Neto, H. C. (2010). Cognitive dysfunction is sustained after rescue therapy in experimental cerebral malaria, and is reduced by additive antioxidant therapy. *PLoS Pathogens*, 6(6), e.1000963. https://doi.org/10.1371/journal.ppat.1000963

Sadavongvivad, C. & Aviado, D. (1969). Pathologic physiology and chemotherapy of *Plasmodium berghei*. VI. Mechanichal properties and histological features of the lung. *Experimental Parasitology*, 24(3), 313-326. https://doi.org/10.1016/0014-4894(69)90170-2

Serghides, L., Kim, H., Lu, Z., Kain, D. C., Miller, C., Francis, R. C., Liles, W. C., Zapol, W. M., & Kain, K. C. (2011). Inhaled Nitric Oxide reduces endothelial activation and parasite accumulation in the brain, and enhances survival in experimental cerebral malaria. *PLoS ONE*, 6(11), e27714. https://doi.org/10.1371/journal.pone.0027714

Sobolewski, P., Gramaglia, I., Frangos, J. A., Intaglietta, M., & Van der Heyde, H. (2005). *Plasmodium berghei* resists killing by reactive oxygen species. *Infection and Immunity*, 73(10), 6704-6710. https://doi.org/10.1128/IAI.73.10.6704-6710.2005

Speyer, C. L., Neff, T. A., Warner, R. L., Guo, R-F., Sarma, J. V., Riedermann, N. C., Murphy, M. E., Murphy, H. S., & Ward, P. A. (2003). Regulatory effects of iNOS on acute lung inflammatory responses in mice. *The American Journal of Pathology*, 163(3), 2319-2328. https://doi.org/10.1016/S0002-9440(10)63588-2

Van Der Heyde, H. C., Gu, Y., Zhang, Q., Sun, G., & Grisham, M. B. (2000). Nitric oxide is neither necessary nor sufficient for resolution of *Plasmodium chabaudi* malaria in mice. *The Journal of Immunology*, 165(6), 3317-3323. https://doi.org/10.4049/jimmunol.165.6.3317

Van Der Heyde, H. C., Nolan, J., Combes, V., Gramaglia, I., & Grau, G. E. (2006). A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. *Trends Parasitology*, 22(11), 503-508. https://doi.org/10.1016/j.pt.2006.09.002

Weiss, M. L. & Kubat, K. (1983). *Plasmodium berghei*: a mouse model for the "sudden death" and "malarial lung" syndromes. *Experimental Parasitology*, 56(1), 143-151. https://doi.org/10.1016/0014-4894(83)90105-4

WHO. World Malaria Report (2016). Geneva: World Health Organization; 2016. http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf?ua=1

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., & Zweier, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proceedings of the National Academy of Sciences*, 93(13), 6770-6774. https://doi.org/10.1073/pnas.93.13.6770

Xia, Y. & Zweier, J. L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proceedings of the National Academy of Sciences*, 94(13), 6954-6958. https://doi.org/10.1073/pnas.94.13.6954

Zanini, G. M., Cabrales, P., Barkho, W., Frangos, J. A., & Carvalho, L. J. M. (2011). Exogenous nitric oxide decreases brain vascular inflammation, leakage and venular resistance during *Plasmodium berghei* ANKA infection in mice. *Journal of Neuroinflammation*, 8(66), 1-9. https://doi.org/10.1186/1742-2094-8-66

Zeidler, P. C, Millecchia, L. M., & Castranova, V. (2004). Role of inducible nitric oxide synthase-derived nitric oxide in lipopolysaccharide plus interferon-γ-induced pulmonary inflammation. *Toxicology and Applied Pharmacology*, 195(1), 45-54. https://doi.org/10.1016/j.taap.2003.10.005