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INHIBITION OF NITRIC OXIDE SYNTHESIS BY DEXAMETHASONE INCREASES SURVIVAL RATE IN *Plasmodium berghei*-INFECTED MICE

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Abstract

Malaria still presents great epidemiologic importance by its high incidence in the world and potential clinical severity. *Plasmodium* parasites are highly susceptible to changes in the redox balance and the relationship between the redox state of the parasite and host cells is very complex and involves nitric oxide (NO) synthesis. Thus, the present study is aimed at evaluating the effects of NO synthesis on the redox status, parasitemia evolution and survival rate of *Plasmodium berghei*-infected mice. Two-hundred and twenty-five mice were infected with *Plasmodium berghei* and submitted to the stimulation or inhibition of NO synthesis. The stimulation of NO synthesis was performed through the administration of L-arginine, while its inhibition was made by the administration of dexamethasone. Inducible NO synthase (iNOS) inhibition by dexamethasone promoted an increase in the survival rate of *P. berghei*-infected mice and data suggested the participation of oxidative stress in brain as a result of plasmodial infection, as well as the inhibition of brain NO synthesis, which promoted survival rate of almost 90% of the animals until the 15th day of infection, with possible direct interference of ischemia and reperfusion syndrome, as seen by increased levels of uric acid. Inhibition of iNOS caused a decrease of parasitemia and increased survival rate of infected animals, suggesting that the synthesis of NO may stimulate a series of compensatory redox effects that, if overstimulated, may be responsible for the onset of severe forms of malaria.

Key words: Nitric oxide, malaria, oxidative stress, dexamethasone, L-arginine, *Plasmodium berghei*, Inducible nitric oxide synthase, parasitemia, survival rate.

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INTRODUCTION

Malaria is an acute febrile infectious disease whose etiological agents are protozoa of genus *Plasmodium*. Five species are known to infect man: *Plasmodium vivax, P. falciparum, P. malariae, P. ovale* and *P. knowlesi.* Although there are evidences of the occurrence of the disease since 2700 B.C. (Cox, 2002), it is of epidemiological importance still today by its high incidence in the world and potential clinical severity, causing considerable social and economic losses in the population at risk, especially to ones in precarious conditions of dwelling and sanitation (WHO, 2011).

According to the World Health Organization (WHO) malaria is a significant public health problem in 108 countries and causes approximately 130 million new cases each year (WHO, 2014), resulting in 445 thousand deaths in 2016 (WHO, 2017). About 90% of these deaths occur in sub-Saharan Africa and it is estimated that the disease kills a child every 30 seconds (WHO, 2011).

Usually, the severe cases of the disease are related to the infection by *P. falciparum*. Among the complications that are worth mentioning are cerebral malaria and pulmonary complications (Botelho *et al.* 1996; Van der Heyde *et al.* 2000; Taylor *et al.* 2006; Penet *et al.* 2007). However, the mechanisms that trigger the pathogeny of malaria and the appearance of severe forms are yet not fully elucidated and additional studies are necessary.

In this regard, several authors recently discuss the involvement of free radicals in the physiopathogenesis of malaria (Huber *et al.* 2002; Dondorp *et al.* 2003; Pabon *et al.* 2003; Omodeo-Salé *et al.* 2003; Jaramillo *et al.* 2003; Becker *et al.* 2004; Yazar *et al.* 2004; Wilmanski *et al.* 2005; Kumar and Bandyopadhyay 2005; Dey *et al.* 2009; Percário *et al.* 2012; Vale *et al.* 2015). This involvement can be related to the pathogenic mechanisms triggered by the parasite (Potter *et al.* 2005), as well as by the production of free radicals (Keller *et al.* 2004), and antioxidant defenses (Sohail *et al.* 2007) by host cells as an attempt to fight the infection.

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During the development of blood stages of *P. falciparum*, trophozoites increase the viscosity of erythrocytes, by causing modifications on the cell surface that allow its adhesion to the endothelial wall of capillaries, which seems to be a mechanism of defense of the parasite, preventing the passage of parasitized erythrocytes by the spleen and its consequent destruction (Luse and Miller 1971). This cytoadherence phenomenon is mediated by expressed parasite proteins, via stimulation of gene *var*, on the surface of infected red cell, interrupting the blood flow and harming the tissues irrigated by the obstructed vessels (Ferreira *et al.* 2004; Pettersson *et al.* 2005), providing the conditions for the participation of ischemia and reperfusion syndrome (IRS), responsible for free radical production and, consequently, causing oxidative stress (Halliwell and Gutteridge 2015).

In fact, the level of oxidative stress is high in patients infected by *P. vivax*, as detected by the elevation of plasma levels of malondialdehyde - biochemical marker of lipid peroxidation (Farombi *et al.* 2003) - even in patients with non-severe forms of the disease (Pabon *et al.* 2003).

Additionally, oxidative changes in erythrocytes infected with *P. falciparum* seem to be associated to the accelerated aging of these cells and contribute to the development of the anemia displayed by these individuals (Omodeo-Salé *et al.* 2003). The development of anemia can promote changes in the circulatory physiology, leading to the existence of moments of alternate hypoxia and tissue oxygenation at basal levels, thus, another inductor factor of IRS.

Moreover, in response to the infection, activated macrophages and neutrophils act as the natural defense mechanism of the host organism and these generate a large amount of free radicals by activation of respiratory burst, causing an imbalance between the formation of oxidant species and the activity of antioxidants. This imbalance triggers the oxidative stress, being an important mechanism of human host in response to microbial infections that, in the case of malaria, can lead to the death of parasites.

In this regard, the levels of oxidative stress markers in parasitized mice and humans are increased in comparison to non-infected controls (Sohail *et al.* 2007).

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In these cases, oxidative stress seems to be the result of an increase in free radicals, and not a consequence of the decrease in the levels of antioxidants, reinforcing the suggestion that oxidative stress is an important mechanism induced by the infection (Pabon *et al.* 2003).

In fact, *Plasmodium* is highly susceptible to alterations in the redox balance, which can contribute to clinical manifestations of the severe cases of the disease, such as cerebral malaria (Narsaria *et al.* 2012). In parallel, the relationship between the redox state of the parasite and host cells is very complex and involves production of nitric oxide (NO; Becker *et al.* 2004; Gomes *et al.* 2015).

Nevertheless, the role of NO in malaria is still controversial. Some researchers say that cerebral malaria results from the production of high amounts of NO in order to promote the death of parasites (Favre *et al.* 1999; Maneerat *et al.* 2000), whereas others defend the suggestion that cerebral malaria arises from a low bioavailability of this gas (Gramaglia *et al.* 2006).

Perterson *et al.* (2007), demonstrated that NO synthesis derived from ingested blood in the digestive tract of the mosquito, induces the formation of toxic derivatives, limiting the development of the parasite. As to protect themselves from the damage induced by these toxic nitrogenated derivatives, the mosquito produces pyridoxines, antioxidant enzymes capable of synthetizing NO in response to parasitemia (Herrera-Ortiz *et al.* 2004).

Nahrevania & Dascombe (2006) identified the increase of NO synthesis in *P. berghei*-infected mice and verified its correlation with the increase of the activity of immunologic cells (lymphocytes CD19, macrophages and monocytes).

In fact, some researchers suggest a protective role of nitric oxide in the development of severe malaria and indicate it as possible adjuvant in malaria drug therapy (Yeo *et al.* 2007, 2008; Dhangadamajhi *et al.* 2009). As suggested by Planche *et al.* (2010), the activation of NOS II is essential for the additional production of NO and elimination of the parasite.

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131 On the other hand, Cabrales *et al.* (2011) associated the development of
132 cerebral manifestations of the inadequacy of NO production, which seems to be
133 essential in maintaining cerebral circulatory hemodynamics.

134 In mice deficient of interleukin 4 (IL-4), it was found that the increase of
135 iNOS expression and the activity of natural killer cells producing IFN-γ, resulted in
136 protection of animals even in the initial phase of infection by *P. berghei* (Saeftel *et*
137 *al.* 2004).

138 Moreover, some parasitic molecules are well known as NO inducers, such
139 as the malarial pigment hemozoin, which associated to IFN-γ, is a potent NO
140 inducer in macrophages, involving the kinase regulated extracellular signaling
141 (ERK) pathway and nuclear factor κappa B (NF-κB). It is also known that in the
142 hepatic stage, the defense mechanisms are strictly related to the production of
143 IFN-γ by NK cells, with posterior synthesis of NO (Saeftel *et al.* 2004). In addition, it
144 was found that hemozoin is also responsible for the activation of macrophages by
145 mechanisms partially dependent on NO (Jaramillo *et al.* 2005) and other ROS,
146 such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2: Brinkmann *et al.* 1984).

147 Similarly, increased levels of iNOS in human monocytes are associated with
148 non-worsening malaria in patients infected by *P. falciparum* (Chiwakata *et al.*
149 2000).

150 Syarifah *et al.* (2003), studying *P. bergh*ei-infected mice susceptible and
151 resistant to the development of cerebral malaria, observed that cytokine expression
152 was increased in resistant animals in relation to susceptible, as well as the
153 expression of NO, worth mentioning the high production of TNF-α in resistant mice,
154 suggesting that the activation of macrophages is significantly greater in those
155 animals.

156 Three isoforms of nitric oxide synthase enzymes (NOS) were described so
157 far, with two constitutive forms and one inducible form. Constitutive forms produce
158 low amounts of NO for a long period, apparently being responsible for the
159 physiological production of NO. The inducible form (iNOS or NOS II) is activated by

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160 factors, such as bacterial lypopolysaccharide (LPS) and cytokines (TNF-α e IFN-γ),
161 producing large amounts of NO in a short space of time (Försterman and Sessa
162 2012).

163 All NOS enzymes use L-arginine as substrate, as well as molecular oxygen
164 ($O_{2)}$ and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-
165 substrates, and flavine-adenine dinucleotide (FAD), flavine-mononucleotide (FMN)
166 and (6R-)5,6,7,8-tetrahydro-L-biopterine (BH4) as cofactors (Försterman and
167 Sessa 2012). The administration of L-arginine has been employed to stimulate the
168 activity of iNOS in several studies, yet with controversial results (Percário *et al.*
169 2012).

170 On the other hand, NOS enzymes can be selectively inhibited. Among the
171 most used inhibitors, N-nitro-L-arginine methyl ester (L-NAME) and N-monomethyl-
172 L-arginine (L-NMMA) inhibit both forms of the enzyme, while aminoguanidine and
173 dexamethasone selectively inhibit iNOS (Walker *et al.* 1997).

174 Dexamethasone is a glucocorticoid drug and acts on nuclear receptors
175 directly interfering in gene expression in a variety of cell types (Katzung and Trevor
176 2017) and modulating the transcription of genes involved in the control of
177 inflammatory process (Barnes *et al.* 1993). Since the beginning of the 1990s, some
178 authors identified the effect of dexamethasone inhibiting iNOS expression in most
179 diverse cell types: mesangial cells (Pfeilschifter and Schwarzenbach 1990), murine
180 macrophages (Di Rosa *et al.* 1990), human endothelial cells (Radomski *et al.*
181 1990), rat hepatocytes (Geller *et al.* 1994), murine fibroblasts (Gilbert and
182 Herschman 1993), and human epithelial cells (Kleinert *et al.* 2004). De Vera *et al.*
183 (1997) attributes this action of dexamethasone by the inhibition of NFκB and to the
184 activation of its inhibitory factor (IFκB). Regardless of the route used by
185 dexamethasone, there is no doubt that NOS inhibition is independent of L-arginine
186 concentration and greatly affects the expression of mRNA for the inducible enzyme
187 (Korhonen *et al.* 2002; Skimming *et al.* 2003).

188 Administering dexamethasone to *P. berghei* infected mice significantly
189 reduces symptoms of cerebral malaria (Neill and Hunt 1995; Sanni *et al.* 1998)

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In the present study it was demonstrated that the selective inhibition of iNOS by dexamethasone reduced the progression of parasitemia in *P. berghei*-infected mice and increased the survival rate of the animals.

METHODS

Two-hundred and twenty-five male Swiss mice (*Mus musculus*), young adults (25-35 g), from the Evandro Chagas Institute (Belem, PA, Brazil) were randomly divided into three groups, each of them further divided into five sub-groups (n=15 each), according to time of animals' euthanasia (one, five, ten, fifteen or twenty days after inoculation), and samples of lung tissue and blood were collected for the evaluation of oxidative stress markers, total antioxidant status, uric acid and assessment of percentage of parasitemia, as follows:

Positive control groups (N=15 for each sub-group): animals were inoculated with *P. berghei*-infected erythrocytes and received 10 µl of sterile distilled water per 25 g of body weight (gavage) two hours prior to the inoculation of *P. berghei* and daily, until the day of animals' euthanasia.

Dexamethasone groups (N=15 for each sub-group): animals were inoculated with *P. berghei* in the same way that groups PC and treated with dexamethasone, as described below, until the day of animals' euthanasia.

L-Arginine groups (N=15 for each sub-group): animals were inoculated with *P. berghei* in the same way that groups PC and simultaneously treated with L-arginine, as described below, until the day of animals' euthanasia.

All animals were assigned into sub-groups by simple randomization using the sub-group sequence generated after sortition (Suresh 2011) and were maintained in the vivarium at the Federal University of Pará (UFPA, Belém, PA, Brazil) in

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polystyrene cages containing five animals each, kept under 12 h light/dark cycles, controlled temperature (25°C), and received rodent chow (Labina™, Presence, Brazil), and tap water *ad libitum* for one, five, ten, fifteen or twenty days after infection and, at the end of each period, animals were submitted to heparin administration (100 UI heparin sulfate, ip.), anesthetized with 50 µl of intraperitoneal ketamine (5%)-xylazine (2%), sample collection, and underwent euthanasia by exsanguination. Absolutely all efforts were made to minimize suffering to animals.

After thoracotomy, blood samples were obtained by cardiac puncture of the right ventricle and both lungs and brain were removed. The project followed the international guidelines for research with experimental animals and procedures were reviewed and approved by the Ethics Committee in Research with Experimental Animals of the Federal University of Pará - CEPAE/UFPA (Report No. MED0126/2013).

Features of the animal model

Swiss mice are widely used as a malaria model and presents the same pattern of infection progression and basic features of lung and cerebral malarias of other mice species. Moreover, *P. berghei* possesses genomic sequences similar to *P. falciparum* (Otto *et al.* 2014) and cause clinical features on animals that mimic human falciparum malaria (Penet *et al.* 2007). Taken together, the histopathological features described are similar to those displayed in severe malaria human cases.

Malaria induction

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Mice were kept in the vivarium for two weeks and underwent clinical examination prior malaria induction through intraperitoneal inoculation of 10^6 *P. berghei* ANKA-infected erythrocytes (in 0.2 mL sterile saline solution). The strain of *P. berghei* was supplied by the Neurochemistry Laboratory of the Federal University of Pará - UFPA and three times replicated in Swiss mice before being used in animals of this study.

Treatments

Dexamethasone (Teuto, Cat # 095214): administered in the dose of 5mg/Kg of animal weight.

L-arginine (Sigma Aldrich, Cat # A5006): prepared in 0.9% PBS, and administered in a dose of 120 mg/Kg of animal weight (Chatterjee *et al.* 2007).

Both dexamethasone and L-arginine were administered 24h prior infection and every 24h henceforth, until the day of animal euthanasia.

Tissue processing

After removal, lungs and brain were perfused with PBS to wash out the blood trapped inside. The tissue was weighed and added to PBS in the ratio of 1:10 (m:v). The homogenization process was performed in an ultrasonic cell disruptor (D Cel; Thornton, Indaiatuba, Brazil). During the process, the glass beaker containing the material was kept on ice to prevent sample damage. The homogenate was centrifuged at 175 x *g* (15 min) and the supernatant collected and stored in a freezer at -20°C until analyzed.

Technical Procedure

Along with blood parasitemia determination, laboratory measurements of trolox equivalent antioxidant capacity (TEAC), thiobarbituric acid reactive substances

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(TBARS), Uric Acid (AU), and nitrites and nitrates (NN) were performed in duplicate on tissue samples. Internal controls and standards were inserted in each batch for the quality assurance of determinations.

Determination of parasitemia

Plasmodium berghei-infected erythrocytes were counted on blood smears obtained by puncture of the caudal vein of animals on the day of euthanasia (one, five, ten, fifteen, and twenty days of infection). After drying at room temperature, the smear was fixed with methanol for 2 min and stained with Giemsa for 10 min. Subsequently, slides were washed in tap water and, after drying, erythrocytes were counted on an optical microscope (Olympus, CX2) with 100x magnification.

Determination of Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Sigma-Aldrich 23881-3) is a powerful antioxidant water-soluble vitamin E analogue. The method proposed by Miller *et al.* (1993) modified by Re *et al.* (1999) was followed, a colorimetric technique based on the reaction between ABTS (2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; Sigma-Aldrich; 1888) with ammonium persulfate potassium ($K_2S_2O_8$; Sigma-Aldrich; 60490), producing the radical cation $ABTS^{\bullet}+$, chromophore of green/blue color. The addition of antioxidants to $ABTS^{\bullet}+$ reduces it again to ABTS, on a scale dependent on antioxidant capacity, concentration of antioxidants and duration of the reaction. This can be measured by spectrophotometry by observing the change in absorbance read at 734nm for five minutes (Fento, Sao Paulo, Brazil; 800 XI). Finally, the total antioxidant activity of the sample is calculated as its relationship with the reactivity of the Trolox as

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284 standard, through the implementation of standard curve under the same
285 conditions.

286 **Determination of Thiobarbituric Acid Reactive Substances (TBARS)**

287 TBARS is a method that evaluates lipid peroxidation and was used as an indicator
288 of oxidative stress. This technique is based on the reaction of malondialdehyde
289 (MDA), among other substances, with thiobarbituric acid (TBA; Sigma-Aldrich
290 T5500), in low pH and high temperature, yielding MDA-TBA complex of pink color,
291 and absorbance peak at 535 nm.

292 The technical procedure was performed according to the protocol proposed by
293 Khon and Liversedge (1944), adapted by Percario *et al.* (1994). In brief: initial TBA
294 solution (10 nM) was prepared in phosphate monobasic potassium (KH_2PO_4 75
295 mM; Synth; 35210) adjusted to pH 2.5 with acetic acid. Two hundred and fifty μL
296 of sample was added to 500 μL of TBA solution, mixed and placed in a water bath
297 (95°C x 60 min); after cooling at room temperature, 2.0 ml of 1-butanol was added,
298 vortex mixed and subsequently centrifuged (175 x *g* x 15 min); 1.0 ml of the
299 supernatant was collected and read at 535 nm (Fento, São Paulo, Brazil; 800 XI).
300 1,1,3,3, tetraethoxypropane (Sigma-Aldrich; T9889) was used for the
301 implementation of the standard curve.

302 **Nitrites and nitrates (NN)**

303 Much of nitric oxide released into the bloodstream is swept by hemoglobin in
304 erythrocytes or converted to nitrite ($NO_2^{\bullet-}$) in the presence of molecular oxygen.
305 Nitrite reacts with oxyhemoglobin, leading to the formation of nitrate ($NO_3^{\bullet-}$) and
306 methemoglobin. Due to its stability, $NO_2^{\bullet-}$ has been widely used to confirm the prior

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existence of NO. 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). Nitrite concentration was calculated based on the absorbance found in the nitrites standard curve.

Uric acid

Performed using the Kit Uric acid UOD-ANA (Labtest, Cat. 51-4/30).

The test is based on the production of H_2O_2 from the reaction of uric acid with oxygen and water, catalyzed by uricase. This H_2O_2 reacts with acid 3,5-dichloro-3-hydroxybenzene sulphonate (DHBS) and 4-aminoantipyrine in the presence of peroxidase, producing dye antipirylquinonimine. Samples were read at a spectrophotometer at 520nm (Biospectro, SP-22, Brazil).

Statistical Analysis

Sample size was calculated by the method proposed by Dell *et al.* (2002). The occurrence of discrepant values (*outliers*) was investigated through calculation of interquartile range, which calculates the difference between the third quartile (Q3) and the first quartile (Q1), called dj. Any value lower than Q1 - 3/2 dj or greater than Q3 + 3/2 dj, was considered as *outlier* and, therefore, removed from mathematical calculations.

Aiming at investigating the existence of statistically significant differences between the studied variables between Groups, we applied ANOVA two factors, when the assumption of normality and homoscedasticity was met, or the Mann-Whitney test,

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when the assumption of normality was not met, which occurred in the case of variable PARASITEMIA. The tests used to access the normality and homoscedasticity of the variables were Kolmogorov-Smirnov and Levene tests, respectively. When the null hypothesis between mean differences between the variables of the study groups was rejected, Tukey's test was applied, and when a statistically significant difference between medians was detected, Dunn's test was applied. In addition, within the same group the differences between the initial values (1 day of infection) and late values (20 days of infection) were studied by the Student's unpaired t test.

The existence of correlation between the variables was also analyzed by Pearson's correlation coefficient, considering all points obtained separately for each group studied. For the statistically significant correlations, intensities were assigned as follows: r up to 0.30 ($r < 0.30$) as weak correlation; r between 0.31 and 0.70 ($0.31 < r < 0.70$), as moderate correlation; r between 0.71 and 1.00 ($0.71 < r < 1.00$) as strong correlation.

For the purposes of tests ANOVA and Mann-Whitney, statistical package SigmaStat version 3.5 was used, whereas for the calculation of correlations the statistical package SPSS version 17.0 was used. All statistical tests were applied considering the significance level of 5% ($p<0.05$).

Availability of Data and Materials

Data and full description of methods and materials are available at Zenodo repository, at https://zenodo.org/record/45202#.VqeqifLVyid.

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RESULTS

As expected, parasitemia of infected animals progressively evolved in all groups, but the rate of progression was lower in dexamethasone-treated animals, which presented lower values than the other two groups at the end of the period of 20 days ($p=2.8x10^{-5}$ *vs.* L-arginine and $p=0.0227$ *vs.* control; Fig. 1). L-arginine-treated animals presented numerically higher values than the control group, but without statistical significance ($p=0.3048$).

Similarly, the survival rate of dexamethasone-treated animals was significantly greater than that of the other groups, which behaved in a similar way, with 60% of animals alive at the end of the period of 20 days of infection (Fig. 2).

TEAC:

For the lung samples, all groups showed a slight decrease of TEAC values along the period of infection, however without statistically significant differences (Fig. 3A). Nevertheless, at the end of 20 days of infection, the group of animals treated with dexamethasone presented statistically lower values than the other two groups ($p=0.0281$ *vs.* L-ARGININE and $p=0.0033$ *vs.* CONTROL). For brain samples a similar behavior was observed, however an important decrease of TEAC after 10 days of infection was identified (1 day *vs.* 10 days, $p=0.0009$ for L-ARGININE and $p=7x10^{-6}$ for DEXAMETHASONE), with both treated groups presenting values lower than the control group ($p=0.0360$ *vs.* L-ARGININE and $p=0.0261$ *vs.* DEXAMETHASONE). However, after the 10^{th} day of infection the DEXAMETHASONE group presented an increase in TEAC values, displaying statistically higher values than the other groups ($p=0.0357$ *vs.* L-ARGININE and $p=0.0005$ *vs.* CONTROL).

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TBARS:

Although none of the groups have presented important variation during the period of infection, Group L-ARGININE presented higher pulmonary TBARS values than the DEXAMETHASONE group at the end of the experiment (p=0.0282). On the other hand, for brain samples, Group L-ARGININE showed progressive evolution over the period of the infection, with higher TBARS values in the 20th day of infection in relation to the first day ($p=4.7x\ 10^{-7}$), but with no differences in relation to the other groups (Fig. 4).

The collective analysis of the values of TEAC and TBARS shows a quite unique pattern: while for lung samples the values of TEAC obtained are found in a high range of absolute values (9-12 µM), brain samples are in a low range (4-8µM), whereas TBARS values presenting opposing behavior, i.e. for lung samples the values are in low range (80-120nmol/mL) and brain samples in high range (160-280nmol/mL).

Nitrites and nitrates:

No significant differences in the evolution of NN levels in any of the groups throughout the period of infection were seen, nor between groups for the lung samples (Fig. 5). However, for brain samples, group DEXAMETHASONE presented lower values than the other two groups during the studied period, culminating with statistically significant differences in the 20th day (p=0.0058 *vs.* L-ARGININE and p=0.0201 *vs.* CONTROL).

Uric acid (AU):

No temporal variation in AU values for lung samples in any of the groups were found (Fig. 6). However, Group L-ARGININE presented lower values than the other

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two groups from the first to the 15th day of infection. Similarly, for brain samples, group L-ARGININE presented lower values than the other groups, with statistical significance at the 20th day of infection (p=0.0395 *vs.* CONTROL and p=0.0407 *vs.* DEXAMETHASONE). In contrast, group DEXAMETHASONE presented progressive behavior over the infection time, with brain AU values significantly greater for the 20th day in comparison to the first day (p=3.9x10^{-4}). Another noteworthy observation is that pulmonary AU values stood in a higher range (40-140mg/dL) than the brain (10-55mg/dL) for all groups.

Correlation studies:

PARASITEMIA vs. TBARS

The correlation between TBARS and PARASITEMIA revealed the existence of a negative and significant correlation only for the lung samples from the group DEXAMETHASONE (Additional file 1 Fig. 1; r=-0.29; p=0.026). The CONTROL group presented a negative correlation, however without statistical significance (r=-0.10; p=0.20), while for group L-ARGININE this correlation showed positive but non-significant values (r=0.06; p=0.673). For brain samples a positive trend was observed for all groups, but only with significance for group L-ARGININE (Additional file 1 Fig. 2; r=0.46; p=0.002).

TBARS vs. URIC ACID

A positive correlation was observed for these parameters in both samples and for groups CONTROL (Additional file 1 Fig. 3-4; r=0.33 and p=0.02, for lung; r=0.45 and p=0.050, for brain), and DEXAMETHASONE (r=0.26 and p=0.041, for lung; r=0.28 and p=0.045, for brain). For group L-ARGININE, in both samples, the

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values of the coefficient of correlation approached zero (r=0.08 and p=0.140, for lung; r=0.03 and p=0.858, for brain).

NN vs. TBARS

For lung samples the existence of significant correlation for any of the studied groups was not observed (Additional file 1 Fig. 5). However, for brain samples, both groups CONTROL and DEXAMETHASONE presented significant positive correlations (Additional file 1 Fig. 6; r=0.30 and p=0.048 and r=0.34 and p=0.014, respectively).

TEAC vs. TBARS

The existence of positive correlation in both samples and for both groups CONTROL (Additional file1 Fig. 7-8; r=0.32 and p=0.024, for lung; r=0.50 and p=0.009, for brain) and DEXAMETHASONE, (r=0.23 and p=0.031, for lung; r=0.27 and p=0.050, for brain) was seen. For the group L-ARGININE, in both samples, the values of the coefficient of correlation were negligible (r=0.05 and p=0.821, for lung; r=0.14 and p=0.374, for brain).

NN vs. PARASITEMIA

No significant correlation was found for any of the groups, nor for any of the samples studied (Additional file1 Fig. 9-10).

Other Correlations

In addition to the studies of correlation presented, we tested the following correlations: TEAC *vs.* NN (Additional file 1 Fig. 11-12); TEAC *vs.* URIC ACID (Additional file 1 Fig. 13-14), TEAC *vs.* PARASITEMIA (Additional file 1 Fig. 15-16), NN *vs.* URIC ACID (Additional file 1 Fig. 17-18), and URIC ACID *vs.* PARASITEMIA (Additional file 1 Fig. 19-20).

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DISCUSSION

Malaria is a disease of high incidence worldwide and infection by *P. falciparum* are responsible for severe manifestations of the disease and the majority of the cases of deaths related to this disease. Cerebral malaria and pulmonary complications are among the noteworthy complications of malaria and are similar to the clinical manifestations associated with *P. berghei* infection on the animal model employed in the present study (Botelho *et al.* 1996; van der Heyde *et al.* 2000; Taylor *et al.* 2006; Penet *et al.* 2007). The mechanisms that trigger the pathogeny of malaria and the appearance of severe forms are not fully elucidated yet. In this context, oxidative stress, through NO synthesis, seems to play a dubious but important role. Nevertheless, survival rate results pointed to a significant increase in the percentage of survival for the groups of mice treated with the iNOS inhibitor dexamethasone in comparison to other groups (Fig. 2), which seems to be correlated with the evolution of parasitemia in these animals, which has changed very little in this group and remained, from the 5th day henceforth significantly lower than the other groups (Fig. 1).

It is important to highlight that dexamethasone is a non-steroid anti-inflammatory drug that acts through iNOS mRNA synthesis inhibition (Korhonen *et al.* 2002). In this sense, it is possible that NO acts oxidatively, both inducing the worsening of the disease, as favoring the increasing of parasitemia.

Therefore, the effect of dexamethasone on the evolution of the parasitemia can promote inhibition of oxidative stress, as may be suggested by the existence of a negative correlation between TBARS and PARASITEMIA found only for the animals of group dexamethasone (Additional file Fig. 1). In the same way, the

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474 effect of L-arginine is consistent with the effect found in this correlation for the
475 animals of Group L-ARGININE, where there is the reversal of this pattern,
476 presenting positive values of correlation.
477 Contrary to that mentioned by some authors (Yeo *et al.* 2007, 2008;
478 Dhangadamajhi *et al.* 2009; Planche *et al.* 2010; Cabrales *et al.* 2011) that attribute
479 a protective role to nitric oxide in malaria, mice treated with L-arginine remained
480 with percentage of parasitemia and survival rate comparable to group CONTROL
481 (Fig.1-2), suggesting that NO synthesis is not involved among the initial
482 mechanisms of host defense and, therefore, may not contribute to the elimination
483 of parasites. However, the mentioned studies have measured the survival rate of
484 animals treated with Dipropylene triamine NONOate, a natural donor of nitric oxide,
485 active in acid PH (common in malaria) whose action, unlike L-arginine, is
486 independent of enzyme activation.
487 **Pulmonary findings**
488 The mechanisms responsible for triggering the syndrome of respiratory anxiety
489 displayed in malaria patients are multifactorial. However, according to some
490 authors there are no doubts about the participation of free radicals (Gachot *et al.*
491 1995; Taylor *et al.* 2006; Gillrie *et al.* 2007), which directly affect the cell
492 membranes, attacking the endothelium and changing vascular permeability.
493 Among the free radicals involved in this process, NO seems to play an important
494 role. However, the paradox of the actuation of this molecule in pulmonary
495 complications is evident: while some authors suggest that the inhalation of this gas
496 is a potential treatment of these complications (Rabkin *et al.* 2001; Schreiber *et al.*
497 2003; McClintock *et al.* 2007; ter Horst *et al.* 2007), others blame nitric oxide

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498 synthesis as responsible for causing the respiratory distress syndrome (Adhikari *et*
499 *al.* 2007), in particular as a result of the activation of iNOS (Mikawa *et al.* 2003;
500 Baron *et al.* 2004).
501 Additionally, superoxide radical, stimulated by substances derived from the
502 inflammatory process, such as TNF-α, IL-1α and lipopolysaccharide (through the
503 increase on NADPH-oxidase activity), are also present in the syndrome, which also
504 seems to be related with the increase of nitric oxide synthesis (Muzaffar *et al.*
505 2003). However, the reaction between these two free radicals (nitric oxide and
506 superoxide) is known to produce a potent third free radical, peroxynitrite (Katzung
507 and Trevor 2017). Thus, nitric oxide seems to play a fundamental role, once that
508 iNOS activation is associated with the induction of NADPH-oxidase and the
509 production of peroxynitrite (Muzaffar *et al.* 2003; Adhikari *et al.* 2007).
510 As a defense mechanism against the cellular damage caused by oxidative stress,
511 there is an increase in the production of antioxidant molecules both from alveolar
512 surfactant production (which is notoriously hyper secreted), as well as by the
513 increase in antioxidant enzyme activity (Rahman and MacNee 2000; Oury *et al.*
514 2002; Christofidou-Solomidou *et al.* 2003; Kinsella *et al.* 2005; Saxena *et al.* 2005;
515 Bein *et al.* 2009).
516 In the present study there was no significant variation in the dosages of TEAC and
517 TBARS levels in the control group along the infection period (Fig. 3-4). However, it
518 was observed the occurrence of a positive correlation between these parameters
519 for both samples tested (Additional file Fig. 7-8), suggesting that the increase in
520 oxidative stress resulting from the infection induced the increase of antioxidant
521 defenses, but could not be reversed by it.

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Additionally, the behavior of the TBARS *vs.* TEAC correlations and TBARS *vs.* Uric acid was similar (Additional file Fig. 3-4), suggesting that uric acid is an important component of the antioxidant defense of these animals, or IRS is associated with the infection. In this sense, the absence of correlation between these parameters found in Group L-ARGININE for both samples is further evidence of the absence of the IRS in animals in this group, probably as a result of vasodynamic effects attributable to NO.

Among the treatments, the only one that showed significant correlation between TBARS and PARASITEMIA, was dexamethasone (r=-0.29, p=0.026; Additional file Fig. 1) and can suggest that the selective inhibition of iNOS, associated to the anti-inflammatory potential of dexamethasone, decrease the lipid peroxidation even with the increase of parasitemia. This suggestion is reinforced by the finding of negative correlations between TEAC *vs.* PARASITEMIA and URIC ACID *vs.* PARASITEMIA (Additional file Fig.15 and 19), since enzymatic antioxidant defenses and IRS suffer direct influence of lipid peroxidation.

In this experimental model, considering that all animals were exposed to the same food supply, high values of uric acid indicate the existence of ischemia and reperfusion syndrome (Halliwell and Gutteridge 2015), and may be caused by the decrease of the caliber of blood vessels, by anemia, or by obstruction of the blood flow by the occurrence of cytoadherence.

It was found that a significant positive correlation for URIC ACID and TBARS levels in both samples and for both groups CONTROL and DEXAMETHASONE, suggesting that IRS arises from the increased oxidative stress in these animals as a consequence of disease progression, as well as that NO synthesis may not exert

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546 important effect in this case. On the other hand, for group L-ARGININE, in both
547 samples, the values of the coefficient of correlation approached zero, suggesting
548 adequate blood supply to these tissues, possibly as a result of NO-attributable
549 vasodilation.

550 The treatment with L-arginine did not promote any modification in the antioxidant
551 capacity during the period studied (Fig. 3). On the other hand, it has significantly
552 increased lipid peroxidation, but only in the first day of infection (Fig. 4A). The
553 decrease in lipid peroxidation in subsequent days can be explained by the
554 decrease in the IRS, justified by the low levels of uric acid for animals of this group
555 during the entire period of infection (Fig. 6A).

556 The high correlations (moderate to strong) for TEAC *vs.* URIC ACID in all groups
557 (Additional file Fig. 13) arises from the simple fact that uric acid, by itself, is an
558 antioxidant, in addition of being a marker of IRS. The same is true for the positive
559 correlations between TEAC *vs.* NN (Additional file Fig.11) and NN *vs.* URIC ACID
560 (Additional file Fig. 17), displayed by most of the groups.

561 Among the most unusual results, it is noteworthy the absence of differences in the
562 levels of pulmonary nitrites and nitrates, independent of the use of inhibitor
563 (dexamethasone) or stimulator of their synthesis (L-arginine; Fig. 5A). The possible
564 explanations for such phenomena arising out of compensatory physiological
565 effects, such as vasoconstriction caused by the NOS inhibition, which seems to
566 stimulate the production of mediators that cause vasodilation such as acetylcholine
567 and bradykinin, which are bronchoconstrictors nonetheless (Silverthorn 2010).
568 Conversely, it is possible that pulmonary hypertension on malaria, reported by
569 Lacerda *et al.* (2009), as caused by the inhibition of NO by treatment with

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570 dexamethasone, along with the need of oxygen as a result of hemolysis, stimulates
571 the synthesis of eNOS, which increases the expression of eNOS receptors in the
572 lungs (Beleslin-Čoki *et al.* 2011).
573 The opposite effect happened for group L-ARGININE, in which it was expected an
574 increase in nitrites and nitrates, but despite the lack of statistical significance, stood
575 numerically below of the other two groups. It is worth mentioning that after formed,
576 L-arginine can follow two paths: the formation of ornithine and urea (action of
577 arginase) or the formation of citrulline and NO (action of NOS). Additionally,
578 interleukins (IL) 13 and 14 act over arginase directing L-arginine to the synthesis of
579 ornithine that is converted, by the action of an aminotransferase, to proline. This
580 route has fibrogenic role, since proline is an essential amino acid in collagen (Lee
581 *et al.* 2001). On the other hand, cytokines interferon γ (IFN-γ), tumor necrosis
582 factor-α (TNF-α) and IL-12, optimize the formation of NO and citrulline from the
583 action of iNOS over L-arginine (Hesse *et al.* 2000). Thus, it is likely that the excess
584 of L-arginine, depending on the profile of cellular response stimulated, follow the
585 arginase route, promoting the clearance of pulmonary nitrites and nitrates (Modolell
586 *et al.* 1995; Chiaramonte *et al.* 1999; Hesse *et al.* 2000; Lee *et al.* 2001), resulting
587 in fibrinogen synthesis, in an attempt to revert pulmonary damage caused by
588 oxidative stress.
589 Another possibility is that the vasodilation produced by NO excess increase
590 availability of O_2, substrate of NADPH oxidase, resulting in greater production of
591 superoxide radical and, consequently, of peroxynitrite (Muzaffar *et al.* 2003;
592 Adhikari *et al.* 2007). According to Wedgwood *et al.* (2012), peroxynitrite levels

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impose a negative feed-back on NOS, i.e., the more peroxynitrite is synthesized, greater inhibition of NOS.

Additionally, the absence of differences between the groups for the values of pulmonary NN may be the result of the existence of a complex system of non-adrenergic non-cholinergic (NANC) neural fibers in the lungs of mammals, capable of producing large quantities of NO (Gaston *et al.* 1994) and, therefore, to masque NO levels arising from malaria in this tissue. This suggestion is reinforced by the absence of correlation between NN and TBARS levels in all groups for lung samples (Additional file Fig. 5). In contrast, for brain samples, both groups CONTROL and DEXAMETHASONE showed significant positive correlations, while group L-ARGININE showed no correlation between these parameters (Additional file Fig. 6). These data suggest that, at least partially, oxidative stress associated with the development of the disease is derived from the production of NO, as pointed out by several authors, in addition to the participation of IRS (Percário *et al.* 2012), which may have been reversed in the animals treated with L-arginine, due to its vasodilator effect.

Cerebral findings

Similar to the pulmonary features of the disease, cerebral edema seems to determine the pathological onset of severe malaria. However, the increase of intracranial pressure due to cerebral edema results in greater risk of death. This abnormality is originated from a set of factors that, despite the apparently derangement, act in order to eliminate infection even without the passage of the microorganism to the cerebral tissue.

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In this context, NO acts as a key molecule in brain infections. However, it is still unknown if the major problem arises from insufficient concentrations of NO acting directly in the elimination of the parasite, and for this reason, by selecting more resistant strains of the parasite (Gramaglia *et al.* 2006), or if the high concentrations of NO, produced as a result of infection by the protozoan parasite, are responsible for the cerebral edema (Favre *et al.* 1999; Maneerat *et al.* 2000).

In the evaluation of brain oxidative parameters, it was noted an increase in lipid peroxidation for mice treated with dexamethasone in relation to the other groups, mainly in first day post-infection. Nevertheless, the opposite happens with the group of mice treated with L-arginine, where TBARS levels are significantly lower than the other groups (Fig. 4B).

The elevation of TBARS levels for the group treated with dexamethasone may result from a technical artifact, as brain tissue is rich in cholesterol and the drug may form cholesterol hydroperoxides, which may react with thiobarbituric acid, greatly increasing the absorbance of brain samples (Lima and Abdalla 2001). A finding that may corroborate this statement are the dosages of TEAC that do not change in the first days of study for all groups (Fig. 3B). The possibility that lipid peroxidation occurs in this initial period by an increase in IRS was eliminated since uric acid values for this group of animals are similar to those of the other groups until the tenth day of infection (Fig. 6B).

Notwithstanding, it seems that the oxidative effect of nitric oxide was overcome by its vasodilator effect, since the production of uric acid in mice treated with L-arginine was significantly lower when compared to other groups (Fig. 6B), notably from the 10^{th} day of infection. However, the probable vasodilation presented by

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group L-ARGININE caused no changes on the survival rate of these animals (Fig. 2).

The re-establishment of antioxidant capacity can be decisive for the survival of mice infected with *P. berghei*. The antioxidant capacity decreases significantly in all tested groups. However, only for group DEXAMETHASONE this antioxidant capacity is significantly reversed from the 10th day (Fig. 3B), reinforcing the idea of Favre *et al.* (1999) and Maneerat *et al.* (2000) that the oxidative stress induced by nitric oxide in cerebral microenvironment contributes to the severity of the disease.

Another point that deserves to be highlighted for the group treated with dexamethasone is that, despite the inhibition of iNOS, there was only significant increase in serum uric acid concentration from the 15th day (Fig. 6B), signaling that the beginning of IRS coincides with the starting point of deaths in this group. The finding of positive correlation between TBARS and NN for group DEXAMETHASONE corroborates this observation (Additional file Fig. 6).

A factor that may have contributed significantly to the late start of the IRS in this group is the inhibition of the inflammatory process, which is necessary for the occurrence of cytoadherence (Ferreira *et al.* 2004; Pettersson *et al.* 2005). Additionally, this is the only group that displays significant positive correlation between URIC ACID and PARASITEMIA (Additional file Fig. 20), reinforcing the idea that IRS occurs on a temporal scale.

The absence of correlation between NN and PARASITEMIA for all groups and both samples strongly suggests that NO levels do not influence the evolution of parasitemia (Additional file Fig. 9-10).

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Considering the different treatments administered, the more promising results were seen with the dexamethasone treatment, since animals exhibited significantly higher survival rate and decreased progression of parasitemia when compared to the other groups. These data suggest that selective inhibition of iNOS, associated to the anti-inflammatory potential of dexamethasone, might decreased lipid peroxidation even with the increase of parasitemia.

In contrast, administration of L-arginine, regardless not significant modification in NN concentrations, promoted vasodilation in both organs, proven by an increase in the concentrations of uric acid, with no effect over the survival rate of these animals.

Nevertheless, the cerebral oxidative changes promoted by the administration of dexamethasone were somehow different from the ones presented by other groups. The re-establishment of the cerebral antioxidant capacity after the 10^{th} day of infection is noteworthy, suggesting the participation of oxidative stress in brain as a result of plasmodial infection, as well as the inhibition of brain NO synthesis, which promoted survival rate of almost 90% of the animals until the 15^{th} day of infection, with possible direct interference of ischemia and reperfusion syndrome, as seen by increased levels of uric acid.

CONCLUSION

Lately, the role of NO in the physiopathogenesis of malaria has been extensively studied. Nevertheless, its precise involvement in the underlying mechanisms of the disease is still controversial. The present study presents the inhibitory effects of dexamethasone on brain nitric oxide synthesis and its relationship to increased

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686 survival in the mice model of malaria. To our best knowledge, it is the first time
687 such results are reported in the scientific literature.
688 Data of the present study showed that iNOS inhibition by dexamethasone
689 promoted an increase in the survival rate of *P. berghei* -infected animals until the
690 point at which it compromised the functioning of the cerebral microcirculation.
691 Indeed, iNOS inhibition by dexamethasone seems to have stimulated a series of
692 redox effects that, if compensatory hyper stimulated, may be responsible for the
693 worsening of the pulmonary symptoms.
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COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOS' CONTRIBUTION

SP, MDG and MFD were responsible for the design of the study, data analysis, and for the critical revision of the text. DRM, ARQG, ACMGU, MESF, RSS and JRSV were responsible for the collection of data, statistical study and drafting of the manuscript.

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FIGURE LEGENDS

Figure 1 – Progression of parasitemia in *Plasmodium berghei*-infected Swiss mice. Animals were pre-treated and received a daily dose of DEXAMETHASONE, L-ARGININE, or PBS (CONTROL). # $p=6.8x10^{-6}$ *versus* L-ARGININE and $p= 7.3x10^{-5}$ *versus* CONTROL; * $p=2.8x10^{-5}$ *versus* L-ARGININE and p=0.0227 *versus* CONTROL.

Figure 2 – Survival rate of *Plasmodium berghei*-infected Swiss mice. Animals were pre-treated and received a daily dose of DEXAMETHASONE, L-ARGININE, or PBS (CONTROL).

Figure 3 - Trolox Equivalent Antioxidant Capacity (TEAC) in lungs (A) and brains (B) of *Plasmodium berghei*-infected Swiss mice. Animals were pre-treated and received a daily dose of DEXAMETHASONE, L-ARGININE, or PBS (CONTROL). # p=0.0401 *versus* DEXAMETHASONE; ***** p=0.0281 *versus* L-ARGININE and p=0.0033 *versus* CONTROL; € p=0.0360 *versus* L-ARGININE and p=0.0261 *versus* DEXAMETHASONE; ¥ p=0.0357 *versus* L-ARGININE and p=0.0005 *versus* CONTROL.

Figure 4 – Thiobarbituric Acid Reactive Substances (TBARS) in lungs (A) and brains (B) of *Plasmodium berghei*-infected Swiss mice. Animals were pre-treated and received a daily dose of DEXAMETHASONE, L-ARGININE, or PBS (CONTROL). € p=0.0294 *versus* CONTROL; # p=0.0282 *versus* DEXAMETHASONE; ***** p=0.0005 *versus* DEXAMETHASONE and p=0.0029 *versus* CONTROL; ¥ p=0.0060 *versus* CONTROL.

Figure 5 - Nitrites and Nitrates in lungs (A) and brains (B) of *Plasmodium berghei*-infected Swiss mice. Animals were pre-treated and received a daily dose of DEXAMETHASONE, L-ARGININE, or PBS (CONTROL). # p=0.0005 *versus* L-ARGININE and p=0.0394 *versus* DEXAMETHASONE; ***** $p=3.1x10^{-5}$ *versus* DEXAMETHASONE and $p=1.5x10^{-4}$ *versus* CONTROL; € $p=3.4x10^{-6}$ *versus* L-ARGININE and $p=5.0x10^{-4}$ *versus* CONTROL; ¥

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1074 p=3.6x10^{-4} *versus* L-ARGININE and p=4.6x10^{-4} *versus* CONTROL; ^{c} p=0.0058 *versus* L-
1075 ARGININE and p=0.0201 *versus* CONTROL.

1076 **Figure 6** - Uric Acid levels in lungs (A) and brains (B) of *Plasmodium berghei*-infected
1077 Swiss mice. Animals were pre-treated and received a daily dose of DEXAMETHASONE,
1078 L-ARGININE, or PBS (CONTROL). $^{\#}$ p=0.0033 *versus* DEXAMETHASONE and p=4.3x10^{-6}
1079 *versus* CONTROL; ***** p=0.00058 *versus* CONTROL; $^{€}$ p=0.00095 *versus* CONTROL and
1080 p=0.00054 *versus* DEXAMETHASONE; $^{¥}$ p=0.00071 *versus* CONTROL and p=0.01167
1081 *versus* DEXAMETHASONE; ^{c} p=0.0080 *versus* DEXAMETHASONE and p=0.0024 *versus*
1082 L-ARGININE; $^{£}$ p=0.0479 *versus* DEXAMETHASONE; $^{\&}$ p=0.0029 *versus* CONTROL and
1083 p=0.0001 *versus* DEXAMETHASONE; ^{ε} p=0.0395 *versus* CONTROL and p=0.0407
1084 *versus* DEXAMETHASONE.

1085

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ADDITIONAL FILE INFORMATION

FILE NAME: ADDITIONAL FILE 1

FILE FORMAT: .docx

TITLE OF DATA: CORRELATION STUDIES

DESCRIPTION OF DATA: Contain Pearson’s correlation studies for all parameters studied

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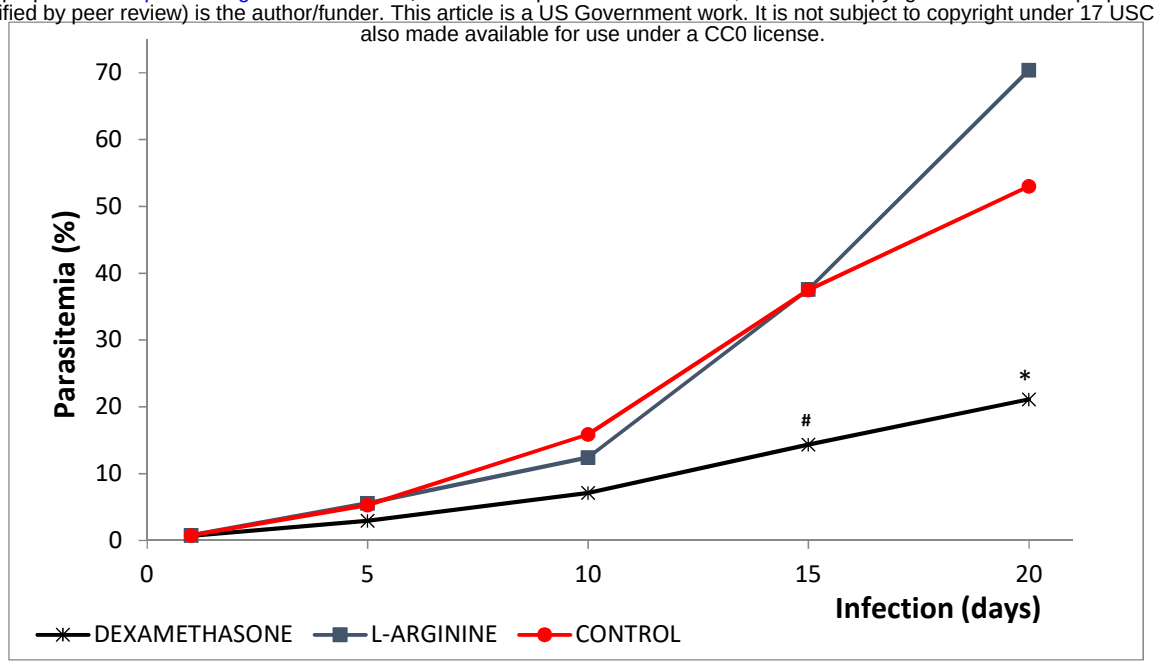

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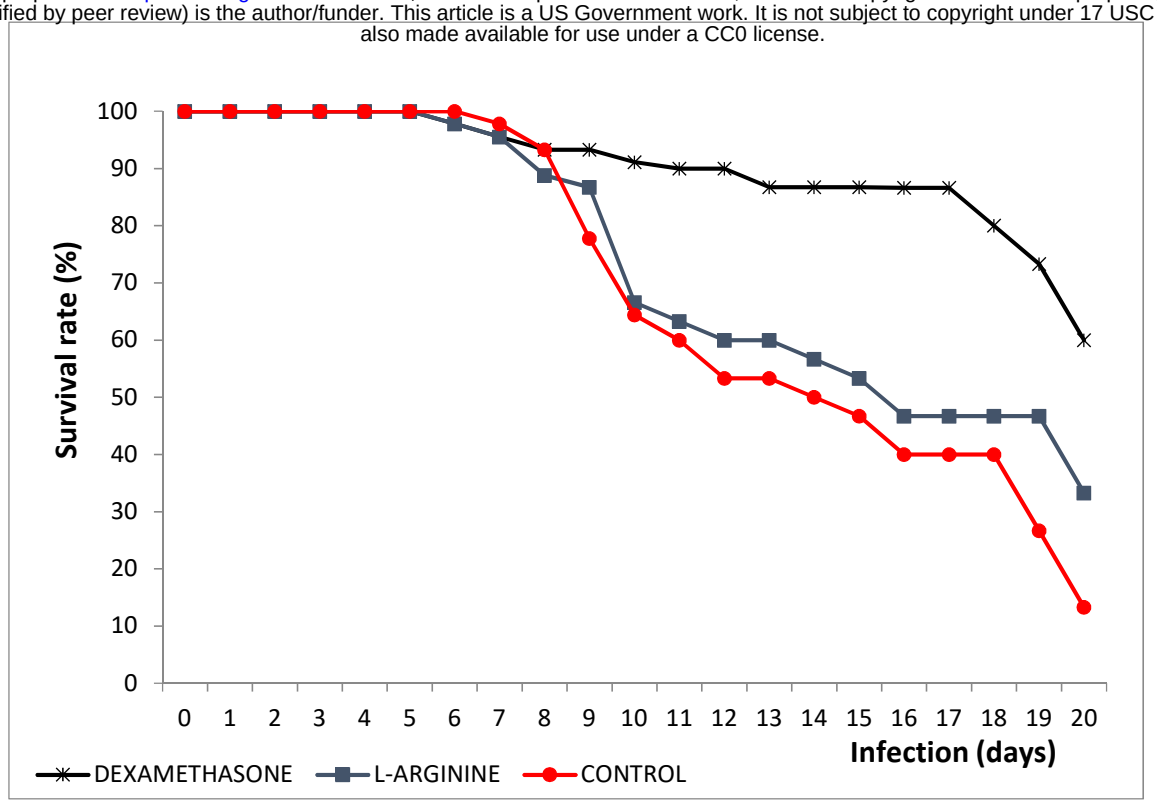

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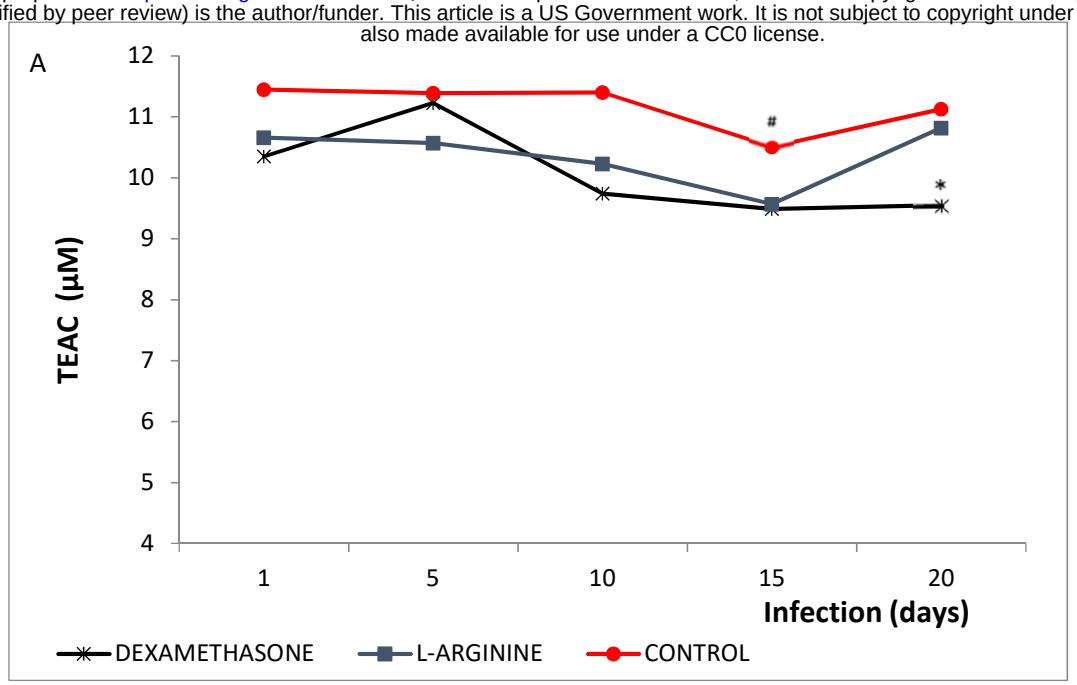

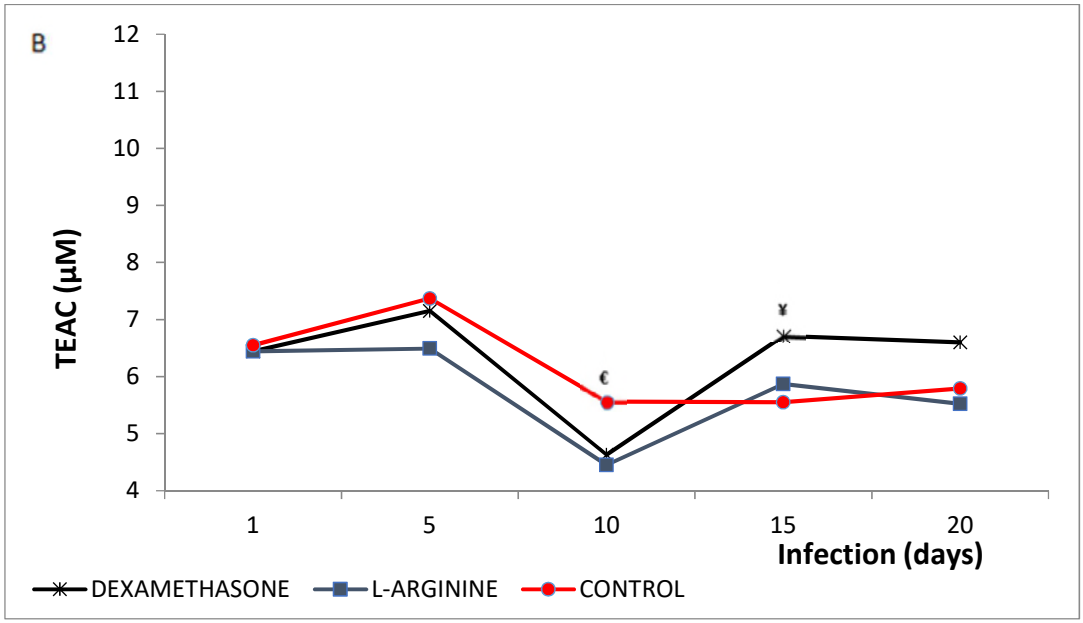

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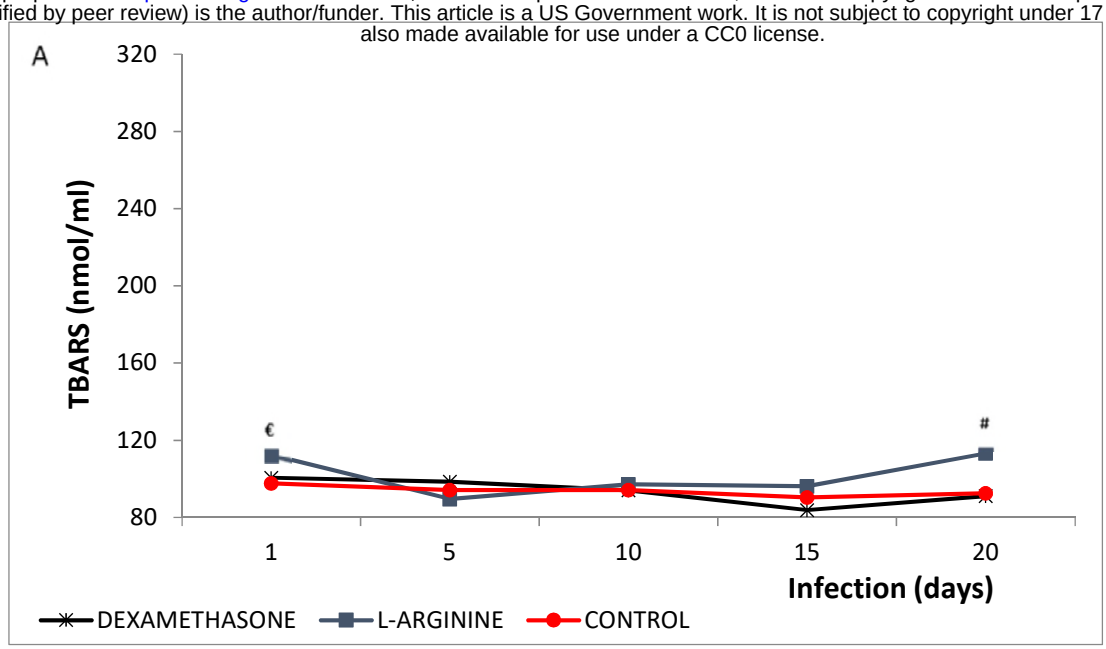

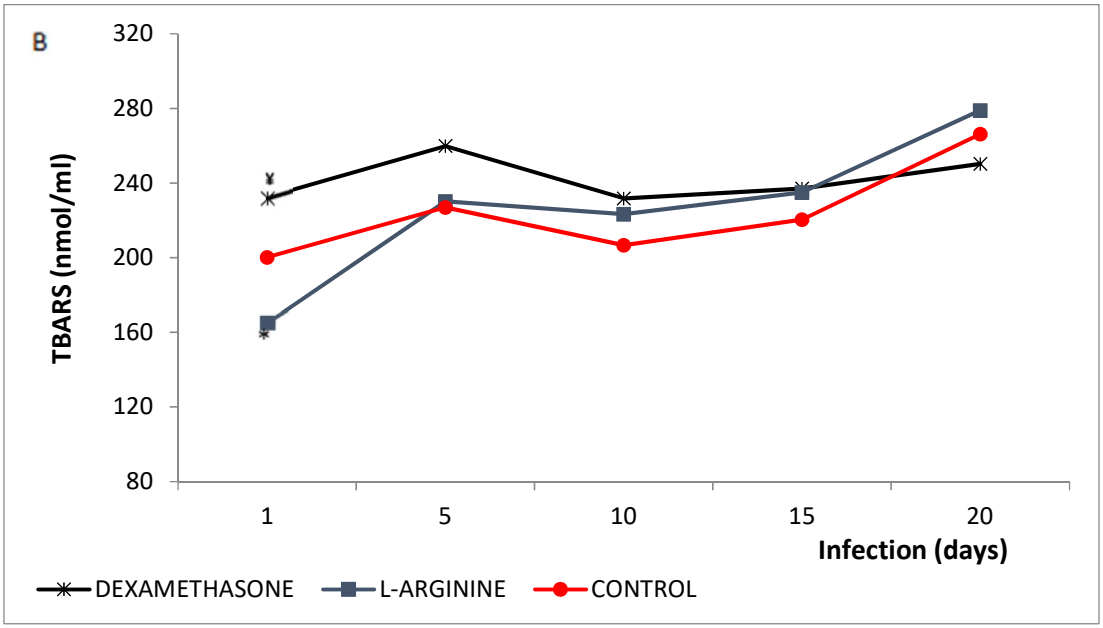

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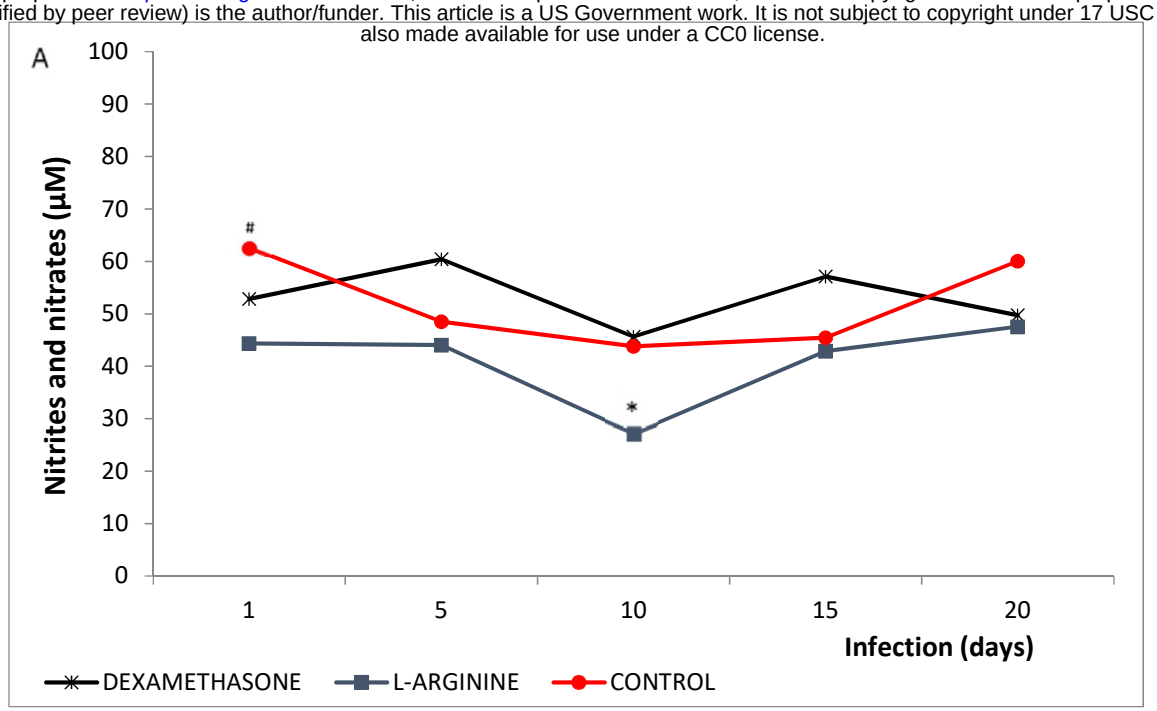

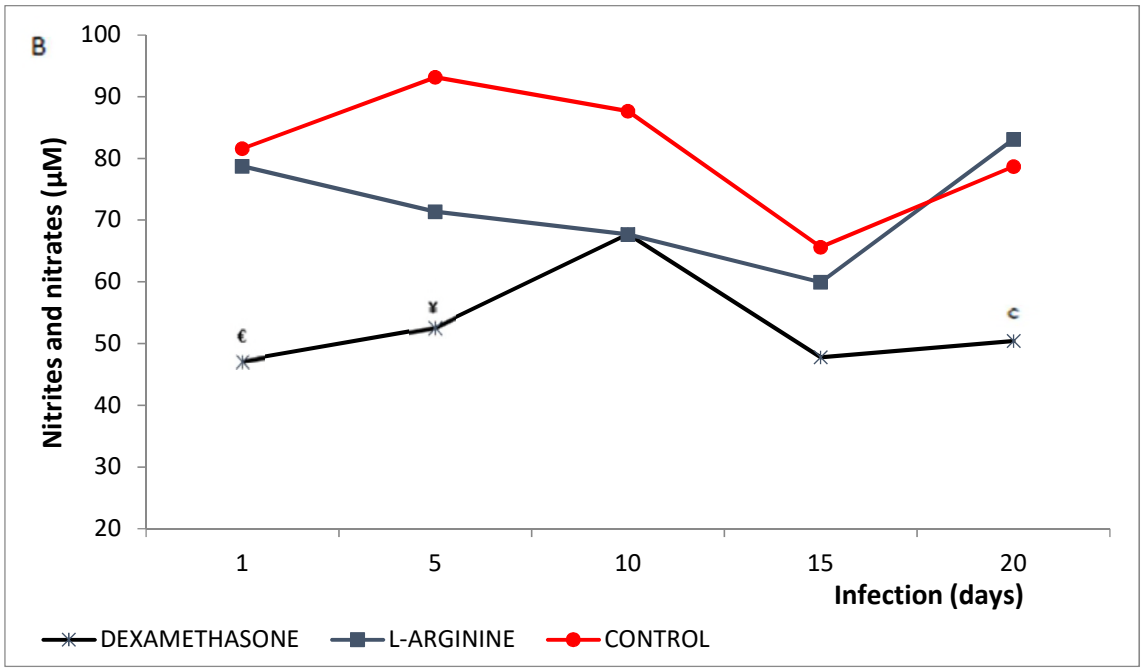

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