

Evans blue staining for the assessment of blood-brain barrier integrity in experimental malaria

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Short Communication

Abstract: Malaria is an endemic disease present mainly in developing regions, and cerebral malaria one of the serious forms of the disease in which there is an accumulation of erythrocytes in capillary network production of pro-inflammatory factors, preventing blood flow and blood-brain barrier (BBB) integrity. Thus, the development of experimental models aimed to study the permeability of BBB can provide important tools to study this important pathology. In this way, 25 mice infected with *Plasmodium berghei* were submitted to the evaluation of the permeability of the BBB through the method of staining with Evans Blue, being submitted to euthanasia after 1, 5, 10, 15 or 20 days of infection. A progressive evolution in parasitemia was observed, but there was just impregnation of dye in infected animals from the 10th day of infection. It is concluded that the proposed method has proved satisfactory for the evaluation of the permeability of the BBB and that infection of mice with *P. berghei* promotes significant changes in the permeability of BBB from the 10th day of infection.

Key words: Cerebral Malaria; Blood-Brain Barrier; Evans Blue; *Plasmodium berghei*; Mice.

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Introduction

Malaria is an endemic disease transmitted by mosquitoes of the genus *Anopheles*. The etiologic agent is a protozoa of the genus *Plasmodium*, and is a significant public health concern that affects approximately 40% of the world's population in 108 countries, causing about 130 million new cases each year and 315 - 689 thousand deaths, most of them in children less than five years of age (1-3).

Most of the deaths from malaria originate from the impairment of the brain, a syndrome called cerebral malaria, frequent among severe cases of *P. falciparum* infection. The exact underlying mechanism of this neuropathology is not yet understood, but it is believed that cytoadherence of parasitized red blood cells to endothelial cells of cerebral microvasculature is implicated as the main process responsible for the development of cerebral malaria in humans (4-6).

With the purpose of clarifying the mechanism of action of the parasite and to promote a better treatment of the disease, researchers often rely on experimental studies. In this regard, to mimic the severe and complicated form of this disease, *P. berghei*-infected mice have been used, since infected animals develop symptoms similar to those presented by humans with *P. falciparum* malaria (7). Likewise, the arrest of macrophages, monocytes and parasitized blood cells also occurs in this experimental model (8-10).

Therefore, the evaluation of the blood-brain barrier (BBB) permeability may provide important knowledge about the pathophysiology of the disease. Hence, studies carried out by Baptista (11) using the dye Evans Blue 2% to evaluate BBB permeability in *P. berghei* ANKA-infected mice treated with the antimalarial medicine pyrimethamine, identified recovery of BBB selective permeability after 6-7 days of infection, showed by the absence of dye leakage to the brain of these animals, whereas non-treated control animals showed extravasation of dye to the brain and, therefore, compromised BBB.

Morrel *et al.* (12), using a similar experimental model, observed high dye extravasation to the brains of animals after five days of infection and that the treatment with IFN- β reduced the extravasation of the dye. In a similar model, treatment with the ligand F1t3, important dendritic cells differentiation and homeostasis cytokine, prevented BBB permeability change in mice (8).

Consequently, the experimental model using *P. berghei*-infected mice is an important model to study human malaria, mainly in regards to the severe form of the disease, cerebral malaria. In this sense, the use of Evans Blue dye may be an important tool for the evaluation of the integrity of the BBB in the pathophysiology of malaria, as well as to study the effect of drugs and of the mechanism of the disease. Thus, this study evaluated the use of Evans Blue dye as an indicator of BBB integrity in *P. berghei*-infected mice.

Methods

Animals

Fifty male Swiss mice (*Mus musculus*), young adults, from the vivarium of the Evandro Chagas Institute (IEC; Belem, PA, Brazil) were randomly divided into 2 groups, as follows:

Group A: composed of *P. berghei*-infected animals. The animals were divided into 5 subgroups (I-V; N=5 each), in which the animals were sacrificed after 1, 5, 10, 15 or 20 days of infection.

Group B: negative control matched animals (sham), which were submitted to the same conditions as group A animals, with the exception of parasite inoculation. This group was also divided into 5 subgroups (VI-X; N=5 each), submitted to euthanasia at the same times of animals of subgroups I-V.

Parasite count was performed at the day of euthanasia (1, 5, 10, 15 and 20 days after infection), prior to intravenous inoculation of 2% Evans Blue dye, procedure performed 2 hours before the animals are anesthetized (xylazine 2%: ketamine 5%, 1:2; 80 μ L/25g of weight). Subsequently, animals were sacrificed by exsanguination through cardiac puncture, followed by removal of the brain. The Ethics Committee in Research with Experimental Animals of the Federal University of Para approved this project (Report CEPAE/UFPA No. 125-13).

Induction of malaria

P. berghei ANKA-infected erythrocytes were originally supplied by IEC and maintained by successive replication at the Experimentation Vivarium of the Oxidative Stress Research Laboratory, Biological Sciences Institute, Federal University of Para (LAPEO/ICB/UFPA). The infection of mice was performed by intraperitoneal inoculation of 10^6 *P. berghei*-infected erythrocytes, diluted in RPMI medium. Animals were kept in appropriate cages for up to 5 mice and maintained with food and water *ad libitum* during the entire period of the experiment.

Evaluation of the Integrity of the Blood-brain Barrier

The permeability of the BBB was evaluated in accordance with technical procedures described by Herbas *et al.* (10), with modifications.

The 2% Evans Blue ($C_{34}H_{24}N_6Na_4O_{14}S_4$) solution (1186; DYNÂMICA, Brazil) was freshly prepared in PBS (pH= 7.2) on the day of use. The volume of solution injected was calculated according to the weight of the mouse (4mL/kg of body weight). After 20 min of anesthesia, the dye solution was injected into the lateral tail vein, followed by another injection of anesthesia 1h later. Subsequently, after 20 minutes, euthanasia was performed and the brain was removed for the assessment of the dye impregnation. This method is based upon the formation of bonds between the dye and plasmatic albumin and, in the event of brain blood vessels leakage due to compromised BBB, the dye-protein complex migrates to the tissue, impregnating it in a tone of blue visible to the naked eye.

Parasite Count

P. berghei-infected RBC were counted on blood smears obtained by puncture of the caudal vein of animals on the day of euthanasia (1, 5, 10, 15 and 20 days after 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, RBC were counted in an optical microscope (Olympus, CX2) with 100x magnification (supplementary file table 1). (8).

Statistical Analysis

The number of parasites in each group (A or B) was assessed by means of ANOVA, followed by Tukey's *post-hoc* test. For the inter-group comparison, distribution normality was verified through the D'Agostin test. Should the distribution was normal, T-test was applied, and otherwise Mann-Whitney test was used. The significance level was set at 5% ($p < 0.05$).

Results and Discussion

Parasitemia

Infected animals showed progressively increasing parasitemia (Figure 1) in accordance with the time of infection, similar result found by Herbas *et al.* (10), who used the same intraperitoneal inoculation of infected erythrocytes in mice C57BL/6J.

Assessment of Blood-brain Barrier Integrity

After injection of the dye solution, all animals in both groups showed blue staining distributed in the body of the animal, being apparent on the paws, tail and in the oral region (Supplementary file Figure 1), and suggesting appropriate distribution of the dye by the circulatory system of animals.

After 1h of dye circulation in anesthetized animals, they underwent euthanasia for brain removal. No impregnation in the brain of infected animals after 1 and 5 days of infection was noticed. Nevertheless, dye impregnation was seen on animal brains of subgroups III, IV and V (10, 15 and 20 days after infection, respectively), suggesting an impairment of the blood-brain barrier, a characteristic that is less intense on the 15th and 20th days post-infection (Figure 2), possibly due to the occurrence of vascular changes and anemia characteristics of advanced disease.

Our data are similar to those found by Herbas *et al.* (10), which detect impairment of BBB on the seventh day after infection in C57BL/6J mice. Moreover, Morrel *et al.* (12) and Tamura *et al.* (8), using same type of rodents, detected impairment of BBB from the 6th day post-infection.

Comparing the evolution of parasitemia and the impregnation of dye in the brain it was observed that BBB

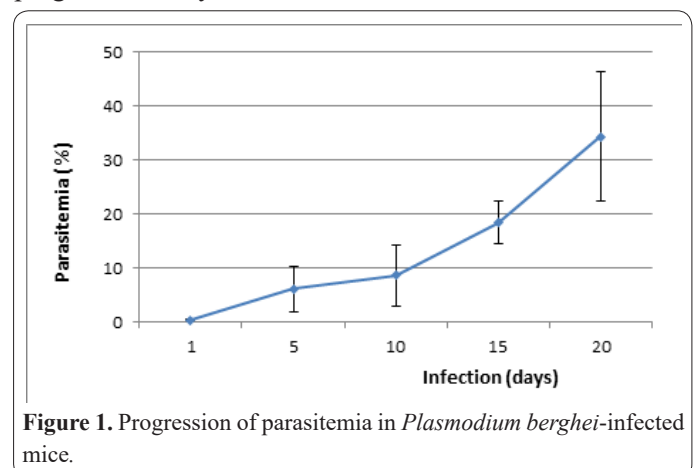


Figure 1. Progression of parasitemia in *Plasmodium berghei*-infected mice.

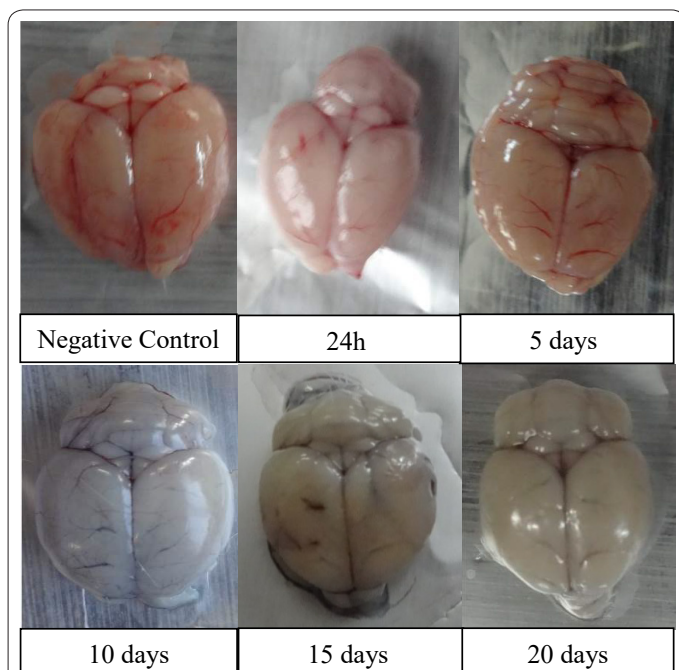


Figure 2. Brains of *Plasmodium berghei*-infected mice (24 h, 5 days, 10 days, 15 days and 20 days post-infection), and after injection of Evans Blue dye. Negative control refers to a brain of a non-infected mouse and, despite the differences in the day of euthanasia, all sub-groups displayed similar colored brains.

impairment does not occur in a progressive fashion as parasitemia does. The brain becomes more impregnated by dye when the animals displayed an average parasitemia of 8.6% on the 10th day after infection. In contrast, 15 and 20 days after infection higher levels of parasitemia were seen (18.4% and 34.3%, respectively), but dye impregnation was far less intense in both groups.

A possible explanation for the change in dye impregnation after the 10th day post-infection lies in the suggestion that cerebral alterations arising from the compromised BBB and plasmodial infection would promote the reduction of cerebral blood flow and, thus, would yield less impregnation of dye. However, this suggestion remains to be further investigated.

To our best knowledge, it is the first time this method is employed in the evaluation of BBB permeability of the Swiss mice model of malaria and, from the results of this study, it can be concluded that Evans Blue is an effective staining method for visualization of changes in the blood-brain barrier permeability in this experimental model of malaria, as well as the impairment of the BBB becomes evident after the 10th day of infection, but is not directly related with the parasitemia, possibly due to the reduction

in cerebral blood flow that progresses with the development of cerebral malaria in animals.

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