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Anxiety-like behavior and proinflammatory cytokine levels in the brain of C57BL/6 mice infected with *Plasmodium berghei* (strain ANKA)

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ABSTRACT

Cerebral malaria (CM) is a severe complication resulting from *Plasmodium falciparum* infection. The underlying mechanisms of CM pathogenesis remain incompletely understood. The imbalance between the release of proinflammatory and anti-inflammatory cytokines has been associated with central nervous system dysfunction found in human and experimental CM. The current study investigated anxiety-like behavior, histopathological changes and release of brain cytokines in C57BL/6 mice infected with *Plasmodium berghei* strain ANKA (PbA). Anxiety-like behavior was assessed in control and PbA-infected mice using the elevated plus maze test. Histopathological changes in brain tissue were assessed by haematoxylin and eosin staining. Brain concentration of the cytokines IL-1β, IL-4, IL-10, TNF-α and IFN-γ was determined by ELISA. We found that PbA-infected mice on day 5 post-infection presented anxiety symptoms, histopathological alterations in the brainstem, cerebrum and hippocampus and increased cerebral levels of proinflammatory cytokines IL-1β and TNF-α. These findings suggest an involvement of central nervous system inflammatory mediators in anxiety symptoms found in CM.

Malaria is associated with at least 2.3 million deaths annually, from an estimated 400 million cases of malaria each year worldwide [28]. Cerebral malaria (CM) is a severe complication resulting from *Plasmodium falciparum* infection [13].

The underlying mechanisms of CM pathogenesis remain incompletely understood [11,24]. High levels of circulating and cerebral tissue proinflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β have been associated with central nervous system (CNS) dysfunction found in human and experimental CM [2,3,14,25].

Overproduction of proinflammatory cytokines, especially TNF-α, induces an increase of adhesion molecule expression on vascular endothelium with subsequent sequestration of leucocytes, parasitized red blood cells (pRBC) and platelets, leading to microvascular obstruction and hypoxia [6]. In addition, the release of anti-inflammatory cytokines, mainly IL-10, seems to have a host-protective role by regulating the synthesis of proinflammatory cytokines in response to the parasite. However, this host-protective mechanism might be deficient in CM disease [12,18]. In this context, an imbalance in the release of proinflammatory and anti-inflammatory cytokines seems to be crucial to the development of CM [6,18].

Animal models have been of great relevance in the study of the mechanisms involved in CM pathogenesis [6,27]. Due to the high degree of reproducibility, easily manageable characteristics and development of histopathological and neurological signs typical of human CM, the murine model using the *Plasmodium berghei* strain ANKA (PbA) has been widely used to better understand this condition [24].

Behavioral and neurological symptoms in PbA-infected mice have been associated with neuroinflammatory processes [7,19,20]. However, to the best of our knowledge, no previous study has investigated the occurrence of anxiety-like behavior associated with CNS inflammation in CM. Thus, the aim of the current study was to investigate anxiety-like behavior and the release of brain cytokines in C57BL/6 mice infected with PbA.

All experiments were approved by the Animal Ethics Committee of the Federal University of Minas Gerais (UFMG). Female C57BL/6 mice (20–25 g), aged 6–8 weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences, UFMG. Animals were

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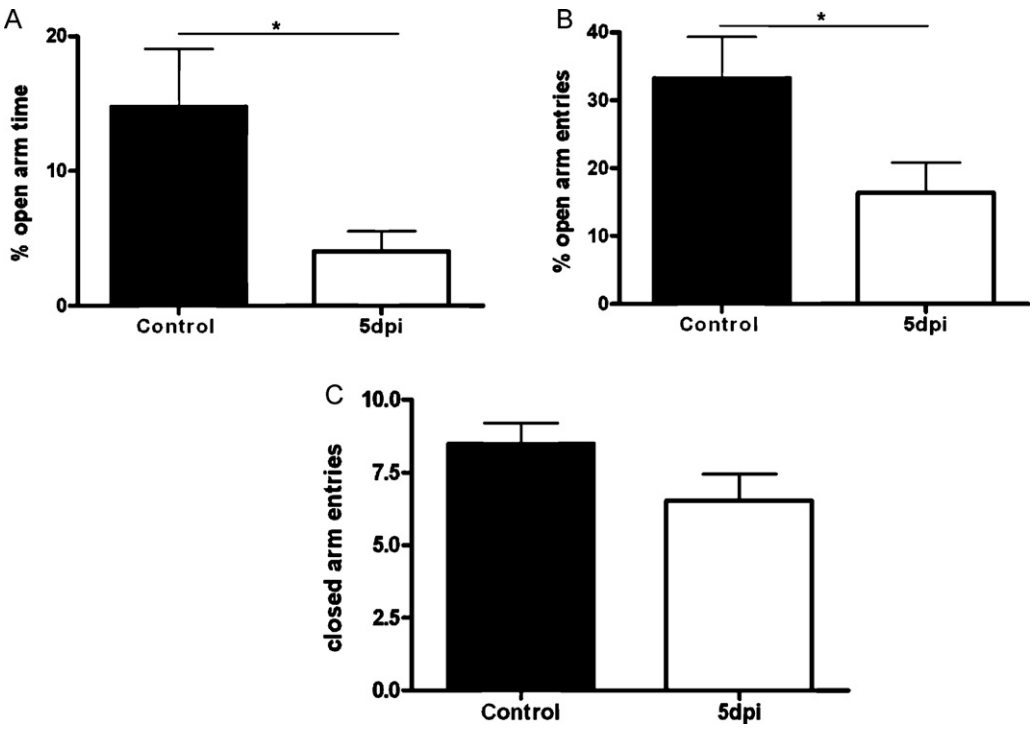

Fig. 1. Anxiety and locomotor measures of PbA-infected mice on day 5 post-infection (dpi) and of control group in the elevated plus maze. (A) % open arm time; (B) % open arm entries and (C) closed arm entries. Results were expressed as the mean ± SEM from at least ten animals per group. Asterisk(s) indicate statistical differences where $^{*}p < 0.05$.

housed in cages in temperature-controlled rooms and received food and water *ad libitum*.

Uncloned parasite line of *P. berghei* (strain ANKA) (PbA) was used in this study. *P. berghei* ANKA pRBC from C57BL/6 mice donor strains were maintained in stabilized liquid nitrogen and were thawed and passed into normal C57BL/6 mice that served afterwards as parasite donors. C57BL/6 mice were infected by intraperitoneal (i.p.) injection of 10^6 pRBC suspended in 0.2 mL PBS [10]. Control animals received the same volume of PBS. The level of parasitemia in infected mice was monitored on Giemsa-stained blood films from day 3 onwards and estimated at 1000 RBCs under immersion oil.

Anxiety-like behavior was assessed in control and infected mice on day 5 post-infection (p.i.) using the elevated plus maze (Insight®, SP, Brazil). Anxiety-like behavior was evaluated on day 5 p.i. in agreement with previous studies describing the onset of behavioral symptoms in CM mice on day 5 after infection with PbA [19,20]. The elevated plus maze (EPM) is a test of unconditioned anxiety-related behavior that involves a conflict between the rodent's desire to explore a novel environment and anxiogenic elements such as elevation and an unfamiliar open area [23]. This is a widely used test for anxiety behavior of rodents [9,23,26]. The EPM test was conducted as previous described by Walf and Frye [30]. Briefly, mice were placed in the center of the maze facing an open arm and were allowed to freely explore the EPM for 5 min. The animal placing all four paws onto the arm was considered to be in the arm, otherwise the animal was in the center of the maze. Behavior that was recorded when rodents were in the EPM included the time spent and entries made on the open and closed arms. The measures of anxiety were the percentage (%) of open arm entries and the percentage (%) of time spent on the open arms. The number of closed arm entries was considered as a locomotor measure. Decreased open arm activities indicate increased anxiety levels in EPM. Between each trial, the maze was wiped clean with a damp sponge and dried with paper towels. Before behavioral assessment, animals were allowed to accommodate to their new environment for 2 days.

For histological and inflammatory analyses the brain tissues of controls and PbA-infected mice on day 3 and 5 p.i. were removed. One hemisphere of the brain was homogenized in extraction solution containing aprotinin and the concentration of the cytokines IL-1β, IL-4, IL-10, TNF-α and IFN-γ was determined by ELISA (R&D Systems, Minneapolis, MN and Pharmingen, San Diego, CA). Another hemisphere of the brain was preserved in 10% buffered formalin. Sections of 5 μm thick were cut and mounted for routine haematoxylin and eosin (H&E) staining. These sections were examined at the optical level (Olympus, Japan, JP). Digital images were acquired for documentation.

Data are shown as mean ± SEM. A one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison post-test was used to analyze the brain concentrations of cytokines measured by ELISA. To analyze the anxiety behavior and locomotor activity in the EPM the Mann Whitney's test and the *t*-student's test were used, respectively. All analyses were performed using Prism 4 software (GraphPad, La Jolla, CA, USA).

CM mice presented a significant decrease in the percentage (%) of time spent on the open arms and in the number of entries into the open arms when compared to the control animals on day 5 p.i. (Fig. 1A and B; $p \leq 0.05$; $n = 10$ per group). No difference was found in the number of entries in the closed arms between the infected mice and controls, indicating that both groups presented similar locomotor activity in the EPM (Fig. 1C; $p = 0.12$).

No lesions were detected in the brain of non-infected group and PbA-infected group on day 3 (Fig. 2A, D and G). Infected mice on day 5 p.i. developed focal meningitis, consisted mainly of lymphocytes and macrophages (Fig. 2B). Sequestration of leukocytes in the microvasculature (vascular plugging) was detected in the cerebrum, brainstem and hippocampus (Fig. 2C and F). The hippocampus of PbA-infected animals on day 5 p.i. presented various shrinkage pyramidal neurons in the medial area of CA1 (Fig. 2H).