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RESEARCH PAPER

Cerebral Malaria Causes Enduring Behavioral and Molecular Changes in Mice Brain Without Causing Gross Histopathological Damage

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Abstract—Malaria, parasitic disease considered a major health public problem, is caused by *Plasmodium* protozoan genus and transmitted by the bite of infected female *Anopheles* mosquito genus. Cerebral malaria (CM) is the most severe presentation of malaria, caused by *P. falciparum* and responsible for high mortality and enduring development of cognitive deficits which may persist even after cure and cessation of therapy. In the present study we evaluated selected behavioral, neurochemical and neuropathologic parameters after rescue from experimental cerebral malaria caused by *P. berghei* ANKA in C57BL/6 mice. Behavioral tests showed impaired nest building activity as well as increased marble burying, indicating that natural behavior of mice remains altered even after cure of infection. Regarding the neurochemical data, we found decreased α2/α3 Na^+,K^+-ATPase activity and increased immunoreactivity of phosphorylated Na^+,K^+-ATPase at Ser^{943} in cerebral cortex after CM. In addition, [^{3}H]-Flunitrazepam binding assays revealed a decrease of benzodiazepine/$GABA_A$ receptor binding sites in infected animals. Moreover, in hippocampus, dot blot analysis revealed increased levels of protein carbonyls, suggesting occurrence of oxidative damage to proteins. Interestingly, no changes in the neuropathological markers Fluoro-Jade C, Timm staining or IBA-1 were detected. Altogether, present data indicate that behavioral and neurochemical alterations persist even after parasitemia clearance and CM recovery, which agrees with available clinical findings. Some of the molecular mechanisms reported in the present study may underlie the behavioral changes and increased seizure susceptibility that persist after recovery from CM and may help in the future development of therapeutic strategies for CM sequelae.

Key words: *Plasmodium berghei* ANKA, chloroquine, cognitive deficits, $GABA_A$ receptor, Na^+,K^+-ATPase, oxidative stress.

INTRODUCTION

Malaria is a serious tropical disease caused by different species of protozoan *Plasmodium* (Phillips et al., 2017). The World Health Organization (WHO) estimates that 212 million cases and 429,000 malaria-related deaths were reported in 2015, affecting mainly people in the sub-Saharan Africa region (WHO, 2016). Cerebral malaria (CM) is a neurological complication which is associated with high rates of morbidity and mortality, even after recovery, and it has been considered the most severe clinical presentation of infection with *P. falciparum* (Hunt et al., 2006; Opoka et al., 2009). In fact, high rates of mortality are observed in patients diagnosed with CM, and about one quarter of survivors presents long-term neurological complications (John et al., 2008; Idro et al., 2010a, 2010b).

Experimental models are useful to investigate mechanisms involved in disease and possible development of sequelae, since clinical studies present limitations including but not limited to ethical issues and the need of invasive techniques (Lacerda-Queiroz et al., 2010). *P. berghei* ANKA (PbA) strain is considered a well-characterized experimental model of CM (ECM) (Hunt et al., 2010). In this context, results obtained with

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PbA infection model may be relevant to a better comprehension of CM complications that occur in humans. For instance, it has been shown that C57BL/6 mice infected with PbA presents inflammatory and neurological manifestations similar to those observed in humans (Lou et al., 2001; Lamb et al., 2006; Reis et al., 2010), including long-term cognitive deficits (Freeman et al., 2016) and seizure susceptibility (Grauncke et al., 2016). However, molecular mechanisms underlying these findings remain unclear. To the present, most studies have investigated acute biochemical, histological and behavioral alterations in this experimental model (Reis et al., 2010; Nacer et al., 2012), and therefore more long-term studies are needed. In the present study, we aimed at potential long-term molecular and histological changes which could be involved in the behavioral changes and increased seizure susceptibility that occur after recovery of CM.

EXPERIMENTAL PROCEDURES

Animals

Adult male mice C57BL/6, 4–6 weeks old (30–40 g) were used to induce ECM. All animals from the central breeding colony of the Federal University of Santa Maria (Brazil) were maintained under conventional conditions, controlled environment (12:12-h dark/light cycle, 24 ± 1 °C, 55% humidity), and free access to food (PuroTrato, Santa Maria, Brazil) and water. All animal experimentation described in this study complied with national and international legislation and with the approval of Institutional Committee on Animal Use and Care of Federal University of Santa Maria (process #020/2014). All protocols aimed to reduce the number of animal used to a minimum, as well as their suffering.

Experimental design

Sixty-Five C57BL/6 male mice were allocated into two independent experimental groups: 45 animals were inoculated intraperitoneally with 0.1 mL suspension of 10^6 parasitized red blood cells (ANKA strain) and 20 age-matched controls received a similar volume of 10^6 non parasitized red blood cells. The animals infected with PbA have a mortality rate above 40% throughout the experimental period. Parasitemia, weight and the CM stage assessment protocol (Reis et al., 2010) were monitored daily. With the CM stage assessment protocol animals received scores related to piloerection, gait, body position, grip strength, positional reflex and spontaneous activity. Low scores, varying from 0 to 1, indicated normal behavior of animals; 2 to 3, altered behavior without severe compromise of general activity; 4 to 5, the most severe condition observed. The treatment with antimalarial chloroquine (Sigma–Aldrich) was initiated when infected animals demonstrated typical neurobehavioral manifestations with a sum of scores equal or greater than 10, being considered positive for CM. On the 15th day post infection (dpi), the animals of control group also started to receive chloroquine. Therefore, animals of both groups received oral chloroquine in a loading dose of 80 mg/kg and were kept until the 45th dpi in a maintenance dose of 25 mg/kg (Reis et al., 2012). The last administration of chloroquine occurred 24 h before behavioral tests or euthanasia.

Behavioral tests

To evaluate persistent behavioral and/or cognitive deficits after rescue of CM, animals were submitted to a behavioral test battery consisting of the following tests: open field (de Oliveira et al., 2016), rotarod (de Oliveira et al., 2016), marble burying (Deacon, 2006), nest building (Deacon, 2012) and sucrose preference (Grigoletto et al., 2016). Behavioral tests were organized from the least to the most aversive and were performed in the same animals during six consecutive days.

Neurochemical assays

In the 46th dpi mice were euthanized to obtain brain samples (cerebral cortex and hippocampus) for neurochemical analyses, such as Western Blot, Dot Blot, Binding and determination of enzymatic activity.

Western blot

Na^+,K^+-ATPase α subunit content and its phosphorylation state at Ser^{943} were carried out according to Funck and collaborators (2015) with minor modifications. In brief, tissues were gently homogenized in ice-cold Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific), supplied with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Homogenates were centrifuged at 10,000×g for 5 min at 4 °C and an aliquot of the supernatant (20 μg protein) was submitted to SDS–PAGE using a 10% gel. After semi-dry transfer the nitrocellulose membranes were blocked with 2.5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.4) for 1 h and exposed to primary antibodies during 3 h at room temperature for α Na^+,K^+-ATPase (1:20000, Santa Cruz sc-28800) or overnight at 4 °C for p-Na^+,K^+-ATPase α (Ser^{943}) (1:5000, Santa Cruz sc-16170-R). Membranes were washed three times with TBS containing 0.01% (v/v) Tween 20 (TBS-T) and exposed to biotinylated anti-rabbit secondary antibodies (1:10000, Sigma–Aldrich) during 1 h at room temperature. Membranes were washed three more times and incubated with streptavidin peroxidase polymer (1:5000, Sigma–Aldrich, S2438) 1 h at room temperature. All antibodies were prepared in TBS-T containing 1.25% BSA. Samples of control and infected mice were processed in parallel on a same gel and were submitted to the same immunoblotting procedures. Ponceau S staining served as the loading control (Romero-Calvo et al., 2010). Immunoreactivity was detected using standard ECL reagent (Thermo Fisher Scientific) and signals were quantified with ImageJ Software.

Dot blot

Oxidative stress markers protein carbonyl and 4-hydroxy-2-nonenal (HNE) were measured according Funck and coworkers (2014). Samples were applied to a nitrocellulose membrane through a vacuum system on Dot Blot

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