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RELEVÂNCIA DO CANAL IÔNICO TRPV1 NO DESENVOLVIMENTO DA MALÁRIA CEREBRAL

DOMINGOS MAGNO SANTOS PEREIRA

São Luis - MA
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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede BIONORTE, na Universidade Ceuma, como requisito parcial para obtenção do título de doutor em biotecnologia.

Orientadora:
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RESUMO

A malária é uma doença infecciosa, sistêmica e parasitária de importância mundial, com elevada taxa de morbidade e mortalidade. A malária grave é uma complicação neurológica associada à inflamação cerebral a qual pode levar a óbito ou causar sequelas neurológicas em pacientes sobreviventes mesmo com o tratamento adequado. A resposta imune inata do paciente apresenta um papel determinante na defesa do hospedeiro e também na patogênese da malária cerebral, uma vez que a presença de eritrócitos infectados sequestrados no endotélio da microcirculação cerebral desencadeia os mecanismos do estresse oxidativo e estresse nitrosativo, com produção excessiva de espécies reativas de oxigênio (ERO) e nitrogênio (ERN), moléculas relacionadas com a peroxidação lipídica, causando danos não somente ao parasita, mas também a células endoteliais e à integridade da barreira hematoencefálica, expondo o sistema nervoso central ao *Plasmodium falciparum* (em humanos) e *P. berghei* ANKA (em roedores). Dentre as diversas alterações que ocorrem no hospedeiro na malaria cerebral, o estresse oxidativo/nitrosativo é essencial para a morte do parasita e para a sinalização da resposta imune, além disso, o mecanismo de ação de diversas drogas antimaláricas disponíveis atualmente tem como alvo o aumento do estresse oxidativo, reduzindo a parasitemia e controlando a infeção. No organismo humano o estresse oxidativo/nitrosativo é regulado por um grupo de receptores de membranas celulares, como o TRPV1. Recentemente, foi demonstrado que o receptor de potencial transitório vanilóide 1 (TRPV1), um sensor do estresse oxidativo, é capaz de modular a resposta imune periférica do hospedeiro contra a malária, no entanto, pouco se sabe acerca de sua relevância nas alterações cerebrais decorrentes da malária grave. Assim, investigou-se a importância do TRPV1 no desenvolvimento da malária cerebral causada por *P. berghei* ANKA em camundongos. Para isto, utilizou-se camundongos C57BL/6 machos, selvagens (*wild type*; WT) e com deleção gênica para o TRPV1 (TRPV1 KO). A contribuição do TRPV1 na malária cerebral foi avaliada ainda, em animais tratados repetidamente com o antagonista seletivo SB366791 (0,5 mg/kg, s.c.). Os resultados obtidos demonstram que a infecção induzida por *P. berghei* ANKA reduz significativamente a expressão do TRPV1 no tecido cerebral. Ainda, animais TRPV1 KO foram protegidos contra a morbidade e mortalidade causadas pela malária cerebral, através da atenuação dos sinais e sintomas da doença e também da mortalidade, sem afetar a carga parasitária. Esta resposta foi associada à redução na formação de edema

cerebral e modulação da expressão gênica de marcadores de integridade da barreira hematoencefálica (claudina-5 e JAM-A), além do aumento na produção de espécies reativas geradas pelo estresse oxidativo tecidual e plasmático; e redução na produção de citocinas sistêmicas e teciduais. O tratamento com SB366791, iniciado pós-indução da malária cerebral, promoveu o aumento da expressão do TRPV1 no cérebro e aumentou a sobrevivência dos camundongos. Os dados da presente tese indicam pela primeira vez que o canal iônico TRPV1 contribue para o desenvolvimento e prognóstico da malária cerebral, através da modulação da inflamação cerebral, portanto, pode ser sugerido como alvo terapêutico para o tratamento de pacientes infectados com o *Plasmodium falciparum*.

Palavras-chave: Malária Cerebral; TRPV1; Inflamação; Estresse Oxidativo.

ABSTRACT

Malaria is an infectious, systemic and parasitic disease of global importance, with a high morbidity and mortality rate. Severe malaria is a neurological complication associated with brain inflammation which can lead to death or cause neurological sequelae in surviving patients. The patient's innate immune response plays a decisive role in host defense and also in the pathogenesis of cerebral malaria, since the presence of infected erythrocytes sequestered in the endothelium of the cerebral microcirculation triggers the mechanisms of the oxidative and nitrosative stress, with excessive production of reactive oxygen (ROS) and nitrogen (RNS) species, molecules related to lipid peroxidation, causing damage not only to the parasite, but also to endothelial cells and to the integrity of the blood-brain barrier, exposing then, the central nervous system to *Plasmodium facliparum* (in humans) and *P. berghei* ANKA (in rodents). Among the several changes occurring in the host in cerebral malaria, oxidative/nitrosative stress is essential for killing the parasite and signaling the immune response; in addition, the mechanism of action of several antimalarial drugs currently available targets the increase of oxidative stress, reducing parasitemia and controlling infection. In the human organism, oxidative/nitrosative stress is regulated by a group of cell membrane receptors, such as TRPV1. It was recently shown that the transient potential receptor vaniloid 1 (TRPV1), an oxidative stress sensor, modulates the peripheral immune response to malaria; however, little is known of its relevance to the cerebral changes caused in the sereve form of the disease. Therefore, the relevance of the TRPV1 in the development of cerebral malaria induced by *Plasmodium berghei* ANKA ($1x10^6$ infected RBCs per animal, i.p) in *wild type* (WT) and *knockout* (TRPV1KO) mice, were investigated. In another set of experiments, the use of the selective TRPV1 antagonist, SB366791, was also studied. The results show that *P. berghei* ANKA-induced infection significantly reduces TRPV1 expression in brain tissue. Furthermore, TRPV1 KO animals were protected against morbidity and mortality caused by cerebral malaria by attenuating the signs and symptoms of the disease as well as mortality without affecting the parasitaemia. This response was associated with reduced cerebral edema formation and modulation of gene expression of blood-brain barrier integrity markers (claudin-5 and JAM-A), as well as increased production of reactive species generated by tissue and plasma oxidative stress; and reduction in the production of systemic and cerebral cytokines. Treatment with SB366791 initiated after induction of the cerebral malaria

promoted enhanced TRPV1 gene expression in the brain and increased mouse survival. Our data from the present thesis indicate for the first time that the TRPV1 ion channel contributes to the development and prognosis of cerebral malaria by modulating cerebral inflammation, therefore, it may be suggested as a therapeutic target for the treatment of *Plasmodium falciparum*-infected patients.

Keywords: Cerebral Malaria; TRPV1; Inflammation; Oxidative Etress.

SUMÁRIO

1. INTRODUÇÃO

A malária é uma doença infecciosa, não contagiosa, considerada como parasitose de importância mundial por apresentar elevada morbidade e mortalidade, com 216 milhões de casos por ano, alcançando 429 mil mortes/ano. A alta mortalidade da malária está associada a evolução da doença para estágios graves, sendo a malária cerebral uma das principais alterações clínico-patológicas destes casos, sendo caracterizada pela presença do estado de coma e convulsões em pacientes infectados pelo *Plasmodium falciparum*. No entanto, apesar da existência de antimaláricos eficazes, pacientes que sobrevivem à malária cerebral podem desenvolver uma série de *déficits* neurológicos, indicando que a erradicação do parasita não previne as consequências clínicas da infecção.

A evolução da malária cerebral está relacionada à presença de uma resposta inflamatória sistêmica e principalmente à uma resposta inflamatória no sistema nervoso central, em parte ocasionada pela intensa geração de espécies reativas de oxigênio (EROs) e nitrogênio (ERNs). Neste contexto, o estresse oxidativo apresenta um papel determinante na malária cerebral. De fato, a produção de EROs e subsequente peroxidação lipídica por diferentes células, possui um impacto direto na evolução da malária e representam um papel central na resposta imune do paciente à infecção pelo *Plasmodium falciparum*, uma vez que a liberação destes mediadores por células endoteliais e células inflamatórias contribui ativamente para a disfunção endotelial e inflamação neuronal, características da malária cerebral.

O TRPV1, um receptor de membrana expresso em células neuronais e não neuronais (como células endoteliais e imunes), hoje é reconhecido como um sensor e modulador do estresse oxidativo em diferentes doenças, principalmente as de origem infecciosas e inflamatórias. Evidências obtidas à partir de modelos experimentais *in vivo* sugerem que a ativação do TRPV1 pode influenciar a resposta imune inata do hospedeiro à infecções, incluindo a resposta contra bactérias e à malária, por mecanismos de regulação de vias imunes e oxidantes.

Fernandes et al., (2014) demonstrou pela primeira vez que o bloqueio do TRPV1 pode influenciar a resposta imune periférica contra a malária, no entanto, apesar dessas informações, o impacto da ativação endógena do TRPV1 na evolução do quadro clínico da malária cerebral ainda permanece não esclarecido. Portanto, através da análise de dados já publicados sobre a relação do TRPV1 com a patogênese da malária e diversas

outras doenças infecciosas e inflamatórias, a hipótese da presente tese é que a deleção ou bloqueio do TRPV1 pode interferir na patogenia da doença e influenciar de forma positiva no prognóstico da malária cerebral. Sendo assim, este projeto visa avaliar a participação do TRPV1 na evolução do quadro clínico da malária cerebral induzida por *Plasmodium berghei* ANKA em camundongos C57BL/6, através do uso de um antagonista seletivo, o SB366791; e animais com deleção gênica para o TRPV1 (TRPV1 *knockout* ou TRPV1KO).

Espera-se, desta forma, adquirir um conhecimento maior acerca deste receptor e suas funções na patogênese da malária grave, principalmente no estresse oxidativo decorrente da infecção pelo *Plasmodium sp.* no tecido neuronal, e seus impactos na evolução clínica e mortalidade da malária cerebral; além de proporcionar um maior entendimento da relevância patofisiológica do receptor TRPV1. Ressalta-se que drogas capazes de bloquear estes receptores vêm sendo desenvolvidas para o tratamento de doenças de curso crônico, como a artrite reumatoide. Assim, faz-se extremamente relevante a obtenção de informações relativas aos potenciais efeitos deletérios do uso repetido destes compostos em um cenário de doenças infecciosas graves.

2. REFERENCIAL TEÓRICO

A malária é uma doença infecciosa, parasitária, sistêmica e não contagiosa que afeta milhões de indivíduos ao redor do mundo, sendo responsável por milhares de mortes por ano (OMS, 2015). É transmitida através da picada da fêmea infectada de insetos dípteros do gênero *Anopheles*, introduzindo os parasitas no sistema circulatório do hospedeiro, os quais migram primeiramente para as células hepáticas para reprodução e maturação (Pimenta et al., 2015). Os sinais e sintomas da malária aparecem cerca de 8 a 25 dias após a picada do mosquito vetor e podem variar de organismo para organismo. Esses sintomas incluem dores de cabeça, febre alta, calafrios, dores nas articulações, vômitos, anemia hemolítica, icterícia, hemoglobina na urina, lesões na retina e convulsões (OMS, 2016). É importante ressaltar que, caso não haja o tratamento durante os sintomas iniciais, a doença tende a evoluir para casos graves de malária, como a a malária cerebral (Ghazanfari et al., 2018).

2.2 Transmissores e agente etiológico

A malária é transmitida através da picada da fêmea de mosquitos do gênero *Anopheles*, pertencentes à família Culicidae e subfamília Anophelinae, apresentando em torno de 400 espécies. Sendo popularmente chamado de mosquito-prego no Brasil (Montoya-Lerma et al., 2011), o *Anopheles* adapta-se à climas tropicais e subtropicais com altos índices de umidade e temperatura variando entre 20ºC a 30ºC. O principal vetor da malária no Brasil é o *Anopheles darlingi*, presente em todo o território nacional (com maior prevalência na região amazônica), sendo responsável pela transmissão do protozoário *Plasmodium sp.* (Emerson et al., 2015).

Diversas espécies de *Plasmodium* infectam o homem e outros animais, incluindo pássaros, répteis e roedores (Delhaye et al., 2018). Conhecidamente, cinco espécies causam a doença no homem: *P. vivax, P. falciparum, P. malariae, P. ovale e P. knowlesi* (Ortiz-Ruiz et al., 2018); no entanto, no Brasil, três espécies são prevalentes, sendo o *Plasmodium vivax* a espécie mais predominante (83,81%) e responsável pelos casos de malária associados à complicações clínicas severas e morte (Costa et al. 2012; Lacerda et al. 2012; Recht et al., 2017). Em relação a outras espécies, a prevalência de *Plasmodium falciparum* (13,15%) foi reduzida na última década, enquanto *Plasmodium malariae* é a espécie menos predominante (0,037%) (OMS, 2015). Apesar disso, o

Plasmodium sp. continua a ser um parasita de importância mundial devido ao seu alto índice de morbi-mortalidade.

2.3 Ciclo de vida

As espécies de *Plasmodium sp*. apresentam similaridades em seus ciclos biológicos, o qual ocorre em duas fases: assexuada ou esquizogônica e sexuada ou esporogônica, ambas discutidas a seguir.

2.3.1 Fase sexuada

O parasita apresenta um ciclo digenético ou heteroxênico, sendo o homem o hospedeiro intermediário e o mosquito *Anopheles*, o definitivo. O ciclo do parasita se inicia durante a alimentação hematófaga do mosquito (fêmea) vetor, o qual, durante ingestão de sangue contaminado do paciente, contrai as formas sexuadas maduras do parasita - gametócitos femininos e masculinos - (Talisuna et al., 2007; Kamya et al., 2015; Sriwichai et al., 2016).

No estômago do mosquito os gametócitos masculinos sofrem exflagelação e originam gametas masculinos (microgametas); enquanto os gametócitos femininos sofrem maturação para gametas femininos (macrogametas). O microgameta fertiliza o macrogameta, formando o zigoto, que após tornar-se móvel, é denominado oocineto (Poolphol et al., 2017). Por sua vez, o oocineto fixa-se na parede do estômago entre as células epiteliais, nessa fase, denomina-se oocisto. Dentro desta estrutura, formam-se esporozoítas, que são células alongadas, móveis e apresentam um núcleo central. Essas formas são liberadas pelos oocistos, então migram para às glândulas salivares, tornado o mosquito infectado e, portanto, pode transmitir o parasita (Pimenta et al., 2015; Sriwichai et al., 2016), reiniciando o ciclo.

2.3.2 Fase assexuada

Nesta fase, durante a picada do mosquito infectado, são inoculados os esporozoítos (presentes nas glândulas salivares do *Anopheles*) na corrente sanguínea. É importante ressaltar que os esporozoítos apresentam proteínas de superfície circum-esporozoíticas (CS) e proteína adesiva relacionada com a trombospondina (TRAP),

responsáveis pela afinidade do parasita pelo tecido hepático, assim, estas formas migram para o fígado e infectam hepatócitos, iniciando um processo de desenvolvimento denominado esquizogonia pré-eritrocítica (Ganter et al., 2017).

No interior dos hepatócitos, os esporozoítos diferenciam-se em trofozoítos pré-eritrocitários, que se multiplicam por reprodução assexuada do tipo esquizogônica, formando uma célula (hepatócito infectado) denominada esquizonte. Posteriormente, o núcleo do esquizonte sofre várias divisões mitóticas. Em seguida, ocorre a divisão citoplasmática do esquizonte, originando diversos parasitas intracelulares em formas denominadas merozoítos, as quais são capazes de expressar proteínas que apresentam especificidade por moléculas presentes na superfície dos eritrócitos. Posteriormente os hepatócitos infectados se rompem liberando os parasitas que ficam livres para invadir a circulação (Mishra et al., 2008; Kamya et al., 2015).

Em infecções causadas por *P. vivax* e *P. ovale*, o *Anopheles* também inocula populações geneticamente distintas de esporozoítos, que não iniciam imediatamente o seu desenvolvimento eritrocítico, então permanecem em estado de latência dentro dos hepatócitos, originando formas denominadas hipnozoítos (Wells et al., 2010; Campo et al., 2015). As reicidivas maláricas ocorrem em decorrência da esquizogonia tardia dos hipnozoítos no interior dos hepatócitos, podendo estes permanecerem quiescentes no fígado por um período de até cinco anos (Shanks et al., 2013; Campo et al., 2015; Mikolajczak et al, 2015). Os parasitas *P. falciparum* e *P. malariae* não desenvolvem hipnozoítos e não desenvolvem reicidivas da doença (Soulard et al., 2015), no entanto, o *P. falciparum* apresenta maior virulência dentre todos eles.

Após a liberação dos merozoítos através da ruptura dos esquizontes, esses parasitas atingem a corrente sanguínea e invadem os eritrócitos (glóbulos vermelhos), reproduzem-se internamente e dão origem a eritrócitos infectados, ou esquizonte eritrocítico. Cada esquizonte por segmentação citoplasmática origina entre 8 e 12 novos merozoítos. Quando ocorre a lise do eritrócito, os merozoítos são liberados na corrente sanguínea e invadem novas hemácias, reiniciando o ciclo. A medida que a doença avança, os merozoítos diferenciam-se em gametócitos, que serão sugados pelo mosquito durante a picada e perpetuar o ciclo do parasita no vetor e transmissão (Keitany et al., 2016).

2.4 Epidemiologia

A malária é de importância mundial, acometendo, principalmente, os países africanos, latino-americanos e os da região sudoeste da Ásia (OMS, 2015). Dados recentes indicam que apesar do número de casos de malária ter declinado, esta patologia ainda apresenta morbidade e mortalidade considerável, afetando em 2015, cerca de 216 milhões de indivíduos ao redor do mundo, e causando aproximadamente 429 mil mortes no mesmo ano (OMS, 2015). Ressalta-se que tais dados podem ser ainda mais expressivos, uma vez que a sub-notificação ainda ocupa papel de destaque nesses países.

No Brasil, os casos de malária encontram-se localizados principalmente na região conhecida como Amazônia Legal, a qual compreende aos Estados do Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins. Nestas regiões, encontram-se quase 100% dos casos notificados (Guimarães et al., 2016), sendo estes, em 2011, cerca de 40 milhões, correspondendo a 20% da população brasileira (OMS, 2014).

A grande ocorrência de malária nessa região pode ser justificada pela presença de floresta tropical úmida e condições climáticas favoráveis ao desenvolvimento do mosquito vetor; pela presença de grupos humanos com atividade ocupacional, como por exemplo, agricultores, garimpeiros e madeireiros, os quais encontram-se vulneráveis à picada do inseto; pela carência de infraestrutura física, social e de atenção básica de saúde; e pelo uso irracional de drogas antimaláricas (Pina-Costa et al., 2014; Sampaio et al., 2015).

Ainda, a malária pode evoluir para malária grave, atingindo cerca de 15% a 35% dos pacientes mesmo recebendo tratamento farmacológico adequado, e quando não tratada é letal. As manifestações clínicas mais frequentes da malária grave incluem malária cerebral, malária placentária e malária pulmonar, podendo desencadear, no paciente, quadros de acidose metabólica, icterícia grave, edema pulmonar, insuficiência renal, danos neurológicos e coma (Turner et al., 2013; Baldeviano et al., 2015).

Uma das principais alterações clínico-patológicas presente na malária grave é a malária cerebral, a qual é caracterizada por coma e convulsões em pacientes infectados pelo *P. falciparum* (Manyiki et al., 2015; Yusuf et al., 2017). A sintomatologia é progressiva, inicialmente caracterizada por cefaleia, alterações de comportamento, desorientação, convulsões e coma. A evolução da malária cerebral está relacionada à presença de uma resposta inflamatória sistêmica e principalmente a uma resposta inflamatória amplificada no sistema nervoso central (SNC), causando inflamação e

edema cerebral (Seydel et al., 2015). Esta resposta é caracterizada por um padrão Th1, responsável pela indução de citotoxicidade e resposta inflamatória decorrente da liberação de IL-2, IFN-γ e TNF-α, com posterior ativação das vias do estresse oxidativo cerebral e dano tecidual nervoso (Dunst et al., 2017). Assim, a malária cerebral apresenta-se como uma manifestação neurológica severa e potencialmente fatal resultante da infecção pelo *P. falciparum* (em humanos) e *P. berghei* (em roedores).

2.5 Malária cerebral

A malária cerebral é uma complicação neurológica grave decorrente da infecção pelo *P. falciparum,* caracterizada como uma síndrome complexa ainda não completamente elucidada, sendo definida como encefalopatia difusa potencialmente reversível (Oliveira et al., 2017; Tamzali et al., 2018). Dentre os grupos de risco mais vulneráveis estão crianças menores de cinco anos e pacientes primíperas, que vivem em áreas endêmicas (Idro et al., 2010; Mutombo et al., 2018). O diagnóstico desta forma clínica da doença depende da presença dos critérios a seguir: 1) estado de coma, onde é definido (em adultos) pela escala de Glasgow e em lactantes pela escala de Blantyre; 2) exclusão de outras neuropatologias como a meningite bacteriana e encefalites virais prevalentes na região; 3) formas assexuadas de *P. falciparum* em exame de gota espessa. Pode haver ou não convulsões nessa fase da doença (Oliveira et al., 2017; Plewes et al., 2018).

A evolução da malária cerebral está relacionada à presença de uma resposta inflamatória sistêmica e principalmente a uma resposta inflamatória no SNC (Ghazanfari et al., 2018). Em condições normais, o SNC encontra-se protegido de respostas imunes e inflamatórias; proteção esta associada à barreira hematoencefálica, uma barreira altamente seletiva que separa o sangue em circulação do líquido cerebral extracelular no SNC (Sharif et al., 2018; Sonar et al., 2018). A barreira hematoencefálica (BHE) é formada por células endoteliais cerebrais, que são ligadas por junções de oclusão intercelulares especializadas com uma resistividade elétrica muito elevada, sendo responsável por controlar o acesso de células e moléculas ao microambiente cerebral (Varatharaj et al., 2017). Vale ressaltar que em doenças como a malária cerebral, a integridade da BHE encontra-se comprometida em decorrência das respostas imune e inflamatória desencadeada nos locais onde há sequestro de eritrócitos infectados no endotélio da microcirculação cerebral. Este evento leva à obstrução da

microvasculatura, além de possibilitar a invasão do parasita no cérebro, resultando em extravazamento plasmático com edema cerebral, e a difusão de células e mediadores inflamatórios para o tecido nervoso (Mosnier et al., 2016; Petersen et al., 2016; Dunst et al., 2017). Estas alterações na homeostase do SNC promove ativação de células da glia, redistribuição de astrócitos, alterações na morfologia da BHE e dano neuronal (Idro et al., 2010; Petersen et al., 2016).

2.6 Resposta do hospedeiro na malária cerebral

Eritrócitos infectados com o *Plasmodium sp.* expressam em suas membranas antígenos específicos do parasito, que podem ser reconhecidos pelas células do sistema imune e desencadeiam uma resposta inflamatória. Particularmente, durante os primeiros estágios da infecção, a imunidade inata envolvendo a proliferação de monócitos e outras células do sistema imune apresentam um papel crucial sobre o controle da parasitemia (crescimento do parasita no sangue), além disso, a fagocitose de merozoitos e eritrócitos infectados após cada esquizogonia é o principal mecanismo de defesa do organismo (Gomes et al., 2016; Gowda et al., 2018).

A resposta imune inicial na malária está associada a diversos parâmetros e envolve a participação do sistema complemento e produção de mediadores inflamatórios, como as citocinas, principalmente interferon-gama (IFN-γ) e fator de necrose tumoral (TNF-α) através da ativação de monócitos/macrófagos, células NK e NKT. As proteínas do sistema complemento, assim como as células NK e NKT são responsáveis pela lise da membrana do parasito e produção de IFN-γ (Wolf et al., 2017; Arora et al., 2018), enquanto monócitos/macrófagos apresentam um papel importante na fagocitose dos plasmódios livres, hemácias infectadas e produção de TNF-α (Ataíde et al., 2011; Matsuzaki et al., 2011; Rogerson et al., 2018).

O glicosilfosfatidilinositol (GPI) é um glicolipídeo presente na membrana do merozoíto do *Plasmodium sp.* e é caracterizado como um PAMP (padrões moleculares associados a patógenos) devido a sua capacidade de ser reconhecido por receptores presentes em células do sistema imune, iniciando assim, uma resposta inata ao microorganismo e induzindo a produção de mediadores inflamatórios como a TNF-α e IL-12 (De Souza et al., 2010; Dunst et al., 2017; França et al., 2017). A IL-12 está relacionada com o aumento da atividade citotóxica das células NK, proliferação de células NK e células T, além da produção de IFN-γ pelas mesmas (Sarangi et al., 2014).

A IL-12 também apresenta uma função importante na resposta inicial entre a imunidade inata e a adquirida devido a sua participação no início de uma resposta do tipo Th1 (Sarangi et al., 2014). Estudos demonstraram que a IL-12 desempenha um papel protetor durante o quadro malárico devido a aumentar a diferenciação de células T $CD4^+$ em células Th1, promovendo uma maior síntese de IFN-γ (Raballah et al., 2017), que age induzindo os monócitos a secretarem TNF-α, promovendo a formação de radicais livres, como o ânion superóxido (e posterior produção de peróxido de hidrogênio) e o óxido nítrico (Weinberg et al., 2016), espécies reativas que podem desencadear a peroxidação lipídica, endoteliotoxicidade e posterior dano tecidual.

É importante rassaltar que o sequestro de eritrócitos na microcirculação cerebral ocorre através da interação de antígenos polimórficos expressos nas membranas das células infectadas, como a P*f*EMP1 e receptores de superfícies de células epiteliais do hospedeiro (CD36 e ICAM-1), sendo ICAM-1 a principal molécula envolvida nesse processo (Brugat et al., 2014; Shabani et al., 2017). Após esse evento, os aglomerados de células infectadas aderidas ao endotélio da microcirculação cerebral desencadeiam uma resposta imune local, recrutando células do sistema imune que fazem o reconhecimento dos antígenos presentes nos eritrócitos e iniciam a produção de citocinas proinflamatórias, como IL-2, IFN-γ e TNFα, estas, por sua vez, induzem essas células a realizar a fagocitose e a produção de espécies reativas de oxigênio (EROs) e nitrogênio (ERNs), gerando o estresse oxidativo e nitrosativo com o intuito de destruir o patógeno e sinalizar a resposta imune (Gomes et al., 2016).

Nesse contexto, o estresse oxidativo apresenta dois papéis distintos: o primeiro relacionado à modulação da fagocitose nos macrófagos e à oxidação de compostos do patógeno (ocasionando morte do parasito) (Gomes et al., 2016); e o segundo relacionado à patogênese da malária cerebral e invasão do SNC pelo *P. falciparum*, uma vez que a produção de ERO e ERN dá origem a produtos oxidantes, como o peróxido de hidrogênio e o óxido nítrico, nos locais onde há sequestro de células vermelhas infectadas, causando a oxidação de componentes estruturais da barreira hematoencefálica (comprometendo sua integridade física) e de fosfolipídios (peroxidação lipídica) presentes nas membrananas das células, causando lesão celular com danos ao endotélio, permitindo, assim, a entrada do agente etiológico no sistema nervoso e a geração de um processo inflamatório neural, responsável pela inflamação e formação de edema cerebral, com posterior morte neuronal como resultado da resposta

imune local e produtos derivados do estresse oxidativo (Wah et al., 2016; Sarr et al., 2017).

Para uma melhor compreensão da patogenia da malária cerebral, há duas teorias: A obstrução mecânica e a inflamação. A primeira relaciona-se com a malaria cerebral como resultado do sequestro de eritrócitos infectados para os capilares cerebrais, obstruindo o fluxo sanguíneo e causando hipóxia cerebral (Saiwaew et al., 2017); e a segunda teoria, que é baseada na resposta imune proinflamatória do tipo Th1, através da produção e liberação de mediadores inflamatórios nos locais dessas células infectadas, especialmente TNF-α e IFN-γ, que induzem as células ao estresse oxidativo, causando lesão tecidual ao endotélio e a barreira hematoencefálica, deixando o sistema nervoso central exposto ao parasito (Dunst et al., 2017), provocando sintomas relacionados à encefalopatias, tais como sonolência, prostração intensa, convulsões, alteração do nível de consciência, coma e morte (Plewes et al., 2018). De acordo com a organização mundial da saúde, a malária cerebral requer a presença de coma profundo com a escala de Glasgow >9 (WHO, 2015). Ainda, é importante ressaltar que os neurônios são células permanentes e, portanto, não se multiplicam, assim, a morte de neurônios no SNC pode ser apontada como a principal causa para o desenvolvimento de sequelas em pacientes sobreviventes.

Além disso, a produção de espécies reativas de oxigênio por células endoteliais e por células inflamatórias, parece estar relacionada à apotose de células endoteliais (Jeong 2016; Zhu et al., 2018). De forma complementar, níveis elevados de H_2O_2 estão relacionados à aderência aumentada de eritrócitos em capilares de órgãos vitais (Isah e Ibrahim, 2014). Dados obtidos de estudos em camundongos com deleção gênica para NADPH oxidase mostram que a falta dessa enzima resulta em uma produção deficiente de superóxido pelos macrófagos, causando assim, a elevação da parasitemia e exacerbação da malária (Sanni et al., 1999). Além disso, a liberação de espécies reativas de oxigênio parece regular a expressão de moléculas envolvidas na adesão de eritrócitos infectados ao endotélio, sendo a CD36 e a CSA as moléculas de maior relevância na adesão de macrófagos e eritrócitos infectados ao endotélio de pacientes com malária (Alister et al., 2012; El-Assaad et al., 2013; Pais et al., 2018).

Além da produção de ROS, as espécies reativas de nitrogênio (ERN) também participam da respota imune na malária cerebral (Mwanga-Amumpaire et al., 2015; Dellavalle et al., 2016). De forma semelhante ao estresse oxidativo, o estresse nitrosativo caracteriza-se pela produção em excesso de radicais livres derivados do

nitrogênio, moléculas responsáveis pela oxidação e nitração de lipídeos, proteínas e ácidos nucleicos (Valez et al., 2013; Mustafa et al., 2018), portanto, também influenciam na patogênese e progressão da malária cerebral e na defesa do hospedeiro. O óxido nítrico (NO) é a principal ERN formada durante a respota imune contra o *P. falciparum* e *P. berghei,* nesse caso, o NO contribui para o dano neuronal por agir como vasodilatador e na oxidação/nitração de lipídeos de membranas celulares, contribuindo para a lesão endotelial e barreira hematocefálica, assim, deixando o sistema SNC vulnerável a entrada do parasita e a ação de moléculas do estresse oxidativo e nitrosativo (Clark et al., 1991; Martins et al., 2012; Cabrales et al., 2011; Mustafa et al., 2018). Além disso, o NO reage com diversos outros radicais livres e moléculas oxidantes, formando produtos mais potentes no que diz respeito a peroxidação lipídica; como a reação que ocorre entre o H_2O_2, formando o peroxinitrito, potente indutor de morte parasitária e dano tecidual (De Melo et al., 2017; Pereira et al., 2018; Surikow et al., 2018). Nesse caso, o conhecimento de mecanismos relacionados à regulação da produção dessas moléculas, como o estudo de receptores envolvidos nesta sinalização, é de extrema importância para o desenvolvimentos de novos alvos terapêuticos para a malária cerebral.

Portanto, de importância para este projeto, encontram-se os receptores de potencial transitório (Transient Receptor Potential), conhecidos como receptores TRP, uma superfamília de receptores constituída por mais de 30 membros, incluindo o TRP Vanilóide 1 (TRPV1) (Fernandes et al., 2012a). Sendo assim, dentro do contexto da resposta imune na malária cerebral, o TRPV1 hoje é reconhecido como um sensor e modulador do estresse oxidativo/nitrosativo em diferentes doenças, principalmente as de origem infecciosas e inflamatórias. Evidências obtidas à partir de modelos experimentais *in vivo* sugerem que a ativação do TRPV1 pode influenciar a resposta imune inata do hospedeiro à infecções, incluindo a resposta contra a malária, por mecanismos de regulação de vias imunes e oxidantes. Para uma melhor compreensão da patofisiologia deste receptor, o mesmo será discutido na próxima sessão.

2.7 O receptor de potencial transitório vanilóide 1 e sua relevância como sensor do estresse oxidativo e modulador de processos infecciosos

O receptor de potencial transitório vanilóide 1 é um canal iônico não seletivo permeável ao Ca^{2+} e pertencente à subfamília TRPV (do inglês, *transient receptor*

potential vanilloid) dos TRPs. São expressos nas fibras sensoriais to tipo C e Aδ (Fernandes et al., 2012a) e em uma série de células e tecidos não-neuronais, incluindo células inflamatórias (Fernandes et al., 2012b) e células da musculatura lisa da aorta e microvasculatura cerebral (Sand et al., 2015). O TRPV1 é codificado pelo gene TRPV1 e foi clonado primeiramente em 1997 em células murinas do glânglio da raiz dorsal de camundongos e está relacionado a uma série de eventos fisiológicos e fisiopatológicos (Caterina et al., 1997).

A nomenclatura "vanilóide" deste receptor foi adotada devido a seu principal agonista exógeno, a capsaicina (8-metil-N-vanilil-trans-6-nonamida), componente ativo de pimentas do gênero *Capsicum* e que apresenta em sua estrutura química o grupamento homovanilil. Esse composto possui características irritantes, agindo como agonista do TRPV1 e produzindo a sensação de queimação, ardência e dor quando entra em contato com tecidos de mamíferos, incluindo os humanos (Darre et al., 2015; Smutzer et al., 2016; Yang, 2017)

O TRPV1 participa de vários eventos fisiológicos e patofisiológicos incluindo a manutenção da homeostase, percepção da dor e progressão de diferentes doenças tais como doenças infecciosas, artrite, colite, asma, dentre outras (Fernandes et al., 2012a; Choi et al., 2018; Christie et al., 2018; Wang et al., 2018; Gorbunov et al., 2019). Recentemente, um papel protetor foi atribuído ao receptor TRPV1 na resposta à infecção bacteriana, uma vez que na ausência da ativação funcional deste receptor leva à perda de função das células inflamatórias, mais especificamente, macrófagos, que tornam-se deficientes no combate à infecção, uma vez que estas células perdem a capacidade de realizar fagocitose e de gerar mediadores como óxido nítrico e espécies reativas de oxigênio (Fernandes et al., 2012b). Além disso, a deficiência da sinalização via o receptor TRPV1 promove aumento da apoptose de macrófagos (Fernandes et al., 2012b). Ainda, evidências sugerem que o TRPV1 pode ser ativado na presença de H_2O_2 (Bose et al., 2015; Uslusoy et al., 2017) e que a produção do ânion superóxido pode afetar a regulação e expressão deste receptor (Fernandes et al., 2014).

Por possuir sítios de ligação ortostéricos e alostéricos, o TRPV1 pode ser ativado por uma larga variedade de substâncias e estímulos exógenos e endógenos (Velisetty et al., 2017; Yang et al., 2017). Importantemente, seus agonistas ou ativadores endógenos participam de respostas inflamatórias, modulando a produção de mediadores da inflamação, a expressão de receptores, a migração celular, dentre outros eventos (Fernandes et al., 2012; Gouin et al., 2017; Kim, 2018). Dentre os ativadores do TRPV1

incluem-se espécies reativas de oxigênio (H_2O_2 e O_2^-), anandamida, N-raquidonoildopamina, 12-S-ácido hidroperoxi-eicosatetranoico, oleoiletanolamida e N-oleoildopamina, 9(s)-HODE e 13(s)-HODE (De Petrocellis et al., 2012; Alsalem et al., 2013; Green et al., 2016; Ramirez-Barrantes et al., 2018). Portanto, a compreensão dos mecanismos de progressão da malária cerebral

Recentemente, foi demonstrado que a ativação endógena do TRPV1 pode influenciar a resposta do hospedeiro à malária (Fernandes et al., 2014). De fato, o antagonismo do receptor TRPV1 é capaz de modular a resposta imune inata em modelo murino de malária cerebral, por reduzir a peroxidação lípica e os níveis circulantes de TNFα; além de inibir a ativação e expansão de monócitos produtores de EROs e TNFα (Fernandes et al., 2014). Estas foram as primeiras evidências de que o receptor TRPV1 pode atuar como importante regulador das alterações sistêmicas decorrentes da malária. Por outro lado, o real impacto da ativação ou bloqueio deste receptor nas alterações neurológicas características da malária cerebral, permanece por ser investigado. Portanto, diversos parâmetros fisiopatológicos relacionados com a progressão da malária para a malária cerebral ainda permanecem não completamente esclarecidos, o que torna os modelos experimentais da malária uma importante fonte de investigação científica para uma melhor compreensão dos mecanismos da doença. Modelos *in vivo* de malária cerebral serão discutiso a seguir.

2.8 Modelo de malária cerebral

Nas últimas duas décadas, estudos laboratoriais em países de clima temperado envolvendo modelos murinos de malária cerebral vêm sofrendo um crescente e notável aumento (Craig et al., 2012; Huang et al., 2015; Minkah et al., 2018), além da utilização de animais geneticamente modificados, com deleção gênica para as diversas proteínas de interesse científico (Huang et al., 2015). Os modelos de malária em animais são ferramentas de investigação para a melhor compreensão da doença humana, visto que os parasitos da espécie *Plasmodium sp.* compartilham diversos parâmetros de invasão nos diferentes organismos (Craig et al., 2012; Huang et al., 2015; Ng et al., 2017; Minkah et al., 2018). Para tal, o *Plasmodium berghei* estirpe ANKA é amplamente utilizado em camundongos C57BL/6 devido apresentarem-se mais susceptíveis do que a linhagem BALB/C, a qual demonstra ser mais resistente à infecção (os animais morrem geralmente de anemia grave e hiperparasitemia, mas não desenvolvem malária

cerebral), neste modelo, em vez de morrer lentamente de anemia, animais infectados com essa estirpe do parasito morrem mais rapidamente devido a uma síndrome cerebral complexa associada a convulsões (Scheller et al., 1994; Baptista et al., 2010; Ghazanfari et al., 2018). Assim, o modelo de malária cerebral através da inoculação intraperitoneal de eritrócitos infectados com *P. berghei* ANKA e posterior evolução da doença é capaz de mimetizar diversos parâmetros maláricos semelhantes aos causados pelo *P. falciparum*, responsável pela malária cerebral em humanos (De Oca et al., 2013; Junaid et al., 2017).

Apesar de ser possível a reprodução da doença em camundongos, a encefalopatia malárica murina apresenta diversas diferenças da humana. O modelo apresenta uma base imunológica no qual as citocinas pró-inflamatórias apresentam o papel principal na evolução clínica (Ghazanfari et al., 2018), apesar disso, eritrócitos murinos infectados com *P. berghei* ANKA são mais sequestrados no pulmão e no tecido adiposo do que na microvasculatura cerebral, embora ambos utilizam a mesma proteína de adesão, a molécula CD36 (De Oca et al., 2013). Camundongos C57BL/6 morrem em torno do 6º e 8º dia após infecção apresentando baixa parasitemia e sinais neurológicos como ataxia e convulsões, sendo a linhagem de animais que mais apresentam sinais graves da doença, deste modo, sendo o mais utilizado por mais se assemelhar com a malária clínica (White et al., 2010; Carvalho et al., 2010; Torre et al., 2018); vale ressaltar que a taxa de mortalidade em animais *wild type* (WT) é de quase 100%, o que não ocorre em humanos (Basir et al., 2012; Pereira et al., 2019). Com base nessas diferenças, diversos questionamentos sobre os modelos experimentais foram levantados. Afinal, é válido ou não o modelo malárico murinho para entender a doença humana? Similarmente à malária humana, em camundongos a doença evolui através da ativação de vias parecidas, estimulando células da resposta inata, como monócitos/macrófagos e células NK e consequente liberação de mediadores pró-inflamatórios, como TNF-α e IFN-γ (Ghazanfari et al., 2018) e espécies reativas de oxigênio e nitrogênio, como o peróxido de hidrogênio e o óxido nítrico (Basir et al., 2012; Al-Shaebi et al., 2018; Kumar et al., 2018), iniciando, assim, o processo inflamatório.

Como foi descrito acima, apesar das disparidades com a doença humana, o modelo de malária cerebral com *P. berghei* ANKA vêm contribuindo bastante para a compreensão dos diversos mecanismos inflamatórios e de invasão dos parasitos do gênero *Plasmodium sp.*, mecanismos os quais são de grande importância para a compreensão e desenvolvimento de novos alvos terapêuticos, pois a malária cerebral

ainda permanece uma patologia com alta taxa de mortalidade e presença de sequelas em pacientes sobreviventes (Oluwayemi et al., 2013; Peixoto et al., 2013; OMS 2016). Com isso, a investigação por novas proteínas e novos bioprocessos envolvidos durante a infecção é de extrema importância, não somente para a comunidade científica, mas também para o desenvolvimento de novas drogas e alvos terapêuticos capazes de tratar ou reverter a progressão da infecção. Há poucas décadas a descoberta da família dos receptores de potencial transitório (TRP) trouxe uma nova luz para o tratamento de diversas doenças infecciosas e inflamatórias, pois muitos membros desta superfamília de receptores de membrana participam diretamente de diversos processos relacionados ao sistema imunológico, como o TRPV1 e o TRPA1.

Fernandes et al., (2014) demonstrou pela primeira vez que a ativação do TRPV1 pode influenciar a resposta do hospedeiro à malária. Os dados dessa pesquisa demonstraram que o antagonismo do TRPV1 modula a resposta imunológica inata em modelo murino de malária cerebral, através da redução da peroxidação lípica e dos níveis circulantes de citocinas inflamatórias, além dos efeitos celulares. Estas foram as primeiras evidências de que o receptor TRPV1 pode atuar como importante regulador das alterações sistêmicas decorrentes da malária. Por outro lado, o real impacto da ativação ou bloqueio deste receptor nas alterações neurológicas características da malária cerebral, permanece por ser investigado. Assim, considerando a relevância da resposta imune e do estresse oxidativo para o desencadeamento e evolução da malária cerebral, e a existência de um mecanismo de retro-alimentação entre a ativação do TRPV1 e a produção de ROS, esta tese de doutorado teve como objetivo investigar a relevância deste receptor e suas inter-relações com a resposta imune e vias pró- e anti-oxidantes, na evolução do quadro clínico da malária cerebral; através do uso do antagonista seletivo para este receptor, o SB366791, e animais com deleção gênica para o TRPV1 em modelo murino de malária cerebral *in vivo*.

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LISTA DE PUBLICAÇÕES:

ARTIGO 1: Oxidative and nitrosative stresses in cerebral malaria: can we target them to avoid a bad prognosis? A ser submetido à revista: British Journal of Pharmacology.

ARTIGO 2: TRPV1 Contributes to Cerebral Malaria Severity and Mortality by Regulating Brain Inflammation. Publicado na revista: Oxidative Medicine and Cellular Longevity, 2019.

ARTIGO 1

Oxidative and nitrosative stresses in cerebral malaria: can we target them to avoid a bad prognosis?

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Running title: Targeting oxidative and nitrosative stresses in cerebral malaria

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Abstract

There is currently a global effort to reduce malaria morbidity and mortality. Still, malaria results in the deaths of thousands of people yearly. Malaria is caused by *Plasmodium sp*., a parasite transmitted through of an infected female *Anopheles* mosquito. Treatment timing plays a decisive role in reducing mortality and sequelaes associated with the severe forms of the disease such as cerebral malaria (CM). The available antimalarial therapy is considered effective but parasite resistance to these drugs has been observed in some countries. Antimalarial drugs act increasing parasite lysis especially through targeting oxidative stress pathways. Here, we discuss the roles of oxidative stress and reactive nitrogen intermediates in CM, by presenting evidences on how drugs targeting these systems have contributed to CM treatment and control.

Introduction

Malaria is an infectious, parasitic, systemic and non-contagious disease of great morbidity and mortality which resulted in over 400,000 deaths in 2015 (WHO, 2016). It is caused by *Plasmodium* sp. which is introduced in to the host circulation through the bite of an infected female *Anopheles* mosquito. The signs and symptoms of malaria appear from day 8 to 25 post-infection and are non-specific in the uncomplicated form of the disease such as headache, fever and chills (Camargo et la., 2018; Hase, 2018); however, as disease becomes severe, one may present with respiratory distress, convulsions, prostration, coma, renal failure, metabolic acidosis, jaundice, hypoglycaemia, neurological sequelae after cerebral malaria, amongst others (Bartoloni et al., 2012; Chaparro-Narváez et al., 2016; Plewes et al., 2018). Despite the global efforts to reduce malaria cases worldwide, a rise in the numbers of infected individuals has been observed in some countries, including Brazil, in the last years (Recht et al., 2017; Sáenz et al., 2017). Also, severe cases of the disease continue to be registered in many countries even when anti-malarial therapy is available (Geleta et al., 2016; WHO, 2016), highlighting the need for a better understanding of the mechanisms of disease and for novel and/or complementary therapeutic approaches.

The definition of severe malaria is associated with mortality due mostly to *P. falciparum* infection. Different clinical syndromes manifest in severe malaria including cerebral malaria (CM). CM is a clinical syndrome of the severe form of the disease, mostly associated with *P. falciparum* and a high lethality (Gething et al., 2016; WHO, 2016). CM is characterized by neurological complications such as coma, convulsions, abnormal muscle tone and posture, and loss of reflexes (Shikani et al., 2012; Yusuf et al., 2017). Most of the surviving patients make a full neurological recover from CM; however, some can present with irreversible neurological and/or cognitive sequelae (Christensen and Eslick, 2015; Mergani et al., 2015; Holmberg et al., 2017; Shabani et al., 2017).

Evidences suggest a role for oxidative and nitrosative stresses in this clinical syndrome, as a result of host-*Plasmodium* interactions; these, will be discussed herein. Pharmacological interventions based on the current knowledge of oxidative stress modulators will be also discussed, in the light of cerebral malaria (CM).

Parasite-host protein interactions

Malaria progression to CM largely depends on the interaction between host and parasite proteins, especially during blood stage infection. Following the infection of erythrocytes, the plasmodium multiplies and maturates, with its proteins becoming expressed on the infected erythrocyte membranes and facilitating the interaction between infected erythrocytes and the endothelial cells of the host (Figure 1). This in turn, contributes to the adherence of infected erythrocytes to the brain microvasculature, with subsequent disruption of the blood brain barrier and brain inflammation.

In this context, we highlight the importance of the erythrocyte membrane protein 1 (P*f*EMP1) family, encoded by about 60 var genes, which contributes to the adherence of infected red blood cells to the brain microvasculature and to further development of CM (Fernandez et al., 1998; Bernabeu et al., 2019; Storm et al., 2019). This is due to the ability of these proteins, to bind to endothelial receptors such as cluster of differentiation 36 (CD36), intercellular adhesion molecule 1 (ICAM-1), integrins αVβ3 and αVβ6, and the endothelial protein C receptor (EPCR) (Fernandez et al., 1998; Chesnokov et al., 2018; Bernabeu et al., 2019; Storm et al., 2019). Interestingly, binding of P*f*EMP1 to CD36 results in the recruitment of integrins such as α5β1 to brain endothelial cells, making erythrocyte adhesion to the microvasculature tighter (Davis et al., 2013). Of note, this integrin is critical to the maintenance of the blood brain barrier (Kant et al., 2019). Indeed, α5β1 was shown to induce disruption of endothelial barriers through breakdown of VE-cadherin and claudin 5 (Huang et al., 2011; Labus et al., 2018).

By binding to EPCR, P*f*EMP1 contributes to endothelial activation by reducing the levels of activated protein C, an inhibitor of thrombin generation (Moxon et al., 2013; Petersen et al., 2015). Excessive binding to EPCR leads to its reduction resulting in thrombin-mediated inflammation and subsequent endothelial dysfunction (Bernabeu and Smith, 2017). Also, during CM, the endothelium over-expresses ICAM-1 further increasing the binding of infected erythrocytes to the endothelium, an effect which has been associated with the availability of nitric oxide (NO) (Pino et al., 2005; Serghides et al., 2011). .

Recently, a subset of the RIFIN family of parasite proteins which also becomes expressed on the membrane of infected erythrocytes, was found to bind to the leukocyte immunoglobulin-like receptor B1 (LILRB1) (Saito et al., 2017) expressed on immune cells such as lymphocytes and monocytes (van der Touw et al., 2017). RIFIN proteins

are encoded by 150-200 *rif* genes and are unique to *P. falciparum* (Craig and Scherf, 2001). Interestingly, infected erythrocytes expressing these proteins were found to cause down-regulation of immune cells and to be more likely to bind to LILRB1 in patients with CM in comparison with non-severe malaria individuals (Saito et al., 2017).

More recently, PbmaLS_05, a plasmodium antigen expressed during the pre- and intra-erythrocytic stages of infection, was shown to contribute to the rupture of the blood brain barrier by activating a specific subset of $CD8^+$ lymphocytes (Fernandes et al., 2018). Interestingly, deletion of PbmaLS_05 conferred protection against CM development; an effect which was associated with reduced inflammation (oedema and microhaemorrhages) and parasite load in the brain tissue of mice with experimental CM (Fernandes et al., 2018).

Overview of oxidative and nitrosative stresses

The close interaction between host and parasite proteins triggers an inflammatory process in to the vasculature, in addition to obstruction of the brain microvasculature by infected and non-infected erythrocytes (van der Heyde et al., 2006; Tripathi et al., 2009; Souza et al., 2015). Both responses contribute to CM progression, resulting in unbalanced inflammatory mediator release, vascular leakage, disruption of blood brain barrier and ultimate leading to cytotoxic effects in the brain tissue (Gramaglia et al., 2006; Narsaria et al., 2012; Ong et al., 2013; Dunst et al., 2017).

In this context, alterations in the levels of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) play important roles in CM.

ROS (Figure 2) are formed as part of the host response to a range of stimuli from oxygen inspiration to exposure to harmful stimuli such as ultraviolet rays or microorganisms. In this process, O_2 can be reduced by NADPH oxidase, an enzyme largely expressed in phagocytes, to superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is then, dismuted to H_2O_2 by superoxide dismutase (SOD) (He et al., 2017; Khazaei et al., 2017; Pizzino et al., 2017). Elevated levels of H_2O_2 can be converted to H_2O and O_2 by catalase or reduced by iron to hydroxyl radicals ($HO^{\bullet}$), highly reactive products which can oxidize carbohydrates, lipids, proteins and DNA (Nita et al., 2016; Liguori et al., 2018). H_2O_2 can be also removed by the glutathione and thioredoxin systems in a dynamic process of oxidation and reduction of glutathione and thioredoxin by the specific enzymes glutathione

peroxidase (GPx) and reductase (GR), and thioredoxin reductase (TrxR); respectively (Ren et al., 2017; Awad et al., 2018).

Of note, not only the host, but also the plasmodium, present antioxidant machinery, although differences have been observed between the host and parasite systems. *P. falciparum* does not express catalase or GPx and expresses two SODs (P*f*SOD-1) and SOD-2 (Müller, 2004; Kavishe et al., 2017). Instead of GPx, the plasmodium expresses glutathione S-transferases (P*f*GST) (Harwaldt et al., 2002; Kavishe et al., 2017) which is suggested to be involved in resistance to antimalarial drugs (Ahmad and Srivastava, 2008; Kavishe et al., 2017).

RNI (Figure 2) include oxidised states and adducts of the nitrogen products of nitric oxide (NO) synthases (neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) including $NO^{\bullet}$, NO_2^-, S-nitrosothiols and peroxynitrite (OONO-); this later, formed by $NO^{\bullet}$ and $O_2^{\bullet-}$, can cause oxidation and nitration of lipids, proteins and DNA (De Melo et al., 2017; Surikow et al., 2018; Triquell et al., 2018; Novaes et al., 2019). This cascade initiates with the formation of NO from arginine by NO synthases; a process largely amplified by iNOS during inflammation (Guzik et al., 2003).

Oxidative and nitrosative stresses in cerebral malaria

ROS role in CM

Oxidative stress has been implicated in CM, occurring as part of the host response to *Plasmodium* sp. (Kavishe et al., 2017) (Figure 3), although its role in this form of the severe disease is not completely understood. The first evidences on the involvement of oxidative stress in CM are dated from the early 80´s. By using *in vitro* and *in vivo* mouse models, the first studies suggested that during host response to infection, activated phagocytes release ROS in order to kill the parasites, but also damage the endothelial cells of the cerebral vasculature, resulting in disruption of the blood brain barrier and bad disease prognosis (Demopoulos et al. 1980; Clark and Hunt 1983; Kontos 1985; Thumwood et al., 1989). Few years later, the administration of pro-oxidants was found to protect against CM in mice by killing the intra-erythrocytic parasite (Levander et al., 1995). In parallel, human studies demonstrated that patients with CM present brain oedema and mononuclear cell accumulation as a result of increased vascular endothelium permeability triggered by high oxidative stress, as

denoted by increased lipid peroxidation in their cerebrospinal fluid in comparison with control subjects; a response that was further exacerbated in fatal cases of the disease (Das et al., 1991). Also, analysis of brain samples obtained post-mortem from CM patients indicated that cells such as intravascular phagocytes, perivascular macrophages and reactive glia are more likely to express the oxidative stress marker hemo oxygenase-1 (HO-1) in haemorrhage areas (Medana et al., 2001). The increased expression of HO-1 mRNA and protein following CM, was also observed in the brains of plasmodium-infected mice (Linares et al., 2013; Imai et al., 2014). Interestingly, HO-1 protein expression is suggested to reach its highest levels of expression at late stages of the disease (stage IV), and this is preceded by increased expression of GPx at stage II (Linares et al., 2013).

Similarly, increased levels of malondialdehyde and conjugated dienes were detected in the brains of mice with CM and associated with cognitive impairment (Reis et al., 2010). Another study by Souza and collaborators (2018) showed that plasmodium infection triggers oxidative stress via protein carbonylation in the mouse hippocampus, thus, contributing to the neurological deficits and seizures observed in CM. Interestingly, cognition was improved in mice treated with the antioxidants desferoxamine or N-acetylcysteine in combination with the antimalarial drug chloroquine; of note, neither desferoxamine nor N-acetylcysteine affected parasitaemia. Also, administration of the antioxidants SOD, catalase or butylated hydroxyanisole was previously shown to reduce blood brain barrier permeability in mice with CM (Thumwood et al., 1989). Another study demonstrated that superoxide and peroxynitrite are deleterious to human endothelial cells incubated with erythrocytes infected with *P. falciparum* (Pino et al., 2003). Indeed, both the incubation with the superoxide dismutase mimetic MnTBAP and the transient supplementation of SOD1 conferred protection to human endothelial cells incubated with red blood cells infected with *P. falciparum* (Pino et al., 2003; Taoufiq et al., 2006).

Of importance, SOD and catalase mRNA expressions have been reported to become down-regulated as the neurological symptoms of CM progress (Linares et al., 2013). More recently, a proteomic analysis demonstrated that antioxidant peptides related to SOD1, glutathione S-transferase kappa 1, peroxiredoxin-6 and peroxiredoxin-5, and mitochondrial isoform A are depleted in the brains of patients with CM (Kumar et al., 2018). On the other hand, analysis of the plasma protein profile of children with uncomplicated and severe (CM and malaria anaemia) disease,

demonstrated that oxidative stress markers are altered in all malaria cases; however, oxidative stress was found to be highly associated with malaria anaemia whilst markers of endothelial damage were associated with CM (Bachmann et al., 2014).

RNI role in CM

Similarly to ROS, changes in the balance of nitric oxide may also contribute to CM outcome, although their definite roles in the disease are debatable. Indeed, studies have reported either a protective, deleterious or a null role for RNIs in CM. A study by Taylor and collaborators (1998) demonstrated no link between the levels of RNI and CM, or its clinical signs such as coma and loss of consciousness. Also, no consistent evidences of iNOS promoter haplotypes were found in Tanzanian CM children with CM (Levesque et al., 2010). However, many evidences have indicated protective or harmful roles especially for NO in CM. The controversial evidences on this gaseous transmitter in CM are based on the different techniques employed to measure RNI/NO, disease time-courses, experimental *versus* clinical studies and used pharmacological tools (knockouts versus blockers/activators of involved pathways); these aspects will be now discussed.

Evidences of a detrimental role for NO in CM

Initial hypothesis suggested that NO is generated during infection following the production of tumour necrosis factor α (TNFα) as a consequence of the contact of infected erythrocytes with the cerebral blood vessels, contributing to neuronal damage and the subsequent neurological signs of CM, in addition to increased intracranial pressure (Clark et al., 1991). Few years later, inhibitors of NO were administered to mice with CM; the study showed these drugs did not stop disease progression (Asensio et al., 1993; Kremsner et al., 1993).

Further studies in humans demonstrated that patients with longer coma duration and those in deeper coma present raised levels of reactive nitrogen species in their serum in comparison with those with lighter coma; also, fatal cases presented higher levels of these mediators than survivors (Al Yaman et al., 1996). In an another study, the cerebrospinal fluid concentrations of NO were found to be higher in children who died from CM in comparison to those who survived the disease (Weiss et al., 1998); the

same study suggested that NO derived from other sources than not leukocytes were detrimental to CM. Supporting a deleterious role for NO in CM, it was suggested that in the absence of TNF, NO levels are reduced and mice are protected from fatal CM (Rudin et al., 1997).

On the other hand, neither the oral administration of the iNOS inhibitor aminoguanidine nor the ablation of iNOS influenced CM in mice (Favre et al., 1999). Also, reports from Thai adults, demonstrated that patients with CM present increased brain iNOS expression during the acute phase of the syndrome (Maneerat et al., 2000). Inhibition of nitric oxide generation was found to protect the endothelial cells against *Plasmodium*-induced apoptosis (Pino et al., 2003).

Evidences of a protective role for NO in CM

Despite evidences on that NO production may be harmful to patients with CM; different studies have shown an opposite role for this transmitter in the syndrome. Accordingly, Tanzanian CM children were found to present with lower levels of leukocyte-dependent NO in their plasma (Anstey et al., 1996). Interestingly, Tanzanian and Kenyan children with polymorphism in the inducible NO synthase (iNOS) gene presented with increased circulating levels of nitric oxide and a protective phenotype against cerebral malaria (Hobbs et al., 2002). This finding was supported by a later study by Trovoada Mde and collaborators (2014) who indentified polymorphisms in the iNOS promoter associated with increased NO levels and protection against CM in Angolan children.

Analysis of the plasma levels of the NO synthesis substrate L-arginine demonstrated it to be reduced in Tanzanian children with CM in comparison with healthy controls; the low levels of L-arginine were associated with limited NO production and fatality (Lopansri et al., 2003). Also, systemic L-arginine levels in children recovered from CM were found to be compatible to that of healthy subjects of similar age; CM children presented low levels of NO at the time of hospital admission (Alkaitis et al., 2016). Of note, the low availability of systemic NO in CM has been attributed to increased conversion of L-arginine to ornithine and urea, instead of NO, by M2 monocytes (Weinberg et al., 2016); and also to low systemic levels of tetrahydrobiopterin (BH_4), an enzyme cofactor required for NO synthesis from L-arginine (Rubach et al., 2015).

In 2006, Gramaglia et al. (2006) showed that low NO availability contributes to CM in mice and that injection of an exogenous NO donor reduces plasma cytokines levels and brain oedema formation. Similarly, endothelial dysfunction in Indonesian individuals with severe malaria was found to be reversed by L-arginine infusion (Yeo et al., 2007). Polymorphism of eNOS enhances it expression and NO production, protecting against cerebral malaria in adult patients (Dhangadamajhi et al., 2009a). The same group also showed that polymorphisms decreasing NO are associated with low NO production and development of CM (Dhangadamajhi et al., 2009b). More recently, it was suggested that both eNOS and nNOS functions are impaired in mouse CM; a response that contributes to cerebrovascular dysfunction and reduced NO availability, and partially restored by BH_4 treatment (Ong et al., 2013). Interestingly, repeated treatment with the NO donor dipropylenetriamine NONOate delayed CM-induced death, improved cerebrovascular blood flow and vascular tone, especially in the microvasculature, and decreased leukocyte accumulation in these vessels (Cabrales et al., 2011). A similar result in survival was found in animals receiving prophylactic inhaled NO (Serghides et al., 2011), or L-arginine infusion (Ong et al., 2018).

Oxidative and nitrosative stress-based antimalarial drug development

Quinolines such as chloroquine and amodiaquine, are antimalarial drugs conventionally used to treat uncomplicated and severe forms of malaria including CM. These drugs bind to ferriprotoporphyrin IX (FP), a product of the digestion of hemoglobin by the plasmodium which becomes polymerized into hemozoin (Postma et al., 1996; Egan et al., 2002; Huy et al., 2017; Olafson et al., 2017). Thus, such therapies impair hemozoin formation leading to the accumulation of an important quantity of FP in the plasmodium digestive vacuole which then, reacts with phospholipids of the plasmodium cell membrane increasing its permeability and causing lysis of the parasite (Olafson et al., 2017). Derivatives of quinolines such as piperaquine also increase free FP (Fitch et al., 1982; Heller et al., 2018), whilst primaquine induces ROS formation in to the erythrocytes leading to lipid peroxidation and cell hemolysis (Bowman et al., 2005).

As a toxic product, free FP needs to be detoxified and this is made by the glutathione systems of both the host and the parasite (Famin et al., 1999; Kawazu et al., 2005). Other derivatives of quinolines include lipophilic quinolone-methanol drugs such

as lumefantrine and halofantrine which act reducing FP detoxification and inducing ROS production (Sullivan et al., 1998; Famin et al., 1999). By augmenting free FP or ROS, quinolines and derivatives demand an increased antioxidant activity especially by the glutathione system in order to the parasite to survive, and; resistance to this group of drugs was linked to increased parasite GST activity (Dubois et al., 1995; Srivastava et al., 1999).

In this scenario, artemisinin and its synthetic derivatives (dihydroartemisinin, artesunate, artemether and arteether) emerged as a novel class of antimalarial drugs; of note, dihydroartemisinin is the active metabolite of this class of drug, meaning the others need to be metabolized in to this compound in order to have antimalarial activity. Artemisinin-based drugs act by inducing free FP and ROS, ultimate leading to parasite death (Pandey et al., 1999; Kannan et al., 2002; Antoine et al., 2014; Wang et al., 2015). They can also, cause rapid depolarization of membrane potential of the parasite, an effect inhibited by ROS scavengers and iron chelators (Antoine et al., 2014). Evidences have also indicated that the excessive induction of ROS by artemisinin drugs can cause destruction and/or inhibition of the parasite mitochondria by acting on molecules involved in the electron transport chain of the plasmodium such as the cytochrome-*c* oxidase and NADH:quinone oxidoreductase (P*f*NDH2) system (Li et al., 2005; Antoine et al., 2014). Importantly, parasites lacking P*f*NDH2 are resistant to artemisinins (Li et al., 2005). Artemisinin-based drugs can also induce parasite dormancy which has been linked to resistance to these therapies (Peatey et al., 2015).

In order to deal with plasmodium resistance, in the 90´s, a combination therapy based on artemisinins was introduced. Five of these combinations were recommended for malaria treatment: i) artemether-lumefantrine, ii) artesunate-mefloquine, iii) artesunate-amodiaquine, iv) dihydroartemisinin-piperaquine and v) artesunate-sulphadoxine-pyrimethamine; still, resistance has been observed even to this combined therapy (Sinha et al., 2014).

In the last years, WHO (2010) has recommended the associations between quinoline derivatives and artemisinin derivatives (artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine or dihyd-roartemisinin-piperaquine). Although these associations have increased treatment efficacy, resistance or loss of sensitivity of *P. falciparum* to such therapies, have also been observed in some countries (Na-Bangchang et al., 2013; Otienoburu et al., 2016; Thanh et al., 2017; Wedam et al., 2018; Ishengoma et al., 2019). The overall effects of

the currently available antimalarial therapies and their effects on ROS are depicted in Figure 4.

Based on the existing therapies and the evidences gathered on the dual role of ROS and NO in CM, it becomes clear that malaria control depends on treatment timing and host´s response to infection, i.e., whilst ROS and free FP are important to kill the parasite, they may also harm the host, causing hemolysis and excessive ROS formation (Bowman et al., 2005; Kurth et al., 2016), as these drugs are not designed to target exclusively the parasite antioxidant system.

Targeting of RNI was also shown to be promising. Artemisinin-NO-donor hybrid compounds have been successfully tested in experimental CM (Bertinaria et al., 2015). Prophylactic treatment with the NO donor S-nitrosoglutathione decreased the incidence of experimental CM by reducing brain oedema formation, leukocyte accumulation and haemorrhage, in addition to inhibiting parasite growth (Zanini et al., 2012). Also, continuous infusion of L-arginine, the substrate for NO synthesis, alone or in combination with artesunate, improved survival of mice with CM (Ong et al., 2018).

In this context, with the discovery of new oxidative stress- and NO-controlling pathways, novel venues have been explored in malaria treatment. For instance, targeting of host proteins such as the transient receptor potential vanilloid 1 (TRPV1), a non-selective cation channel expressed on neuronal and non-neuronal cells of the host (such as immune and endothelial cells) (Fernandes et al., 2012a), and a recent discovered oxidative stress sensor and regulator of NO (Fernandes et al., 2012b), protected *P. berguei* ANKA-infected mice from CM (Pereira et al, 2019). This response was associated with increased oxidative stress (high levels of H_2O_2 and increased protein nitrotyrosine and carbonyl protein residues). Although blockage of TRPV1 was promising in experimental malaria, the clinical use of such drugs was suggested to be limited as it increases mortality in bacterial infections (Clark et al., 2007; Guptill et al., 2011; Fernandes et al., 2012a), which are frequent in areas with high malaria incidence (Bhattacharya et al., 2013; Church and Maitland, 2014; Park et al., 2016; Aung et al., 2018).

Overall, clinical and experimental data suggest that anti-ROS and FP therapies, and modulators of NO availability are historically and highly important to malaria treatment, and have proven to be important tolls in reducing disease associated mortality and morbidity in the last years. This has been associated with increased surveillance and timing of treatment in susceptible areas. Still, mortality and sequelae remains a problem

in patients infected with plasmodium strains resistant to the available drugs, and also those not able to effectively fight infection through a balance between oxidant and antioxidant-dependent responses.

All these evidences indicate that controlling of CM highly depends on a fine regulation of treatment timing, targeting of host/parasites responses and more importantly, policies for vector (mosquitoes) control.

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Figure legends

Figure 1. Parasite-host protein interactions. Following the infection of erythrocytes, plasmodial proteins become expressed on the infected erythrocyte membranes, facilitating the interaction between infected erythrocytes and the endothelial cells of the host. Through protein binging, infected erythrocytes become adhered to the brain microvasculature, causing subsequent disruption of the blood brain barrier and brain inflammation. Erythrocyte-expressed proteins include the erythrocyte membrane protein 1 (P*f*EMP1) family which can bind to host endothelial receptors such as cluster of differentiation 36 (CD36), intercellular adhesion molecule 1 (ICAM-1), integrins αVβ3 and αVβ6, and the endothelial protein C receptor (EPCR). Binding of P*f*EMP1 to CD36 additionally recruits α5β1 integrins to brain endothelial cells, making erythrocyte adhesion to the microvasculature tighter, and inducing disruption of endothelial barriers through breakdown of VE-cadherin and claudin 5. By binding to EPCR, P*f*EMP1 activates the endothelium causing thrombin-mediated inflammation and subsequent endothelial dysfunction. P*f*EMP1 can also bind to ICAM-1 further increasing the adhesion of infected erythrocytes to the endothelium. Other parasite proteins such as those of the RIFIN family and PbmaLS_05 interact with immune cells of the host contributing to the rupture of the blood brain barrier.

Figure 2. Overview of oxidative and nitrosative stresses. The close interaction between host and parasite proteins triggers an inflammatory process in to the vasculature, contributing to cerebral (CM) progression. Plasmodium infection causes alterations in the levels of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS), which therefore, play important roles in CM. ROS are formed from O_2 reduction by NADPH oxidase, an enzyme largely expressed in phagocytes, to superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is then, dismuted to H_2O_2 by superoxide dismutase (SOD)

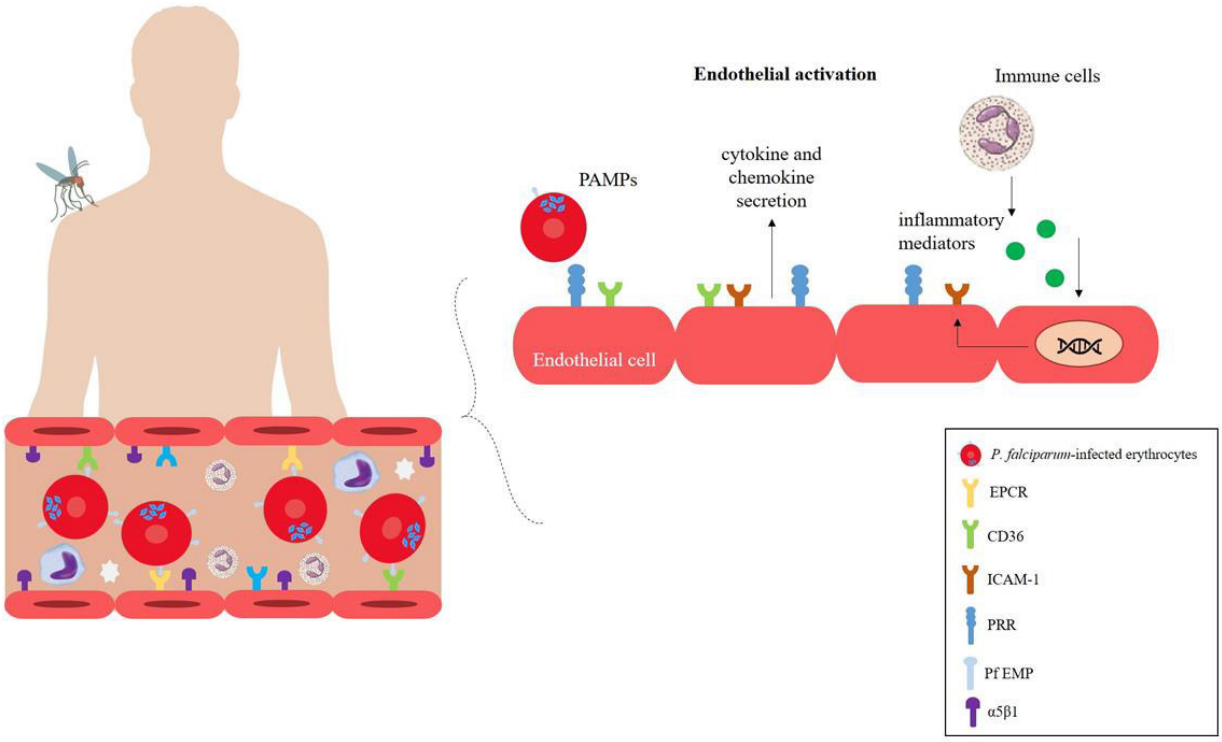
Elevated levels of H_2O_2 can be converted to H_2O and O_2 by catalase or reduced by iron to hydroxyl radicals ($HO^{\bullet}$), highly reactive products which can oxidize carbohydrates, lipids, proteins and DNA. H_2O_2 can be also removed by the glutathione and thioredoxin systems expressed by both the host and parasite. RNI production cascade initiates with the formation of NO from arginine by NO synthases. RNI products include oxidised states and adducts of the nitrogen products of NO synthases (neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) including $NO^{\bullet}$, NO_2^-, S-nitrosothiols and peroxynitrite (OONO-); this later, formed by $NO^{\bullet}$ and $O_2^{\bullet-}$, can cause oxidation and nitration of lipids, proteins and DNA.

Figure 3. The role of reactive oxygen species in cerebral malaria. Oxidative stress has been implicated in cerebral malaria (CM), occurring as part of the host response to *Plasmodium sp*.. During host response to infection, activated phagocytes release reactive oxygen species (ROS) in order to kill the parasites, but also damage the endothelial cells of the cerebral vasculature, resulting in disruption of the blood brain barrier. ROS markers (lipid peroxidation, superoxide, protein carbolynation and heme-oxygenase (HO-1)) are observed in the brain tissue in severe and fatal cases of the disease. Also, superoxide dismutase (SOD) and catalase are down-regulated during the progression of CM.

Figure 4. Oxidative stress-based antimalarial drugs. Quinoline, artemisinin and its derivatives have been used as antimalarial drugs for treating both uncomplicated and severe forms of malaria. Quinolines bind to ferriprotoporphyrin IX (FP), a product of the digestion of hemoglobin by the plasmodium which becomes polymerized into hemozoin. These drugs impair hemozoin formation leading to the accumulation of an important quantity of FP in the plasmodium digestive vacuole which then, reacts with phospholipids of the plasmodium cell membrane increasing its permeability and causing

lysis of the parasite. Derivatives of quinolines also increase FP or induce ROS formation in to the erythrocytes leading to lipid peroxidation and cell hemolysis. By augmenting free FP or ROS, quinolines and derivatives demand an increased antioxidant activity in order to the parasite to survive. Artemisinin and its synthetic derivatives act by inducing free FP and ROS, or by causing rapid depolarization of membrane potential of the parasite, or destruction and/or inhibition of the parasite mitochondria by acting on molecules involved in the electron transport chain of the plasmodium such as the cytochrome-*c* oxidase and NADH:quinone oxidoreductase (P*f*NDH2) system. A combination therapy based on artemisinins and quinoline derivatives has been used in to the clinics. However, plasmodium resistance or loss of sensitivity to the antimalarial drugs has been observed in some countries.

Figure 1


Endothelial activation
Immune cells
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Endothelial cell
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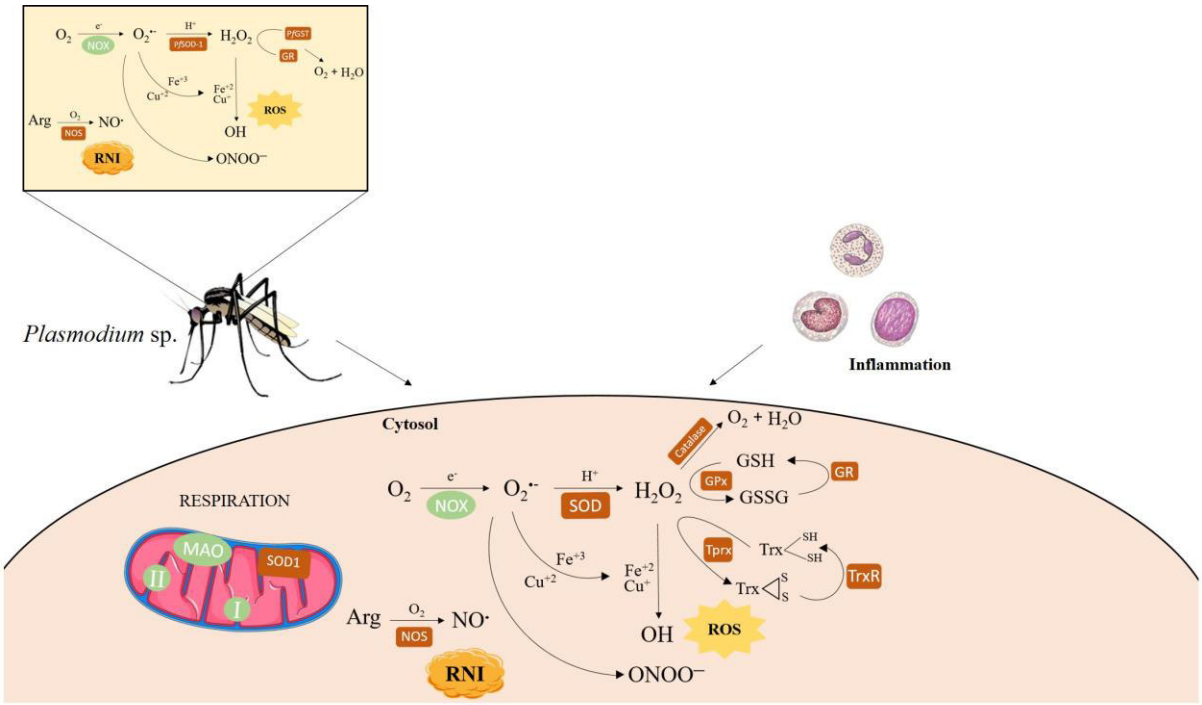
EPCR
CD36
ICAM-1
PRR
Pf EMP
α5β1

Figure 2


Plasmodium sp.
Inflammation
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SOD
Catalase
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Figure 3

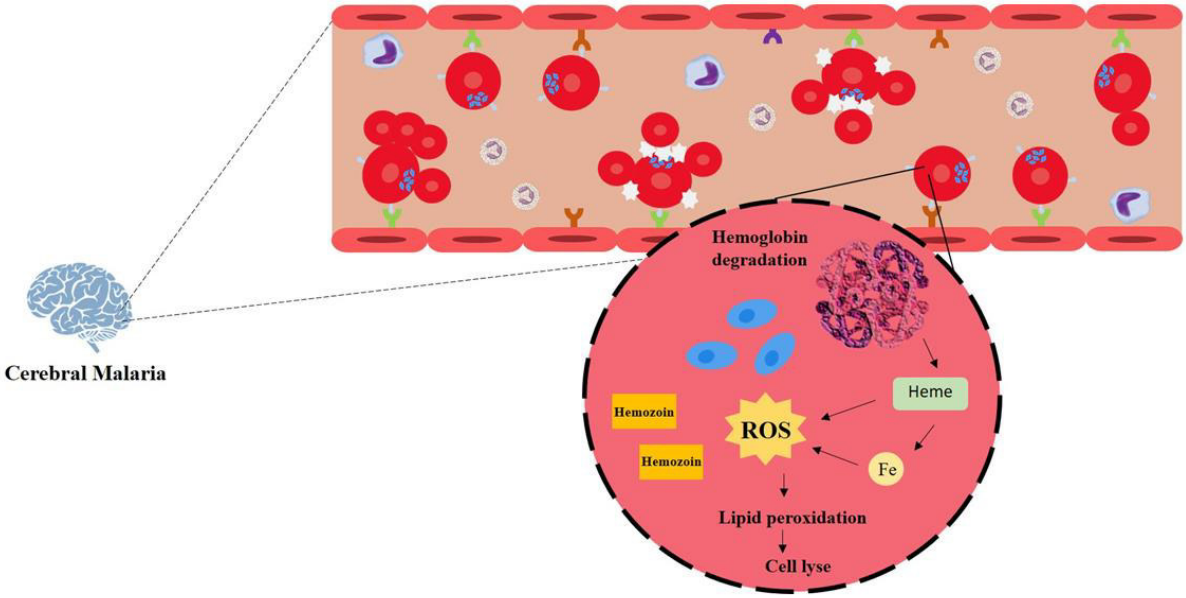
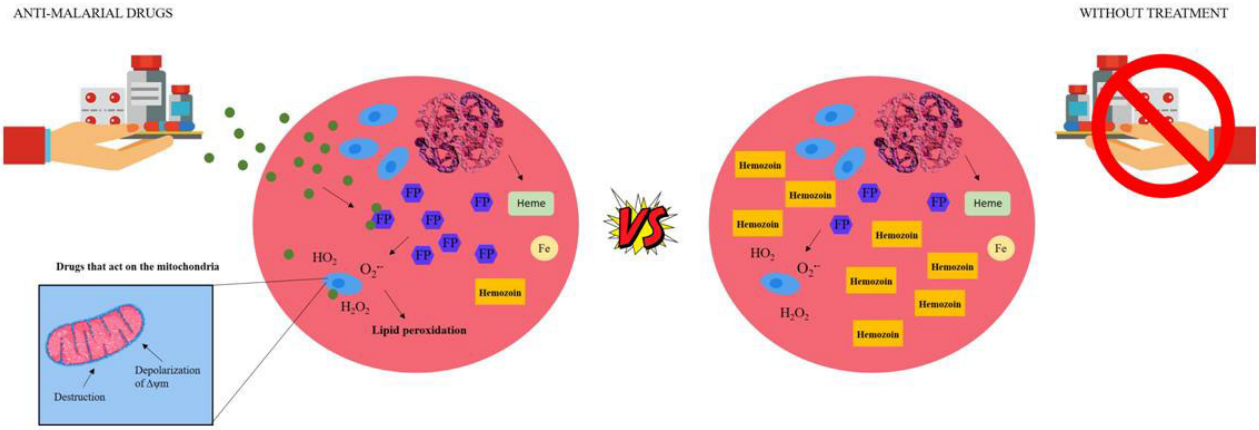

Cerebral Malaria
Hemoglobin degradation
Heme
Hemozoin
ROS
Hemozoin
Fe
Lipid peroxidation
Cell lyse

Figure 4


ANTI-MALARIAL DRUGS
WITHOUT TREATMENT
FP
Heme
Fe
Hemozoin
HO_2
$O_2^{\cdot-}$
H_2O_2
Lipid peroxidation
Drugs that act on the mitochondria
Depolarization of Δψm
Destruction
VS

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Moreover, a **pre-acceptance Editing Service (comprising English language editing, translation services, manuscript formatting and figure preparation) is available** and can provide you with expert help to ensure your manuscript is ready for submission. Japanese authors can also find a list of local English improvement services at **http://www.wiley.co.jp/journals/editcontribute.html** . All services are paid for and arranged by the Author, and use of one of these services does not guarantee acceptance or preference for publication.

Tips for authors on how to navigate the peer review process can be found **here**.

Instructions for preparing specific article types

Research papers

- Supply a structured abstract of no more than **250 words.**
- Please submit no more than a combined maximum of ten (10) figures and tables.
- Research papers should be no more than **4,000 words.**
    - 'Methods' should not be included in the word count.
    - The Discussion and Conclusions should be a maximum of 1,500 words.
- References: No more than 60.
- Scope should be pharmacological, i.e. focus on drugs and/or drug targets by characterising novel effects or mechanisms, or by validating new analytical approaches, methods or models and must constitute a significant contribution to pharmacological knowledge. Papers that reassess pharmacological concepts based on earlier results, and purely theoretical papers, will be considered. Papers describing new methods in pharmacology that embody new principles are also welcome.

Review articles

- Review articles must be no more than 5,000 words (excluding reference and figure legends).
- Supply a non-structured Abstract of no more than 250 words.
- There should be a minimum of two figures that summarise the major findings discussed.
- There should be no more than a combined maximum of five additional figures and tables (combined).
- The use of explanatory figures in the form of cartoons, flow diagrams, etc. is encouraged.
- Professional assistance with diagrams can be provided for review articles on request.
- Authors should break up their review into headed sections.

Un-commissioned review will undergo a preliminary Editorial decision.

Authors of unsolicited reviews must submit them directly to ScholarOne, following the standard submission procedure.

Letters to the Editor

Any correspondence is limited to specific comments or responses relating to a recent BJP paper, the authors of which will be invited to reply. Criteria for Letters to the Editor are as follows: no abstract or any internal structuring; fewer than 800 words; no figures; fewer than 5 references; no new or unreviewed data

Commentaries

Criteria for commentaries are as follows: no abstract; fewer than 1200 words; no figures; fewer than 5 references; there should be no new or unreview data and no internal sectioning.

Manuscripts should be submitted via the Scholar One (S1) Website in the form of a Word document. Submission is now accomplished by uploading a single Word document, which should follow the sequence laid out below in these Author Guidelines.

BJP *has strict requirements for reporting experimental design, statistical analysis and experiments involving animals or animal tissue*. See editorials**McGrath & Lilley, (2015)** and **Curtis *et al.*, 2015.**

MANUSCRIPT PREPARATION AND SUBMISSION

For submission you will be asked to write your manuscript in a Word document in the sequence recommended below.

The Word document should consist of

- Title page
- Abstract (structured)
- Introduction
- Methods
- Results
- Discussion
- Author contributions
- Acknowledgments
- References
- Figures and figure legends
- Tables (if applicable).

Word Limit

To facilitate complete transparency and reproducibility, "Methods" will not be included in the word count: the text in the remainder of the manuscript should contain no more than 4000 words (it should, however, be as succinct as possible). Legends to figures and tables are excluded from this limit.

Units and Symbols

SI units and symbols should be used. Negative index notation (e.g. mg kg^{-1}, pmol mm^{-2} min^{-1}) should be used rather than solidus notation (e.g. mg/kg, pmol/mm^2/min). 'dL' is not an SI unit; this is the most common mistake.

Should you have any queries regarding preparation of your manuscript, its submission or the peer review process, please do not hesitate to contact us at **BJPedoffice@wiley.com**.

SUMMARY OF SUBMISSION REQUIREMENTS

TITLE

Your title should not exceed 150 characters (including spaces). Please **optimise your title for search engines**.

- The Title must clearly indicate the subject matter of the paper, why the work is important and any assertions it contains must be justified by the results presented in the paper. Cumbersome chemical names, technical details, and unfamiliar abbreviations should be avoided in the title.
- On the Title page (only), if you are using author initials and not full names, include spaces in between an author's initials, e.g. 'A E Smith'. If an author's initials do not appear on the title page of your manuscript according to these guidelines, there may be a delay or error in archiving in PubMed.

RUNNING (SHORT) TITLE

Please supply a short title of no more than 60 characters, which will be used as the running head of your paper.

AUTHOR INFORMATION

For each author, please supply

- Full name
- Full institutional affiliation
- The Contribution of that author to the paper (please see guidance on authorship and contribution in the Journal's Ethics Policy. (In a nutshell, authors must have made a real contribution to the paper and must fulfil all four ICJME authorship criteria.)

ABSTRACT

Your Abstract should convey clearly the key messages of the work, and why the work is important.

It should not exceed 250 **words** (including subheadings). For Research Articles, the Abstract **must be structured** as follows:

- Background and Purpose
- Experimental Approach
- Key Results
- Conclusion and Implications.

Minimise abbreviations (see more below) and do not include any references.

NON-APPROVED ABBREVIATIONS

There is a list of **Approved Abbreviations** that do not need to be defined in the manuscript or abstract. All other abbreviations must be defined here, as an alphabetical list, and when first used in the text.

PHARMACOLOGICAL NOMENCLATURE

Nomenclature used in your article should follow that of the **IUPHAR/BPS Guide to PHARMACOLOGY**. Should your manuscript be accepted, the main pharmacological targets discussed in your manuscript will ultimately be highlighted in your published article. Until the end of February 2017, the main pharmacological targets should be presented as two tables of links (for targets and ligands).

From March 1st 2017, however, you will be asked to apply linking to these ligands and targets within the body of your manuscript. You will also be asked to provide a simple list of ligands and targets you have hyperlinked so that the press editors can check you have included all major targets. This list will not be published.

You will also be asked to supply a standard ' **'Nomenclature of Targets and Ligands'** statement.

You are not required to supply these items at submission, this will be requested should your manuscript move forward in the review process. You will be given full and clear instructions on how to fulfill these requirements by the editorial office. Please see more below, within the section 'FURTHER INFORMATION'.

ACKNOWLEDGEMENTS: FUNDING STATEMENT

Where there has been funding or financial support, authors must publish a statement in the Acknowledgements section. Please see below for guidance.

CONFLICTS OF INTEREST STATEMENT

All papers publish a Conflicts of Interest statement. Please see below for guidance.

TABLES, FIGURES AND LEGENDS

Please state the numbers of figures and of tables submitted as part of your manuscript.

Please note

- Reviews: There should be a minimum of two figures that summarise the major findings discussed. There should be no more than a maximum of five additional figures or tables.
- Research articles: There should be a combined maximum of ten figures and tables.
- Each figure and table should be embedded in the manuscript or placed at the end of the manuscript. **You do not need to upload them individually on initial submission.** If your manuscript moves forward through the review process you will be asked to upload figures individually as separate files at the first revision stage.
- Non-essential figures and tables can and should be added as supporting information.

SUPPORTING INFORMATION

BJP does not accept 'unpublished data' or 'data not shown'. All data that is essential for a manuscript should be contained within the main document. However, any additional data that provides supplementary information that aids interpretation such as demonstrations of selectivity of an antibody, demographic data of animal or human cohort, typical western blots or immunohistochemical images should be submitted as supporting information, with an explanation of why it is considered supporting, rather than essential to the paper. Please state the number of figures and tables submitted as supporting information (including zero - please add 'None supplied').

What is supporting information?

Supporting information is peer-reviewed material **directly relevant but not essential** to the conclusion of an article, such as control experiments, supporting data tables or movies. The article must be complete and self-explanatory without this additional information. It is not edited, so before submission, consider carefully how any additional data supports the paper.

File sizes must be as small as possible, so that they can be downloaded quickly, so please submit supporting information as PDFs where possible. When not possible, accepted formats are HTML files (.html), movie files (.mov/.mpg), and audio files (.wav/.mp3/.wma).

Supporting data for an article appears in the *Supporting information* section of both the html and PDF version of an article on the journal website; it is accessible via a hyperlink and can be downloaded separately.

Open data

BJP supports authors who wish to publish their raw data in open repositories. Please include any details on this with your submission.

MANUSCRIPT PREPARATION

INTRODUCTION

In the Introduction state the background to your work and its purpose. State your hypothesis and questions asked. Provide only essential background. 500 words is generally more than sufficient.

METHODS

To facilitate complete transparency, 'Methods' will not be included in the word count of Research Articles.

Your Methods must be described in sufficient detail to allow the experiments to be interpreted and repeated by an experienced investigator. Where published methods are used, references should be given, together with a brief outline: any references must provide the full description and not be a signpost to another reference. If this is a problem please provide the full description of the method in your own manuscript.

For experimental studies, the methods should be presented in sections and should cover

- Test systems used (animal preparations, isolated tissues, cultured cells, in vitro systems, etc.) and the measurements made (with technical details) for each system;
- Where animals have been used as a test system the Journal has strict requirements for the reporting of experiments involving animals or animal tissue (adherence to ARRIVE and BJP guidelines should be stated in this section).
- Experimental protocols and design (adherence to BJP guidelines should be stated in this section).
- Data and statistical analysis
- Materials.

For all studies, **experimental design, data analysis and statistical procedures** should be consistent with the principles explained in the editorial **Experimental design and analysis and their reporting: new guidance for publication in BJP,** and the major points detailed below. (For a precise bullet list, please see the **Declaration of Transparency and Scientific Rigour**.)

Authors should ensure that for each test system used the following information is provided within the methods. Authors should follow the guidance provided at the bullets.

For the rationale, please read **Curtis et al., 2015.**

Group sizes

- Explain how you have determined/designed group sizes. These should be equal by design, and any variation owing to experimental losses or violation of predetermined exclusion criteria must be explained.
- BJP accepts the use of post-hoc statistical tests designed to identify outliers within datasets. These tests should be appropriate for the type (distribution) of data being analysed. Examples of such tests include Grubb's or ROUT outlier tests. Where outliers are identified a clear indication of number of data points excluded must be provided with a robust explanation for the type of test used.
- The principle is that you should provide the exact group size (n) for each experimental group/condition, not a range; and *n* refers to independent values, not replicates. Data subjected to statistical analysis should have a group size (n) ≥ 5. If *n* is less than 5 **anywhere** in the study, please provide an explanation, and please do not undertake statistical analysis of the dataset.

Randomisation

- Please state whether animals or human subjects were randomised for treatment. If randomisation was not carried out, state that randomisation was not used, and please supply an explanation for why not.

Blinding

- Please state whether how the operator and data analysis were blinded. If blinding was not undertaken, or not feasible, please state why.

Normalisation

- When normalisation is employed (e.g. expression of values as '% of baseline' or 'fold mean control') please provide a valid scientific justification (i.e. to control for unwanted sources of variation).
- If employing normalisation that generates control or baseline values with no variance (SEM = 0), please explain with a valid scientific justification and do not subject such data to parametric statistical analysis.
- Please explain any data transformation (such as log transformation) with a valid scientific justification (i.e. to generate a Gaussian-distributed data set amenable to parametric analysis).

Data and Statistical analysis

- This section is mandatory in all manuscripts and should include the statement that 'the data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).'
- Provide details of any statistical package or program employed, including manufacturer and model number and details of which tests (and which options) and which program(s) (with full version number) were used.
- If an experiment (e.g. assay) is undertaken in duplicate, triplicate etc., please state that technical replicates were used to ensure the reliability of single values. This reliability can be quoted as a coefficient of variation. In data analysis and data presentation use the single values (i.e. 5 samples each run in triplicate is n=5 not n=15).

When comparing groups, and if a level of probability (P) is deemed to constitute the threshold for statistical significance, define this here in Methods, and do not vary it later in Results (by presentation of multiple levels of significance). Thus if $P<0.05$ is defined as threshold, $P<0.01$ etc. should not appear in the results. However, setting P at a lower value such as $P<0.01$ or 0.001 is quite acceptable, provided that this is defined as constituting statistical significance, and is not varied. It is not necessary to state the exact level of P.

Studies employing animals, animal tissues or primary cultures from animal tissues must provide additional detail covering the **requirements for reporting experiments involving animals or animal tissue**, as detailed below.

Requirements for reporting experiments involving animals or animal tissue

***This information is required only if animals, animal tissue or primary cultures** are involved.* It allows you to comply with BJP Policy on reporting experiments involving animals and with the principles of**ARRIVE** and the United States NIH. Please ensure that this section contains the details described as follows.

Enter the general principles that you followed in this section. You may already have put some details in the main section of Methods, and we apologise for the duplication. Please do not worry about minor repetition.

Validity of animal species or model selection

- Provide a scientific justification for the animal species and each model selected for study. For instance, 'this model of pain in rats has been in use for several years (reference)' (refer to review on pain models).

Ethical statement

- Make a statement of ethical approval for experimentation that will be recognised worldwide. Indicate the nature of the ethical review permissions, and national or institutional guidelines for the care and use of animals, that cover the research. Include application approval numbers and web addresses of the approving organisations, if available.

For further details, please see the Ethics Section below.

Animals

- Please note source, species, strain, sex, age range, weight and any additional data that are relevant to the study.

Housing and husbandry

- Standard animal housing and care does not need to be explained in detail as long as these meet the standards required by relevant local guidance or law
- Provide details of non-standard housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish).

Experimental procedures

Provide details, as appropriate, of behavioural tests, anaesthesia and analgesia, surgical procedures, how the animal was killed and, if there is recovery following surgery, the methods of asepsis, and post-operative care. Include welfare-related assessments, measurements and interventions (e.g. humane end points) that were carried out prior to, during, or after the experiment.

Materials

- Finally, please end your Methods section with the Materials sub-section. Provide the suppliers (names and addresses) of drugs and other chemicals, reagents and other materials.
- For new compounds, the synthesis and physicochemical characteristics of the compound(s) must be summarised here unless these have already been published in another journal or in a patent.

- Please note BJP will not consider manuscripts concerning or using compounds of undisclosed structure or undefined mixtures of compounds e.g. plant extracts.

RESULTS

In this section, please do not repeat numerical values of any data presented in tables or figures. Each value should be shown EITHER in the Tables or Figures OR in the text, NOT both. When a change is statistically significant, there is no need to show $P<0.05$ etc. in the text, as this level will have already been given in the Methods (data analysis) and ONLY ONE VALUE of P should be used throughout the manuscript.

Do not interpret, compare or discuss the data reported in the Results section; it is more appropriate to do this in the Discussion and Conclusions.

DISCUSSION AND CONCLUSIONS

Please note this section is normally restricted to 1,500 words and Senior Editors will ask for justification when this limit is exceeded.

Explain how your hypothesis or initial questions have been addressed by your results and why this is important.

Make a statement concerning the possible clinical relevance of the study.

If your study has any implications for the 3Rs (replacement, refinement or reduction), please make a statement on this in the Discussion, e.g. 'When used in signalling assays, the above procedure results in one rat pup yielding approximately 8 data points for neurons and 24 data points for glia. This is a significant enhancement over previous studies examining cAMP signalling, where approximately two rat pups have yielded a single data point for trigeminal ganglia-derived neurons' (Walker *et al*., *British Journal of Pharmacology*. 2014)

REFERENCES

The number of references for a Research paper is normally no more than 60. Editors may ask for reduction of references when this limit is exceeded.

Use **Harvard style**.

In the text, references to other work should take the form '(Connor and Kitchen, 2006)' or 'Connor and Kitchen (2006) showed that…'. For more than six authors, it is 'Zamora *et al*. 2006 showed…' Reference to 'unpublished observations' or 'personal communications' should not be included in the list of references and in general should be avoided (because they cannot be verified). Papers in preparation or those that have been submitted but not yet accepted for publication must not be included in the list of references.

In the reference list, arrange alphabetically according to the surname of the first author, and include the following crucial information: journal title, author surnames and initials, year of publication, volume and page numbers - or DOI (digital object identifier) number if a paper is in press and not yet published in an issue (please see example below). When the surnames of first authors are identical, the alphabetical order of the surnames of subsequent authors takes precedence over the year of publication. If more than one paper by the same authors in one year is cited, 'a', 'b', 'c', etc. are placed after the year of publication, both in the text and in the list of references. All authors should be quoted for papers with up to six authors; for more than six authors, quote the first six followed by '*et al.*'. Please see examples below.

Follow these examples but do not worry about minor variations introduced by your source of references, such as punctuation, as long as the essential elements are present.

Journal Reference:

Connor M, Kitchen I (2006). Has the sun set on κ3-opioid receptors? Br J Pharmacol 147: 349–350.

Journal Reference: Early View or Accepted Article :

van Goethem NP, Schreiber R, Newman-Tancredi A, Varney M, Prickaerts J (2015). Divergent effects of the 'biased' 5-HT1A receptor agonists F15599 and F13714 in a novel object pattern separation task. Br J Pharmacol. DOI: 10.1111/bph.13071.

Book Reference :

Meesmann W (1982). Early arrhythmias and primary ventricular fibrillation after acute myocardial ischaemia in relation to pre-existing coronary collaterals. In Early arrhythmias resulting from myocardial ischaemia. Ed Parratt, JR McMillan: London, pp 93–112.

E-book Reference:

Sadler P (2003). *Strategic Management*. [Online] Sterling. VA Kogan Page. Available from: http://www.netlibrary.com/reader/. [Accessed: 6th May 2015].

Meeting Abstract Reference :

Wenger TL, Lederman SN & Strauss HC (1985). Effects of flecainide in dogs with coronary occlusion and reperfusion. Circulation, 72 (suppl. III):225.

Website Reference :

Links to websites may be included in manuscripts, but these links must, where possible, terminate on a permanent data repository, such as those of the host platforms used by the journals. Links to private author's web pages/sites are not permitted. The text accompanying links should be constructed so that in the event of link failure the text can be used in a search engine to locate the website. For links to databases that are not permanent repositories please cite the date of access.

IUPHAR/BPS Guide to Pharmacology . [Online] Available from **http://www.guidetopharmacology.org/**). [Accessed: 9th May 2015].

Equivalent Permanent Repository:

Alexander SPH, Benson HE, Faccenda E, Pawson A J, Sharman JL, McGrath JC, *et al.* (2015), The Concise Guide to PHARMACOLOGY 2015/16: Overview. Brit J Pharmacol, 172: 5729–5743. doi: 10.1111/bph.13347

Data Archive Reference :

Brown LJ (20XX). *Dataset title*; Data repository or archive; Version (if any); Persistent identifier (e.g. DOI).

Other (e.g. government guideline) :

FDA (2002). ICH Draft Consensus Guideline, S7B Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals: US Department of Health and Human Services.

TABLES, FIGURES AND LEGENDS

Figures and Tables can be embedded in your manuscript at the appropriate point or placed at the end of the paper. They should be uploaded as separate files to Scholar One only on revision. Figure legends should go below each Figure and Table titles should be at the top of each Table. (If you find embedding complicated, after the references

simply place all the Tables with their associated legends first, followed by the Figure Legends in one sheet and then the figures.)

Non-critical data, e.g., structure of primers, should be put in a supplementary file and submitted as supporting information (see below) rather than as figures or tables in the manuscript.

Figures

To avoid unnecessary figures, only critical data should be presented in the figures. Non-critical data (e.g. positive /negative controls) should be put in a supporting file and submitted as supporting information.

Figures should be numbered consecutively with Arabic numerals and may comprise several parts (Fig 2A, 2B, 2C etc.). Authors must ensure that all parts are fully legible including lettering in the labels of axes or data sets, when the figure is printed to a maximum of A4 page size.

Keys to the different symbols, bars and lines should be placed in the whole figure and not repeated in the legend.

Figure Legends

A legend should be provided for each figure. Figure legends should be placed below each figure if embedded or in a list just prior to the figures themselves if placed at the end of the manuscript. These legends should include a brief title to indicate the content of the figure.

Abbreviations may be used in the figure (e.g. for drug treatments) but must be explained in the legend. Approved abbreviations or abbreviations already defined in the manuscript are permitted.

Tables

Each Table needs a title, which appears above the Table, and a footnote, which appears below the Table. The title should describe briefly the content of the Table. The footnote explains the characteristics of the data shown, such as treatment groups, times of sampling, numbers of samples, along with significant differences, statistical methods used. Tables should be self-explanatory and should be numbered consecutively with Arabic numerals. The number ('Table X') should be followed by a short title ('Effect of Y on haemodynamics''), occupying not more than two lines, at the head of the table. Any necessary explanations of the nature of values (e.g. % or mean ± SEM, * $P < 0.05$, compared with what, etc.) and the sources of any material not your own, or material published elsewhere should be placed in a Footnote, which will appear below the Table. Use superscript letters (not symbols) for callouts from the Table, e.g. data from another publication, and a single symbol (*, #, † etc.) to show significant effects.

Technical guidance on tables and figures:

- Save line art such as charts, graphs and illustrations in EPS format.
- Save photographic images in TIFF format.
- Save figures containing a combination of photographic images and text (eg annotated photographic images with text labels) as EPS.
- For large file sizes, zip or save in another compressed format such as .rar to reduce the file size.
- Resolution for all illustrations (graphs, annotated artwork, micrographs and photographs) must be 300 dpi.
- Use hatching rather than shading in graphs.

- Use colour only if it enhances the clarity of figures. The meaning of the figure must remain clear even if viewed in black and white.
- If using colour pairs, blue/yellow is preferable to red/green.
- Text and labelling in standard fonts at 8-10 point font size; Line Width at 0.3 to 1 point size.
- When submitting, please name your Figure files according to the convention "figure1.jpg", "figure2.tif" etc. (using the correct file extension), and label the figures themselves with the name of the corresponding author and the figure number (jones_fig1, etc.).
- 'Box style' figures are not in keeping with the Journal style; line drawings, etc., must have only left-hand and bottom axes.
- Please note that text, tables (usually) and legends to figures can be corrected by the Press Editors, but figures requiring changes need to be returned to the author for correction and re-submission.

See: **http://authorservices.wiley.com/bauthor/illustration.asp** for full details on preparing artwork.

That concludes the list of items that should be included in the Manuscript Submission Word Document.

FURTHER INFORMATION

PEER REVIEW AND PUBLICATION PROCESS

The journal operates a single-blind peer-review process using a body of expert peer reviewers; papers are normally reviewed by one Senior Editor, one Editor and three reviewers. Correspondence related to published research in BJP is not usually subject to peer review, but is shared with the authors of the original paper prior to any publication, with a right to reply. All substantive papers — i.e. original research, reviews (commissioned and non-commissioned), including those published under our open access programme *OnlineOpen* — undergo the same rigorous and consistent peer review process.

Once submitted, a manuscript will first be checked by the Editorial Office to ensure all elements have been submitted. If any required documentation is not present, the Editorial Office will return the manuscript to the authors and request any missing information or material.

Manuscripts are then given a first review by a Senior Editor (triage), who may return the manuscript to the authors to avoid delay if (s)he judges that it is out of scope or has little chance of being accepted after review. Otherwise it will be sent for review, usually by an Editor and three referees.

The referees' comments and Editor's recommendation will be reviewed by the appropriate Senior Editor, who will communicate the decision to the corresponding author. This process takes one month on average from submission to the initial decision, but can take longer.

Authors may be asked to revise their manuscript before a final decision is made.

Once your paper is accepted, you will be asked by the editorial office to send any outstanding essential material. This will include a request that you create your Tables of Links (to the end of February 2017) or - **from March 1st 2017** - apply hyperlinking within the body of your manuscript (just to the main ligands and targets discussed in your article). **You will be supplied with full instructions.**

Once all material is received, your article moves into production and your paper is then published within a few days on the journal website in the format of the final accepted version (i.e. as a PDF of the Word version). This final accepted version, known as an 'Accepted Article' is fully citable. Simultaneously, the accepted paper is re-formatted into the journal style and then the language and scientific content are checked by a Press Editor. The authors are then sent proofs and asked to agree final changes. Finally, the article is published in Early View, and then in an issue of the journal. This can take a couple of weeks but depends on how quickly the authors respond at proof stage.

Transfer to other British Pharmacological Society (BPS) journals

Pharmacology Research & Perspectives is jointly edited on behalf of the BPS and the American Society of Pharmacology and Experimental Therapeutics (ASPET). The Editors of BJP might consider that a submitted manuscript is out of scope and more suitable for consideration by its sister journals *British Journal of Clinical Pharmacology* (*BJCP*) or the open access journal *Pharmacology Research & Perspectives*. If so, the Editors will offer authors the opportunity to transfer the manuscript to the editorial office of its sister journals for consideration, with no need to reformat the manuscript.

After Acceptance

Video Abstracts

A video abstract can be a quick way to make the message of your research accessible to a much larger audience.
In 2017, Wiley, our Publishers, and its partner Research Square, will be offering, on a trial basis, a service of professionally produced video abstracts, available to authors of 5 articles selected by the journal. You can learn more about it at **www.wileyauthors.com/videoabstracts** . Authors of papers selected for this service will be contacted through the editorial office.

Accepted Articles

Within days of acceptance in BJP, manuscripts are made available online as 'Accepted Articles' – an unedited but peer reviewed version of a manuscript. They have not been subject to copy or press-editing, composition or proof correction, so do not have the professional appearance of the final article, but they are citable. This service provides for the earliest possible dissemination of research data following article acceptance.

Accepted Articles appear in PDF format only and are given a Digital Object Identifier (DOI), which allows them to be cited and tracked. The DOI remains unique to a given article in perpetuity and can continue to be used to cite and access the article following its publication in an issue of the Journal in its final format. More information about DOIs can be found online at **http://www.doi.org/faq.html**. Accepted Articles are indexed in PubMed.

Press editing and Copy-editing are carried out after publication of the Accepted Article. In rare occasions, it is possible that the press edit will identify some fundamental concern regarding the research article, which may require further action with respect to additional editorial changes to be implemented by the authors. If the editorial intervention results in identifying irredeemable problems with the article then the authors and Editorial team may decide that a retraction is required. Neither the British Pharmacological Society nor John Wiley & Sons Ltd. can be held responsible for errors or consequences arising from the use of information contained in Accepted Articles; nor do the views and opinions expressed necessarily reflect those of the British Pharmacological Society or John Wiley & Sons Ltd.

Early View and publication in an issue

Following publication as an Accepted Article, manuscripts are press edited and, after any required changes have been made, are then sent for typesetting. Page proofs will be sent electronically to the corresponding author, who will receive an e-mail alert containing a link to a secure web site for the proofs. A working e-mail address must therefore be provided for the corresponding author. In the absence of the corresponding author, please arrange for a colleague to access the e-mail to retrieve the proofs. Please note that you (i.e. ALL authors) have final responsibility for what is stated in the proofs of your manuscript.

Corrections to the proofs must be returned within **3 days of receipt** and instructions on how to do so will be provided in the email. Significant textual alterations are unacceptable at proof stage without the written approval of the Editor, and they are likely to result in the delay of publication. **Authors should not make changes to the nomenclature at proof stage**.

Fully reviewed, revised and edited articles (except for Letters) will appear as Early View papers around 45 days after acceptance in BJP and can be found in the Early View section of the Wiley Online Library. Early View articles replace the Accepted Article and are complete full-text articles published online in advance of their publication in an issue. They are complete and final, and because they are in final form (fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated), no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. Early View articles are then usually assigned to the next available issue of the journal.

The DOI of the article will remain the same from Accepted Article, through to Early View, to publication in an issue, and can continue to be used to cite and access the article.

The date of publication of the article is the date of its first appearance online as an Accepted Article.

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Research Article

TRPV1 Contributes to Cerebral Malaria Severity and Mortality by Regulating Brain Inflammation

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Transient receptor potential vanilloid 1 (TRPV1) is a Ca^{+2}-permeable channel expressed on neuronal and nonneuronal cells, known as an oxidative stress sensor. It plays a protective role in bacterial infection, and recent findings indicate that this receptor modulates monocyte populations in mice with malaria; however, its role in cerebral malaria progression and outcome is unclear. By using TRPV1 wild-type (WT) and knockout (KO) mice, the importance of TRPV1 to this cerebral syndrome was investigated. Infection with *Plasmodium berghei* ANKA decreased TRPV1 expression in the brain. Mice lacking TRPV1 were protected against *Plasmodium*-induced mortality and morbidity, a response that was associated with less cerebral swelling, modulation of the brain expression of endothelial tight-junction markers (junctional adhesion molecule A and claudin-5), increased oxidative stress (via inhibition of catalase activity and increased levels of H_2O_2, nitrotyrosine, and carbonyl residues), and diminished production of cytokines. *Plasmodium* load was not significantly affected by TRPV1 ablation. Repeated subcutaneous administration of the selective TRPV1 antagonist SB366791 after malaria induction increased TRPV1 expression in the brain tissue and enhanced mouse survival. These data indicate that TRPV1 channels contribute to the development and outcome of cerebral malaria.

1. Introduction

Malaria is an infectious disease of great morbidity and mortality, which claimed the lives of more than 400 thousand people worldwide in 2015 [1]. Cerebral malaria is a clinical syndrome of the severe form of the disease and is characterized by neurological complications (coma and convulsions) associated with brain inflammation (for review, see [2]) which can be lethal or cause irreversible neurological and/or cognitive sequelae in surviving patients (for review, see [3]).

Several mechanisms were found to contribute to cerebral malaria including alterations in nitric oxide availability, unbalanced oxidative stress responses, changes in the pattern of expression of inflammatory molecules, vascular leakage, and blood brain barrier disruption, amongst others [4–10]. However, its treatment has proven to be difficult and of low efficacy depending on timing and parasite resistance [3, 11],

with nearly 50% of the infected patients presenting this syndrome [3]. Importantly, 10-40% of the children with cerebral malaria die and a significant percentage develop sequelae [3, 12, 13]. In this context, the host response to infection plays a decisive role in the clinical evolution of malaria and therefore influences disease outcome.

The transient receptor potential vanilloid 1 (TRPV1) is a Ca^{+2}-permeable channel expressed on neuronal and non-neuronal cells such as brain endothelial and immune cells [14–18], which plays a role in the inflammatory response of different pathologies (for review, see [16, 19]) and an emerging role in neuroinflammation (for review, see [20]). It was found that TRPV1 is protective against bacterial infection [21–24] and modulates the innate immune response to malaria [25]. These studies also indicate that TRPV1 is detrimental to macrophage/monocyte-mediated responses, including their ability to produce inflammatory mediators, especially those related to oxidative stress [23, 26–30], in addition to regulating body temperature [21, 23]. However, the relevance of TRPV1 to the brain inflammation and symptoms of cerebral malaria has never been investigated.

Here, we used TRPV1 wild-type (WT) and knockout (KO) mice to evaluate the role of TRPV1 in cerebral malaria. Disease progression and brain inflammation were assessed in mice infected with *Plasmodium berghei* ANKA. It was found that TRPV1 contributes to disease severity and mortality, by mediating brain inflammation.

2. Methods

2.1. Mice. Nonfasted male C57BL/6 wild-type (WT) and TRPV1 knockout (TRPV1KO) mice (2-3 months of age; 22-28 g) were used. Animals were obtained from the animal's facility of the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP). Mice (n = 3-4/cage) were housed in a climatically controlled environment (room temperature of 22 ± 2°C) and humidity of around 60%, on a 12-12 h light/dark cycle (lights on at 07:00), with free access to water and food. All experiments were conducted in accordance with the Brazilian Society for Animal Welfare (SBCAL), following approval by the Ethics Committee of USP. Animals were randomly assigned into groups, and the experimenter was blinded towards the genetic background of animals during the experiment. In some cases, C57BL/6 mice received the selective TRPV1 antagonist SB366791 (0.5 mg/kg, twice a day; Sigma-Aldrich, Brazil) or vehicle (10% DMSO in saline) for up to 14 days, starting at 24 h postmalaria induction. All assays were conducted in a blinded manner.

A total of 16 infected TRPV1 WT and 14 KO mice were used for analysis of survival rates, disease stage, and severity score; these data were obtained from two independent experiments. For performing the different biochemical, qPCR, and cytokine measurement experiments, samples were collected from 12 TRPV1 WT (5 noninfected and 07 infected) and 16 TRPV1 KO (5 noninfected and 11 infected) mice with stage III/IV malaria, in three independent experiments.

In two separate experiments, 20 WTs were used for assessment of mortality rates with the TRPV1 antagonist (10 vehicle-treated and 10 SB366791-treated). Disease stage and severity score experiments included 17 WT mice (8 vehicle-treated and 9 SB366791-treated), in two independent experiments. For experiments in animals with stage III/IV malaria, 11 TRPV1 WTs were used (5 vehicle-treated and 6 SB366791-treated) in two independent experiments.

2.2. Induction of Cerebral Malaria. Malaria was induced by a single intraperitoneal (i.p.) injection of 10^6 red blood cells infected with *P. berghei* ANKA$^{GFP/HSP7}$ as previously described [31, 32]. Parasitaemia and disease progression were evaluated from day 1 postinfection, by daily recording of parasitaemia and clinical neurological signs of cerebral malaria.

2.3. Blood Parasitaemia. The percentage of parasitaemia was determined by flow cytometry. For this, a drop of blood from the tail was collected directly into 2 ml of PBS for flow cytometry analysis. Each sample was run on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer with a 488 nm argon laser and BD CellQuest™ Pro software version 6.0.1 (Becton Dickinson, San Jose, CA, USA). Erythrocytes were identified on the basis of their specific forward (FSC) and side (SSC) light-scattering properties, and a total of 100,000 events were counted for each sample.

2.4. Analysis of the Clinical Neurological Signs of Cerebral Malaria and Mortality Rates. The neurological signs were evaluated as described by Linares et al. [33], in order to determine disease progression (stages I-IV) as follows: stage I—presence of parasitaemia and absence of neurological symptoms; stage II—presence of head deviation or hemi- or paraplegia, in the absence or presence of piloerection, altered gait or ambulation, muscle weakness, tremor, rollover response, and/or anaemia; stage III—presence of significant neurological symptoms, including head deviation, paraplegia/hemiparaplegia, immobility, muscle weakness, piloerection, anaemia, pelvic elevation, lack of responses to external stimuli, tremor, and swollen eyes; and stage IV—presence of exacerbated neurological symptoms in comparison to those observed at stage III.

Disease severity was analysed by using the Rapid Murine Coma and Behavior Scale as previously described by Carroll et al. [34], with minor modifications. Briefly, a score from 0 (normal) to 2 (severe alteration) was attributed to each one of the following parameters as follows: (i) coordination (gait and balance), (ii) exploratory behavior (motor performance), (iii) strength and tone (body position and limb strength), (iv) reflexes and self-preservation (touch escape, pinna reflex, toe pinch, and aggression), and (v) hygiene-related behavior (grooming). The summation of the scores attributed to each of the parameters for each animal was taken as severity score index, with the highest scores corresponding to the worst outcome of disease.

The animals were observed for up to 14 days postinfection and were culled by anaesthetic overdose (90 mg/kg ketamine + 2 mg/kg xylazine; i.p.) as soon as they reached stage III/IV (premortality end point). Blood samples were collected, and the plasma was obtained. Brain samples were also

collected and weighted. Collected plasma and brain tissue samples were immediately frozen and stored at -80°C until further processing for analysis of different parameters, except those used for qRT-PCR to which RNAlater was added according with the manufacturer's instructions (Sigma-Aldrich, Brazil).

Also, body weight and temperature were registered before (baseline) and at stage III/IV postinfection. All those which did not reach stage III/IV during the observation period were culled, and their measurements and samples were collected at the 14^{th} day postinfection. Noninfected mice were used as controls.

In a separate series of experiments, mortality rates were evaluated over 14 days following induction of cerebral malaria, in independent groups of mice.

2.5. Brain Parasite Load. Tissue parasite load was evaluated in brain samples (left hemisphere) collected from infected TRPV1 WT and KO mice, as previously described [33], and modified. Tissue parasite loads were determined by quantitative PCR and expressed as copy numbers of *P. berghei* ANKA 18S DNA per milligram of host tissue. For this, RNA was extracted in RNeasy Microarray Tissue Mini Kit, according to the manufacturer's instructions (Qiagen, Brazil). Then, the cDNA was prepared by reverse transcription of 2 μg of RNA with ImProm-II Reverse Transcriptase (Promega, USA). The cDNA was assayed by qRT-PCR using the TaqMan® system (Applied Biosystems, USA) with *P. berghei* probes (AI 38261, PN 4332079). GAPDH levels were assessed by TaqMan Mouse GAPDH System (TaqMan®, Applied Biosystems, USA) and were used as housekeeping gene controls.

2.6. TRPV1, Junctional Adhesion Molecule-A (JAM-A), and Claudin-5 Gene Expression by Real-Time qPCR. qRT-PCR was performed using GoTaq qPCR Master Mix (Promega, USA) and a Rotor-Gene 6000 real-time PCR machine (Corbett Life Science, Australia) in a final volume of 12 μl (hold: 2 min at 95°C; cycling: 40 cycles: 15 s at 95°C and 30 s at 60°C; melt: 68-90°C). The following primers were used: TRPV1 (forward 5′-GCGACCATCCCTCAAGAGT-3′, reverse 5′-CTTGCGATGGCTGAAGTACA-3′; 109 bp; accession number NM_001001445.2), JAM-A (forward 5′-GGTCAGCATCCACCTCACTGT-3′, reverse 5′-AGGTCAGCACTGCCCTGTTC-3′; 94 bp; accession number NM_172647), claudin-5 (forward 5′-GTGCCGGTGTCACAGAAGTA-3′, reverse 5′-GTACTTGACCGGGAAGCTGA-3′; 147 bp; accession number NM_013805), and GAPDH (forward 5′-AAGGTCATCCCAGAGCTGAA-3′, reverse 5′-CTGCTTCACCACCTTCTTGA-3′; 138 bp; accession number NM_008084.2). For each gene in each sample, $2^{-\text{efficiency}\times Ct}$ values were calculated and divided by the corresponding value of $2^{-\text{efficiency}\times Ct}$ obtained for GAPDH. In order to normalize the data, all the individual results were divided by the average value obtained for the control group (noninfected WT mice). Efficiencies were of 0.47, 0.59, 0.68, and 0.8 for TRPV1, claudin-5, JAM-A, and GAPDH primers, respectively.

2.7. Cytokine Measurements. Brain samples (right hemisphere) were prepared, and the supernatant was obtained as previously described [35] and used in the assays. The tissue and plasma levels of TNFα, IFNγ, and IL-6 were evaluated by using mouse cytometric bead array (CBA) cytokine kits according to the manufacturer's instructions (BD Biosciences, Brazil). Data analysis was performed on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems). Results were calculated in FCAP Array Software version 3.0.1 (BD Biosciences, Brazil) and expressed as picograms of cytokine per mg of tissue protein (pg/mg of protein) or picograms per milliliter of plasma (pg/ml).

2.8. Tissue Sample Preparation for Biochemical Analysis of Oxidative Stress Pathways. Brain samples (100 mg; right hemisphere) were homogenized in 1000 μl of 0.05 M $NaPO_4$ (pH 7.4) containing ethylenediaminetetraacetic acid (EDTA, 1 mM) and centrifuged at 10,000g, for 10 min, at 4°C, and then the supernatant was collected and stored at -80°C for analysis of enzyme activities.

2.9. Superoxide Dismutase (SOD). SOD activity was measured as described by Abreu et al. [36]. Briefly, 10 μl of each sample was incubated with 260 μl of sodium carbonate buffer (50 mM; pH 9.4 containing 3 mM EDTA), 10 μl of 3 mM xanthine, 10 μl of 153 mU/ml of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), and 10 μl of 1.87 mU/ml xanthine oxidase. Then, 200 μl of the mixture was added per well in 96-well plates and the absorbance was read at 470 nm for 20 min. Blank reactions were prepared for each sample by boiling them for 5 min in order to inactivate SOD. Results are expressed as milliunits (mU) of SOD/mg of protein. Enzyme activity was defined as the ability of one unit of SOD to dismutate 1 μmol of O_2^-/min.

2.10. Catalase. Catalase activity was measured as previously described [36], by incubating 30 μl of brain homogenates or plasma samples with 500 μl of hydrogen peroxide (H_2O_2, 10 mmol/l) for 20 min, at 25°C. Reactions were stopped with 500 μl of sodium azide (1 mmol/l), and the concentration of the remaining H_2O_2 was determined by the oxidation of *o*-dianisidine. For this, 20 μl of each reaction was incubated with 200 μl of phosphate buffer (5 mM; pH 6.0) containing 0.167 mg/ml *o*-dianisidine and 0.095 mg/ml horseradish peroxidase (HRP). The absorbance was immediately read at 460 nm (SpectraMax Plus 384, Molecular Devices Inc., Sunnyvale, EUA) for 10 min. The remaining reactions were incubated at 60°C for 2 h, in order to inactivate catalase, and used as controls. A standard curve of H_2O_2 (11.3-8820 μM) was used for comparison. Results are expressed as international units (IU) of catalase per milligram (mg) of protein. One IU of catalase was defined as the amount of H_2O_2 (in μmol) degraded per minute.

2.11. Glutathione Peroxidase (GPx) and Reductase (GR). GR activity was assessed by measuring the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor in the reduction of oxidized glutathione (GSSG) to reduced GSH [36]. For this, 10 μl of the sample was

incubated with 190 μl of a solution containing 2 mg/ml GSSG and 0.4 mg/ml NADPH, at 37°C. Absorbances were then recorded for 30 min (incubation period), at 340 nm. The results are expressed as μmol of NADPH per min normalized per mg of protein (μmol of NADPH/min/mg of protein).

GPx activity was determined as previously [36]. For this, 30 μl of sample per well (diluted 1:3) was incubated for 5 min at 37°C, with 145 μl per well of 0.05 M phosphate buffer (pH 7.4) containing 0.1 M EDTA, 5 μl of glutathione (GSH, 80 mM), and 5 μl glutathione reductase (0.0096 U/μl). After incubation, 5 μl of 0.46 % *tert*-butyl hydroperoxide solution and 10 μl of 1.2 mM NADPH were added to each well. Absorbances were monitored at 340 nm for 10 min. The results are expressed as μmol of GSH/min/mg of protein.

2.12. Thioredoxin Reductase (TrxR). TrxR activity was determined by incubating 20 μl of the sample with 140 μl of assay buffer (0.05 M phosphate buffer (pH 7.4) containing 0.1 M EDTA, 50 mM potassium chloride, and 0.2 mg/ml bovine serum albumin), 20 μl of 2 mM NADPH, and 20 μl of 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), in the presence and absence of a TrxR inhibitor (sodium aurothiomalate; 20 μM) [37]. Absorbances were read at 412 nm for 5 min. The results are expressed in IU of TrxR per mg of protein (IU/mg of protein). Enzyme activity was defined as the NADPH-dependent production of 2 μmol of 2-nitro-5-thiobenzoate per min at 22°C.

2.13. Protein Nitrotyrosine and Carbonyl Levels. For analysis of protein nitrotyrosine and carbonyl levels, 2.5 μg of each sample was assayed by slot blotting. The presence of proteins containing 3-nitrotyrosine residues was analysed in the samples as previously described [38]. After sample derivatization by addition of Laemmli buffer (0.125 M Trizma, pH 6.8; 4% SDS and 20% glycerol; 20 min at room temperature and boiling for 2 min), the membrane was incubated with mouse monoclonal anti-nitrotyrosine primary antibody (1 : 2,000; Merck Millipore Co., Germany) overnight at 18°C.

Carbonylated proteins were determined according to the method described by Robinson et al. [39]. After the derivatization reaction by addition of 2,4-dinitrophenylhydrazine (DNPH) solution (0.1 mg/ml in 2N HCl, 5 min), the membrane was incubated with anti-DNP primary antibody (1 : 25,000 in blocking buffer, Abcam, UK) overnight at 18°C.

Immunoreactive bands were detected by chemiluminescence, and their intensities were estimated by densitometric analysis (ChemiDoc Image Systems, Bio-Rad, USA). Results were normalized by the band intensity values obtained after staining with Ponceau red.

2.14. Plasma and Tissue Hydrogen Peroxide Measurements. The levels of H_2O_2 were measured in brain homogenates and plasma samples by using a H_2O_2/peroxidase assay kit (Amplex Red H_2O_2/peroxidase assay kit; Molecular Probes, Invitrogen, Brazil) according to the manufacturer's instructions. Results were obtained by comparison of each sample with a H_2O_2 (0–10 μM) standard curve and are expressed as H_2O_2 levels in μM (plasma) and in picomoles of H_2O_2 per mg of protein (brain samples).

2.15. Statistical Analysis. The results are presented as mean ± standard error (SE). The percentage of inhibition is reported as the mean for each individual experiment. For multiple statistical comparisons between groups, data were analysed by repeated-measures analysis of variance (ANOVA) or one-way ANOVA followed by the Bonferroni test with FDR correction. Paired and unpaired t tests were used when appropriate. Survival curves were analysed by the nonparametric Mantel-Cox test. All data were analysed in GraphPad Prism 5.0. $p < 0.05$ was considered significant. All n numbers are indicated on the graphs.

3. Results

3.1. P. berghei Infection Reduces TRPV1 mRNA Expression in the Mouse Brain, a Response Attenuated by TRPV1 Antagonism. We initially investigated whether infection with *P. berghei* ANKA, a plasmodium strain known to cause cerebral malaria in mice, influences TRPV1 expression in the mouse brain. Infected WT mice expressed lower TRPV1 mRNA levels (56%) in their brain tissue than non-infected controls did (Figure 1(a)). On the other hand, the systemic administration of SB366791 in C57BL/6 mice with malaria increased TRPV1 expression (2.1-fold increase) in comparison with vehicle controls (Supplementary Material Figure S1a).

3.2. Loss of TRPV1 Signaling Protects against Cerebral Malaria. We next assessed whether the ablation of TRPV1 influences cerebral malaria progression and mortality. Data depicted in Figure 1(b) show that infected TRPV1KO mice exhibit attenuated disease in comparison with WT controls. Of note, TRPV1KOs only presented parasitaemia without any other sign or symptom of cerebral malaria, suggesting they do not develop this syndrome. Accordingly, malaria was less severe and remained at stage I in these mice whilst it progressed into stages III and IV in the majority of the WT animals over the 14-day observation period (Figures 1(b) and 1(c)). Mortality was markedly prevented by TRPV1 ablation as 90% of the TRPV1KO mice survived infection in contrast with WT animals (19% survival; Figure 1(d)). Mice treated with SB366791 presented similar disease severity and course to those receiving vehicle until day 6 postinfection, improving their condition over the 14-day observation period (Supplementary Material Figures S1b and S1c). Twenty percent of those receiving the TRPV1 antagonist survived (Supplementary Material Figure S1d). As lack of TRPV1 was previously shown to exacerbate hypothermia in mice with bacterial infection [23], mouse body temperatures were registered. At baseline conditions, both genotypes exhibited similar body temperatures; however, hypothermia was only observed in stage III/IV WT but not TRPV1KO mice (Figure 1(e)). A similar response was registered in those receiving SB366791 (Supplementary Material Figure S1e).

Blood parasitaemia was similar in both genotypes, although WT mice exhibited higher parasitaemia than TRPV1KOs did at days 6 and 7 postinfection (Figure 2(a)). On the other hand, *P. berghei* ANKA 18S levels were elevated

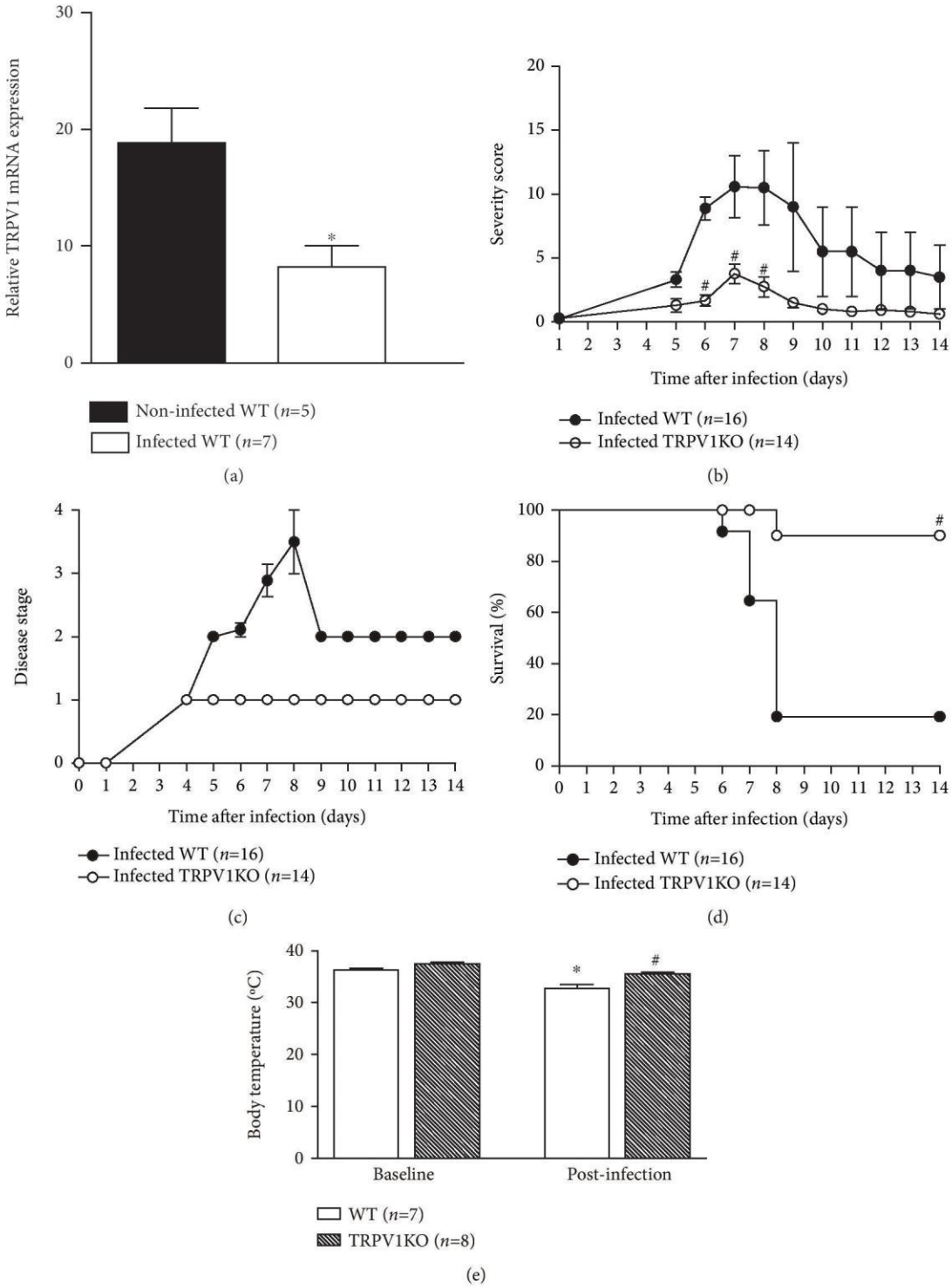

FIGURE 1: Brain TRPV1 mRNA expression and cerebral malaria progression. (a) TRPV1 mRNA expression in brain samples of noninfected and infected (at stage III/IV) TRPV1 wild-type (WT) mice. Disease progression (b) and stage (c); survival rates (d) and body temperature (e) recordings from TRPV1 WT and knockout (KO) mice infected with *P. berghei* ANKA. Disease progression, stage, and survival rates were registered over 14 days postinfection. Mouse body temperatures were evaluated at baseline and postmalaria induction (at stage III/IV or at day 14 for those that survived the observation period). Results represent the mean ± SEM of all mice per group, obtained from two-three independent experiments. n is indicated on each graph. Data were analysed by repeated-measures analysis of variance (ANOVA) followed by the Bonferroni test with FDR correction (panels b and c). Paired and unpaired t tests were used when appropriate (panels a and e). Survival curves were analysed by the nonparametric Mantel-Cox test (panel d). $^{*}p < 0.05$ differs from noninfected WTs or baseline readings; $^{\#}p < 0.05$ differs from infected WT mice.

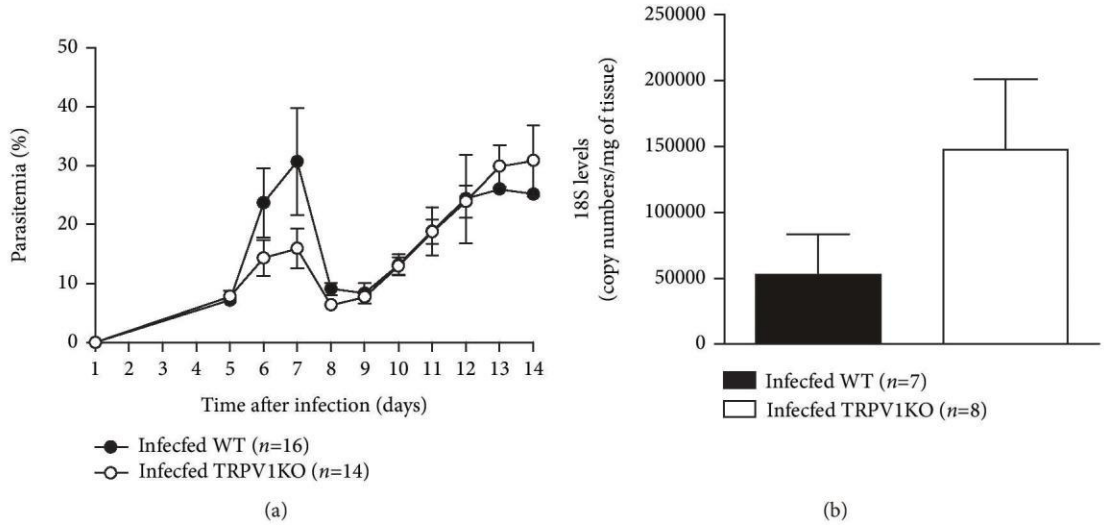

FIGURE 2: Blood and brain parasitaemia. (a) Blood parasitaemia and (b) brain 18S levels in TRPV1 wild-type (WT) and knockout (KO) mice infected with *P. berghei* ANKA. Blood parasitaemia data was collected over 14 days postinfection; brain samples were collected at stage III/IV or at day 14 for those that survived the observation period. Results represent the mean ± SEM of all mice per group, obtained from two-three independent experiments. *n* is indicated on each graph. Data were analysed by repeated-measures analysis of variance (ANOVA) followed by the Bonferroni test with FDR correction (panel a). Unpaired *t* test was used when appropriate (panel b).

in the brain samples of TRPV1KO (2.8-fold) in comparison with those obtained from WT mice (Figure 2(b)).

3.3. Lack of TRPV1 Increases the mRNA Expression of Blood Brain Barrier Integrity Markers and Attenuates Oedema Formation in the Brains of Infected Mice. Loss of integrity of the blood brain barrier is a hallmark of cerebral malaria, contributing to increased oedema formation and neuronal damage as disease progresses [40, 41]. Possible effects of TRPV1 ablation in brain oedema formation and in the gene expression of the tight junction components claudin-5 and JAM-A [41, 42] were then, investigated. Data depicted in Figure 3(a) demonstrates that infection with *P. berghei* ANKA promotes brain swelling in WT (1.7-fold) and TRPV1KO (1.2-fold) mice in comparison with their respective noninfected controls; however, this response was reduced by 25% in those lacking TRPV1. Additionally, analysis of claudin-5 and JAM-A mRNA levels revealed that infected WT mice express diminished levels of both genes (49% and 80%, respectively), in comparison with noninfected controls, a response that was attenuated in infected TRPV1KO mice (Figures 3(b) and 3(c)). Genotype did not affect brain weight/body weight ratios or claudin-5 mRNA expression in noninfected mice (Figures 3(a) and 3(b)). However, noninfected TRPV1KOs presented with lower expression of JAM-A (47%) in comparison with their WT counterparts (Figure 3(c)).

3.4. H_2O_2, Protein Nitrotyrosine and Carbonyl Residues Are Raised in Infected TRPV1KO Animals. Oxidative stress normally occurs as part of the host response to malaria [5, 43]. TRPV1 is an oxidative stress sensor [28], which not only does modulate oxidative stress [23, 27] but also can have its expression regulated by endogenous oxidant molecules [26]. Therefore, the impact of TRPV1 ablation in malaria-associated oxidative stress was investigated. Higher levels of H_2O_2 and protein nitrotyrosine residues (indicative of NO-dependent oxidative stress; [44]) were detected in infected mice of both genotypes in comparison with their noninfected controls (Figures 4(a) and 4(b)). WT mice presented 4.8-fold and 3.7-fold increases and TRPV1KOs 6.0-fold and 2.7-fold increases for tissue H_2O_2 and protein nitrotyrosine residue levels, respectively. Protein carbonyl residues (indicative of lipid peroxidation-dependent oxidative stress; [45]) were only increased (1.9-fold) in brain samples of infected mice lacking TRPV1 (Figure 4(c)). Analysis of plasma H_2O_2 levels, and protein nitrotyrosine and carbonyl levels indicated these were raised in TRPV1KO but not WT mice infected with *P. berghei* ANKA (Figures 4(d)–4(f)). TRPV1KOs presented greater levels of plasma H_2O_2 (13.9-fold increase), protein nitrotyrosine (1.5-fold increase), and carbonyl (1.4-fold increase) residues in comparison with those observed for WT animals with cerebral malaria (Figures 4(d)–4(f)).

As TRPV1KO mice presented with an exacerbated production of oxidants, the activity of antioxidant enzymes was then, investigated. The tissue activity levels of SOD, GPx, and GR were attenuated (by ~35%, ~20%, and ~34% of reduction, respectively) in infected mice irrespective of genotype when compared to noninfected controls (Figures 5(a), 5(d), and 5(e)). Also, TrxR activity was enhanced in both infected genotypes (1.5-fold increase; Figure 5(f)). On the other hand, brain catalase activity was markedly diminished (49%) in infected TRPV1KO but not WT mice (Figure 5(b)). Infected TRPV1KO mice also displayed lower levels of catalase activity (70% less) in their plasma in comparison with WT controls (Figure 5(c)).

3.5. Diminished Cytokine Production Is Detected in Infected TRPV1KO Mice. Cytokines are involved in neuronal survival [46, 47] and therefore may affect cerebral malaria

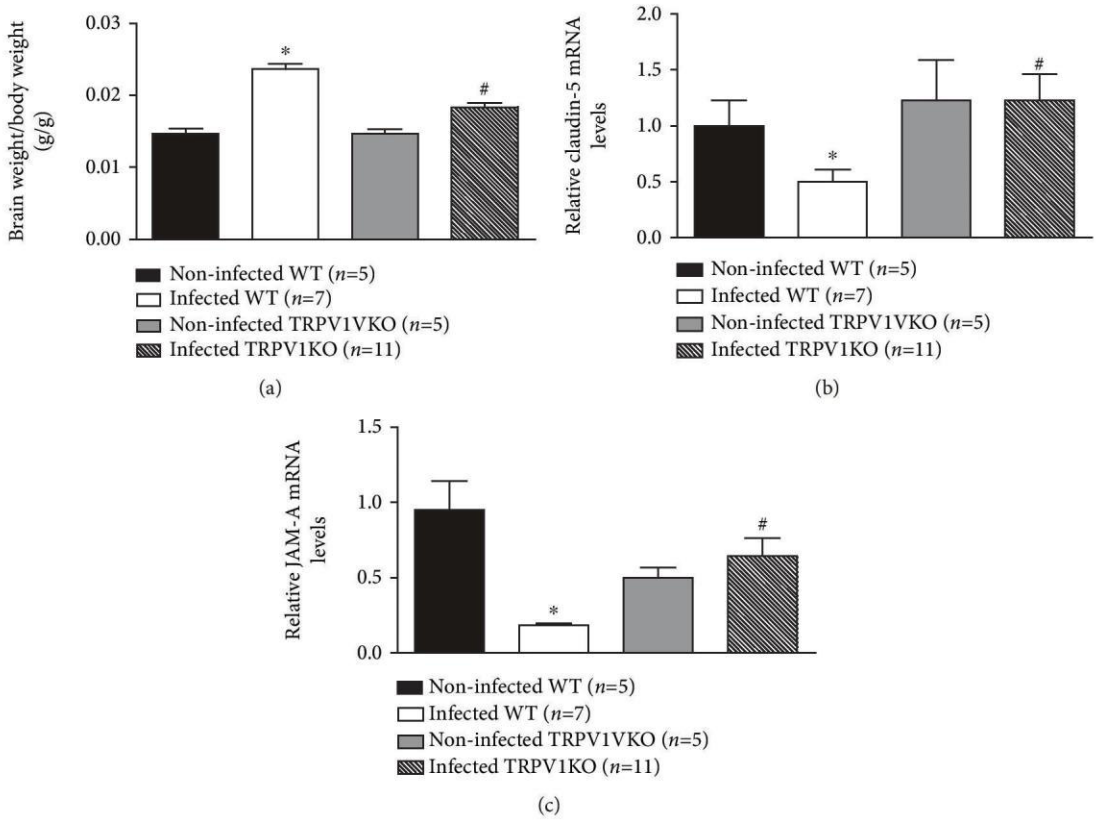

FIGURE 3: Brain swelling and expression of blood brain barrier integrity markers. (a) Brain weight/body weight ratios and mRNA expression levels of claudin-5 (b) and JAM-A (c) in brain samples of TRPV1 wild-type (WT) and knockout (KO) mice infected with *P. berghei* ANKA. Brain samples were collected at stage III/IV or at day 14 for those that survived the observation period. Samples from noninfected mice were used as controls. Results represent the mean ± SEM of all mice per group, obtained from three independent experiments. n is indicated on each graph. Data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni test with FDR correction. $^{*}p < 0.05$ differs from noninfected WTs; $^{\#}p < 0.05$ differs from infected WT mice.

progression. Thus, the levels of both tissue and plasma IFNγ, TNFα, and IL-6 were assessed in WT and TRPV1KO mice with malaria. Tissue and plasma TNFα production was markedly reduced (52% and 64%, respectively; Figures 6(a) and 6(d)) in TRPV1KO in comparison with WT controls. A similar profile was observed for IL-6 as mice lacking TRPV1 exhibited significant lower levels of this cytokine at both tissue (65% reduction) and plasma (86% reduction) levels (Figures 6(b) and 6(d)). Genotype did not affect IFNγ levels in a significant manner (Figures 6(c) and 6(f)).

4. Discussion

Since its discovery, the TRPV1 channel has been pointed out as an essential receptor in a variety of physiological and pathological responses. This is due to its wide expression and ability to transduce signals in both neuronal and nonneuronal cells, therefore participating in responses that range from cell differentiation to death [16, 23, 48–50]. Novel and recent findings on its role indicate that the endogenous activation of TRPV1 protects mammals from bacterial infections [21–24]. More recently, a nonselective TRPV1 antagonist (capsazepine) was found to modulate the peripheral immune response to malaria [25], but no studies have reported to date, a role for TRPV1 in cerebral malaria development and outcome. Here, we show for the first time that in the absence of TRPV1, *P. berghei* ANKA infection does not progress into cerebral malaria in the majority of the infected mice, protecting them from death and from the development of any disease symptoms and signals apart from blood parasitaemia. Protection was also observed in mice receiving the TRPV1 antagonist SB366791 repeatedly after malaria was induced. Of note, this effect was more pronounced in TRPV1KOs than in mice treated with SB366791. Although these results suggest that an intervention with a TRPV1 antagonist may be an alternative to avoid malaria progression, its use should be carefully considered as it may increase mortality upon bacterial infection [23]. Interestingly, although TRPV1 ablation exacerbates hypothermia in bacterial infection [21, 23], it was found herein that TRPV1KO mice and WTs treated with the selective TRPV1 antagonist SB366791 are protected from this condition in comparison with infected WTs.

P. berghei ANKA-infected mice treated with capsazepine were previously demonstrated to present similar blood parasitaemia to those treated with vehicle [25]. Here, we show

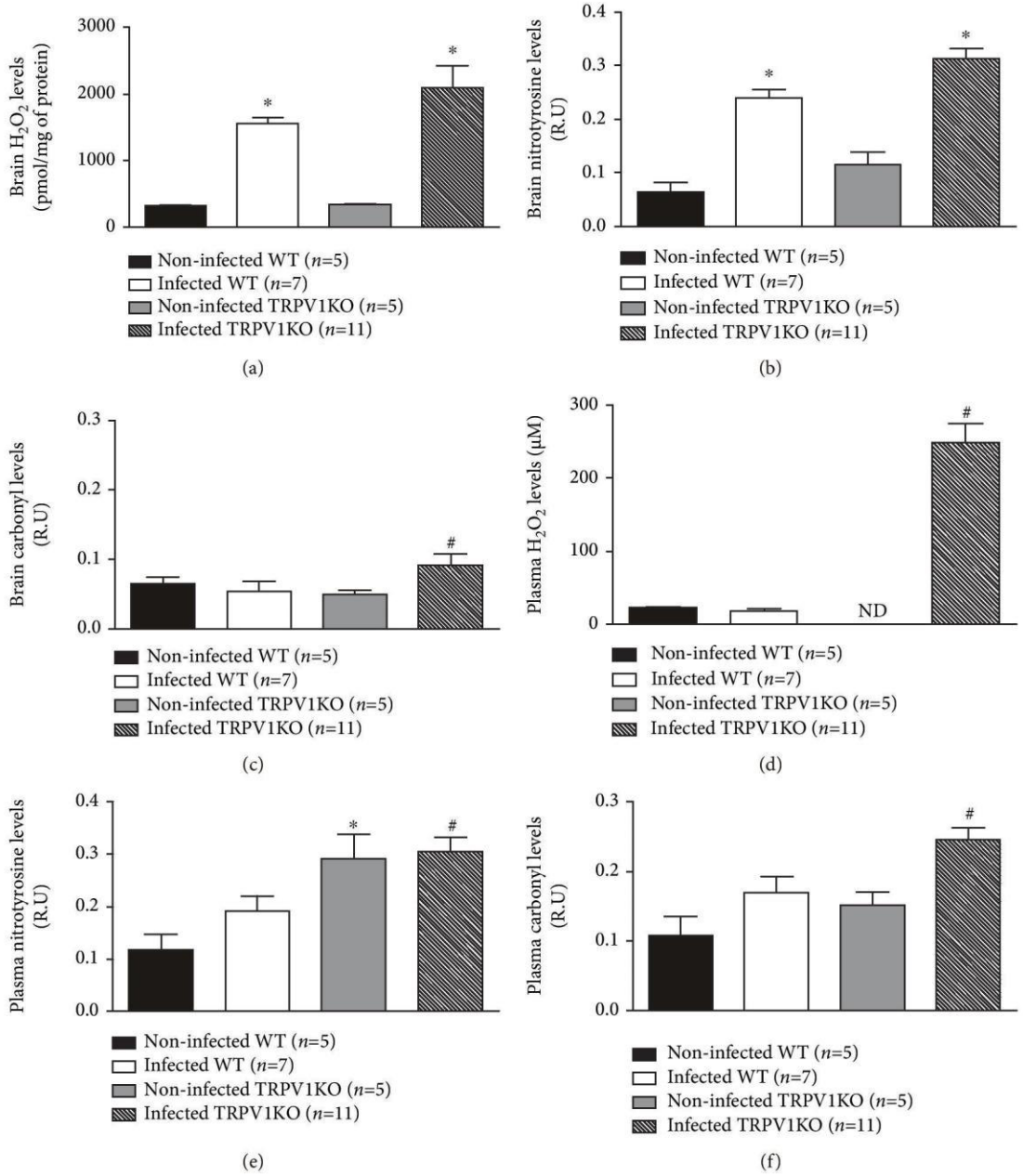

FIGURE 4: Levels of H_2O_2, protein nitrotyrosine, and carbonyl residues. (a) H_2O_2 concentrations, protein (b) nitrotyrosine and (c) carbonyl residues in brain samples obtained from TRPV1 wild-type (WT) and knockout (KO) mice infected or not with *P. berghei* ANKA. (d) H_2O_2 concentrations, protein (e) nitrotyrosine, and (f) carbonyl residues in plasma samples obtained from TRPV1 wild-type (WT) and knockout (KO) mice infected or not with *P. berghei* ANKA. Samples were collected at stage III/IV or at day 14 for those that survived the observation period. Results represent the mean ± SEM of all mice per group, obtained from three independent experiments. *n* is indicated on each graph. Data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni test with FDR correction. $^{*}p < 0.05$ differs from noninfected WTs; $^{\#}p < 0.05$ differs from infected WT mice.

that infected mice lacking TRPV1 present with similar blood parasitaemia to those expressing this receptor. On the other hand, at days 6 and 7 postinfection, infected WTs presented higher parasitaemia than TRPV1KOs did. Despite that, surviving TRPV1KO mice exhibited higher levels of plasmodium 18S in their brain samples than WTs did with cerebral malaria at stage III/IV. Of note, the techniques used to measure blood parasitaemia and brain parasite load are different as peripheral parasitaemia comprises the detection of live parasites whilst brain 18S expression does not discriminate between live and dead plasmodium. However, it is possible that TRPV1KO mice are able to kill the parasites that reach the brain more efficiently than WTs are, therefore protecting those lacking TRPV1 from death.

Brain oedema formation in patients with cerebral malaria is indicative of a bad disease prognosis, especially in children [3, 51]. In adults, brain oedema is not as usual but affects 25% of these patients [52]. Brain swelling results from increased

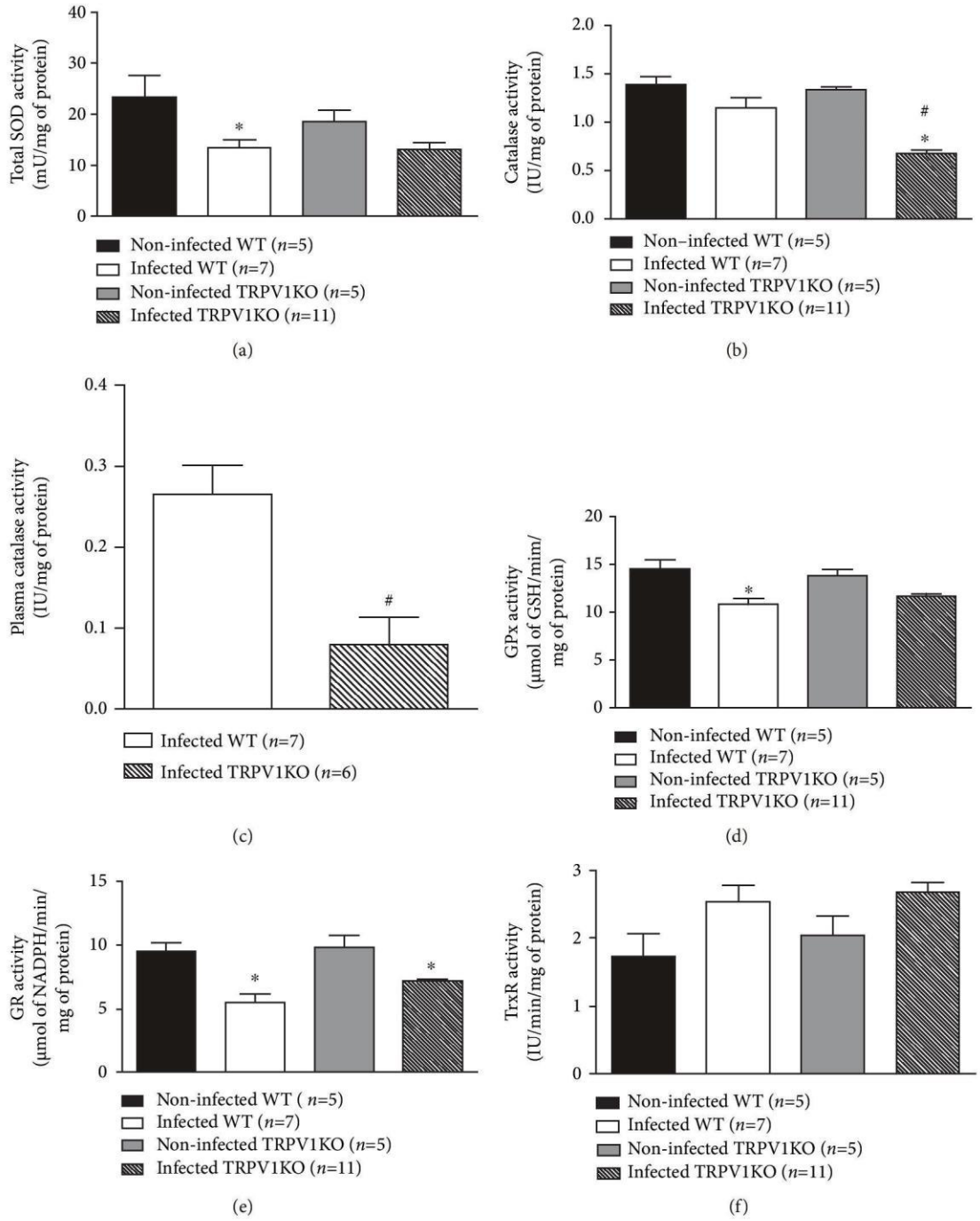

FIGURE 5: Activity levels of antioxidant enzymes. (a) Superoxide dismutase (SOD), (b) catalase, (d) glutathione peroxidase (GPx), (e) glutathione reductase (GR), and (f) thioredoxin reductase (TrxR) activity levels in brain samples obtained from TRPV1 wild-type (WT) and knockout (KO) mice infected or not with *P. berghei* ANKA. (c) Activity levels of catalase in plasma samples of infected TRPV1 WT and KO mice. Samples were collected at stage III/IV or at day 14 for those that survived the observation period. Results represent the mean ± SEM of all mice per group, obtained from three independent experiments. n is indicated on each graph. Data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni test with FDR correction (panels a, b, d, e, and f). Unpaired t test was used when appropriate (panel c). $^{*}p < 0.05$ differs from noninfected WTs; $^{\#}p < 0.05$ differs from infected WT mice.

vascular leakage and disruption of the blood brain barrier [8, 9]. TRPV1 activation promotes vasodilation and oedema formation [53, 54]. Then, the contribution of TRPV1 to brain oedema formation was assessed in infected mice. Infected WT mice exhibited brain swelling and decreased mRNA expression of the markers of blood brain barrier integrity JAM-A and claudin-5 [41, 42]. However, in the absence of TRPV1, there was higher JAM-A and claudin-5 mRNA expression. This response was associated with less brain oedema formation, suggesting that mice lacking TRPV1 are

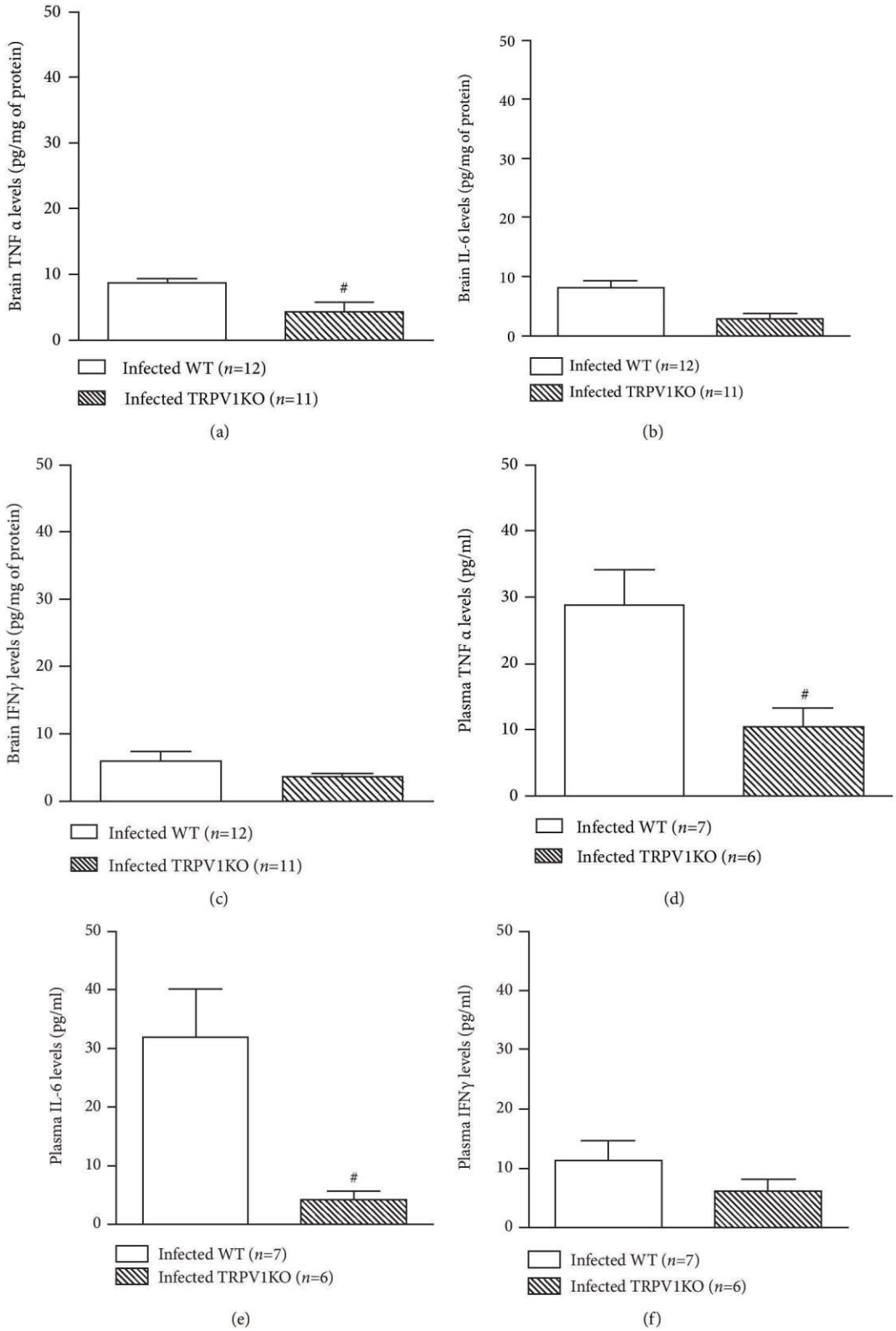

FIGURE 6: Brain and circulating levels of cytokines. Brain levels of (a) tumor necrosis α (TNFα), (b) interleukin-6 (IL-6), and (c) interferon γ (IFNγ) and plasma concentrations of (d) TNFα, (e) IL-6, and IFNγ (f) in TRPV1 wild-type (WT) and knockout (KO) mice infected with *P. berghei* ANKA. Samples were collected at stage III/IV or at day 14 for those that survived the observation period. Results represent the mean ± SEM of all mice per group, obtained from three independent experiments. n is indicated on each graph. Data were analysed by unpaired t test. $^{\#}p < 0.05$ differs from infected WT mice.

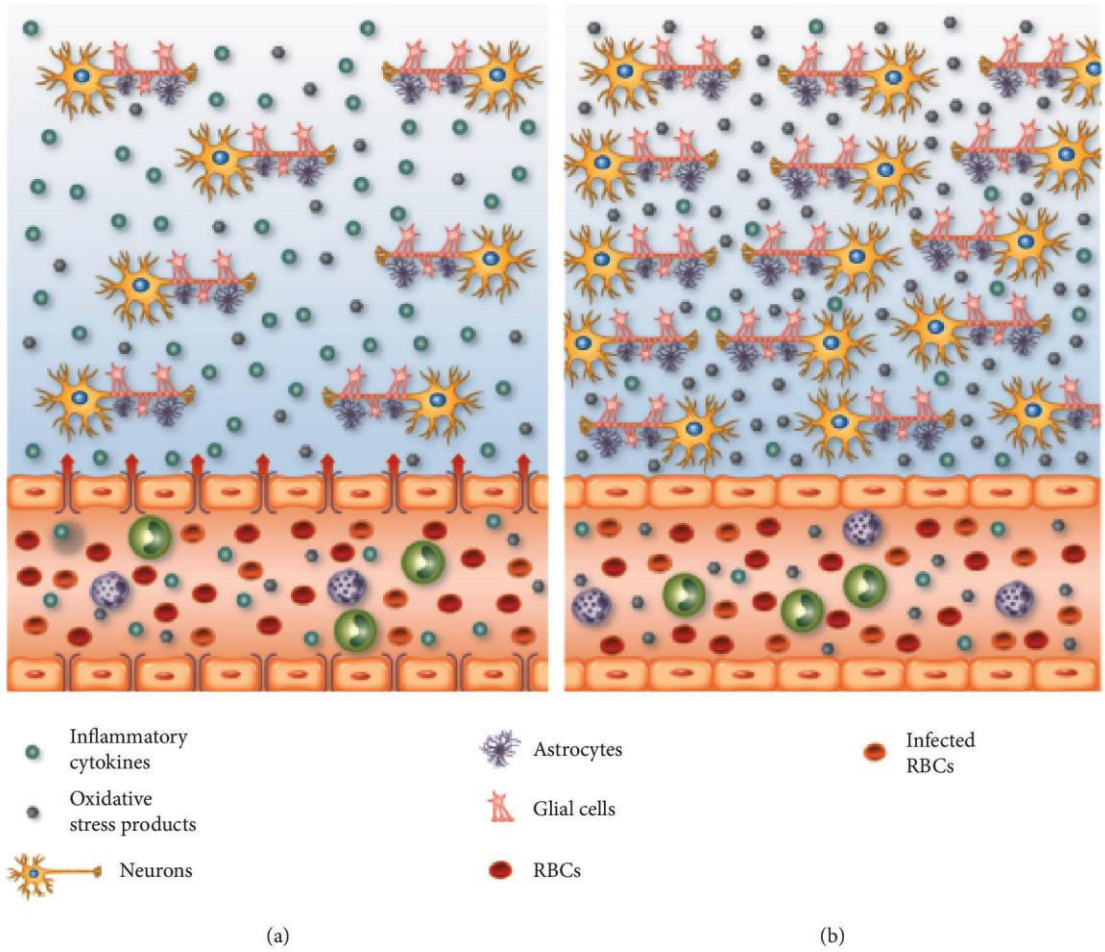

Figure 7: Brain and vascular changes in cerebral malaria in TRPV1 WT and KO mice. (a) Several alterations occur in the brain tissue and vasculature during cerebral malaria. Wild-type (WT) red blood cells (RBC) infected with *Plasmodium berghei* ANKA reach the brain vasculature and trigger the accumulation of leukocytes in the vascular space. As a result of this close interaction between infected RBC, leukocytes, and the endothelium, oxidative stress products (H_2O_2, nitrosylated and carbonylated proteins) and cytokines (TNFα, IL-6, and IFNγ) are detected in the circulation and in the brain tissue; H_2O_2 levels are a lot higher in the brain tissue in comparison with the circulation. Plasma extravazation is increased in the brain and this is associated with reduced mRNA expression of the tight-junction endothelial markers claudin-5 and JAM-A. These alterations may culminate with neuronal death, thus, contributing to the increased morbidity and mortality observed in WT mice following infection with *P. berghei* ANKA. (b) Infected mice lacking TRPV1 (TRPV1KO) present increased levels of H_2O_2 and nitrosylated and carbonylated proteins than WT animals at both brain tissue and circulation. TRPV1KOs also exhibit lower concentrations of plasma and brain cytokines, especially TNFα and IL-6, and less plasma extravazation than WT mice, a response that is accompanied by higher expression of claudin-5 and JAM-A in their brain vasculature. The inflammatory response profile observed in TRPV1KO mice may reflect in less neuronal damage, as these animals are protected from *P. berghei* ANKA-induced death and symptoms.

protected from the brain damage, coma, and death associated with protein leakage into the brain tissue secondary to plasmodium infection.

Intravascular oxidative stress is a common phenomenon in malaria which has been associated with alterations in the endothelium that in turn, facilitate the parasite accumulation into the brain tissue and/or vasculature [55, 56]. Additionally, decreased NO availability was recently linked to increased cerebral-vascular dysfunction in cerebral malaria [6]. A feedback between TRPV1 expression/activation and oxidative stress pathways has been previously demonstrated [23, 26–28]. Of note, the activity of oxidative stress enzymes has been investigated in neurons under inflammatory conditions and may influence neuronal survival [57–61]. Therefore, the influence of TRPV1 on brain oxidative stress was evaluated.

Our data show that infected WTs present higher levels of H_2O_2 and protein nitrotyrosine residues (indicative of excessive NO- or peroxynitrite-dependent oxidation; [44]) in their brain tissue than noninfected mice do. Interestingly, these markers were present at even higher concentrations in mice lacking TRPV1. Of note, the elevated production of these oxidant products was observed not only in the brain tissue but also systemically. In comparison with infected WTs, TRPV1KO mice injected with *P. berghei* also exhibited increased protein carbonylation

(indicative of lipid peroxidation-dependent oxidative stress; [45]) in brain and plasma samples. The higher levels of H_2O_2 in infected TRPV1KO were accompanied by a significantly lower catalase activity in comparison with WT animals. These results reinforce the idea that TRPV1KO mice may be able to deal with the parasite load more efficiently than WTs. This is supported by data showing that TRPV1KO mice present higher H_2O_2 and NO production which may lead to increased parasite killing.

High levels of cytokines have been linked to severe malaria in both humans and mice as their production contributes to cerebral-vascular dysfunction and even neuronal death [46, 47, 62–65]. Of note, lipid peroxidation is suggested to cause suppression of NF-κB activation (for review, see [66]), a key molecule in the generation of pro-inflammatory cytokines. Here, plasma and cerebral TNFα and IL-6 production was markedly diminished by TRPV1 ablation, thus evidencing, once more, that TRPV1 signaling is involved in the tissue damage associated with cerebral malaria. Although not significant, a similar profile was observed for IFNγ in the same mice. Interestingly, IFNγ and TNFα have been linked to cerebral malaria progression by acting on brain endothelial cells, thus promoting their activation and/or apoptosis [67, 68]. Recently, the TRPV1 antagonist AMG9810 was found to confer neuroprotection by attenuating TNFα production in a rodent model of stroke [54]. These evidences and the gathered data allow us to suggest that the diminished cytokine generation by TRPV1KO mice contributes to the diminished brain swelling and damage observed in *P. berghei* ANKA-infected mice, a response that is associated with a greater ability of these mice to produce higher amounts of oxygen/nitrogen-derived oxidant species which in turn may enhance their capacity of killing this parasite.

Figure 7 summarizes the inflammatory events that occur in the brain of TRPV1 WT and KO mice during cerebral malaria. Overall, the data presented here, indicate that TRPV1 channels contribute to the development and outcome of cerebral malaria. Although antagonists targeting this receptor may be useful to preventing the development of the cerebral syndrome caused by *Plasmodium* sp., their clinical use may be limited as they worsen sepsis outcome.

Data Availability

The datasets used to support this study will be made available upon request. Requests should be sent to the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

D.M.S. Pereira, S.A. Teixeira, O. Murillo, E.P.M. Peixoto, M.C. Araújo, and N.C.F. Sousa performed the experiments and data analysis; C.R.F. Marinho, M.N. Muscará, and E.S. Fernandes designed the experiments and supervised the study; E.S. Fernandes secured the funding support and originally drafted the manuscript; V. Monteiro-Neto, J.B. Calixto, T.M. Cunha, C.R.F. Marinho, M.N. Muscará, and E.S. Fernandes critically revised the manuscript.

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Supplementary Materials

Figure S1: Effect of the selective TRPV1 antagonist SB366791 in the brain expression of TRPV1 mRNA and in cerebral malaria progression. (a) TRPV1 mRNA expression in brain samples of infected (at stage III/IV) TRPV1 wild-type (WT) mice. Disease progression (b) and stage (c); survival rates (d) and body temperature (e) recordings from TRPV1 WT mice infected with P. berghei ANKA. Disease progression, stage, and survival rates were registered over 14 days postinfection. Mouse body temperatures were evaluated at baseline and postmalaria induction (at stage III/IV or at day 14 for those that survived the observation period). Mice received the TRPV1 antagonist SB366791 (0.5 mg/kg, s.c., twice a day) or vehicle (10% DMSO in saline), from 24 h postinduction of malaria. Results represent the mean + SEM of all mice per group, obtained from three independent experiments. n is indicated on each graph. $^{*}p < 0.05$ differs from baseline readings; $^{\#}p < 0.05$ differs from infected WT mice treated with vehicle. *(Supplementary Materials)*

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SUPPLEMENTARY MATERIAL

TRPV1 contributes to cerebral malaria severity and mortality by regulating brain inflammation

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Running title: TRPV1 deletion attenuates cerebral malaria

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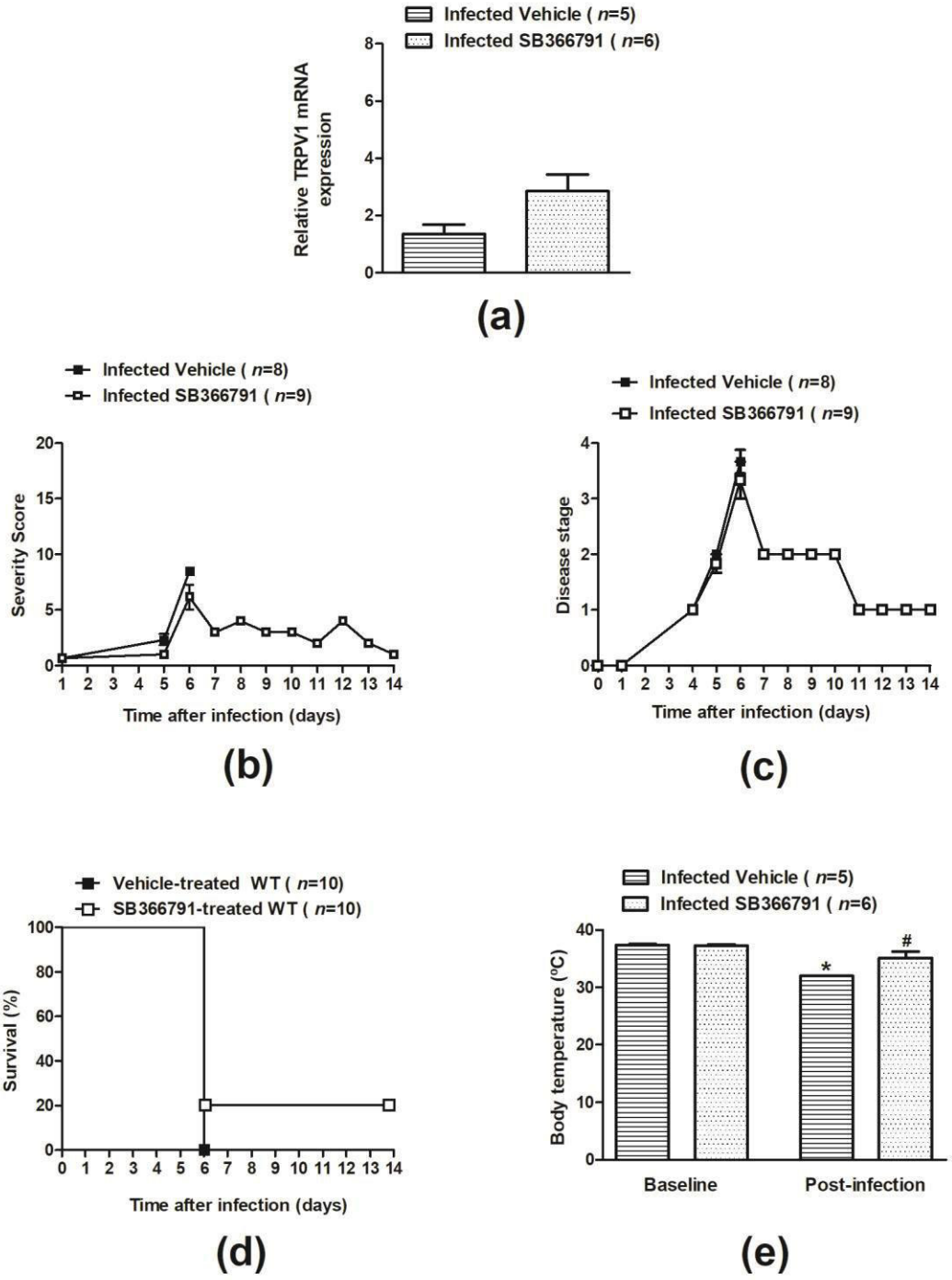

Figure S1. Effect of the selective TRPV1 antagonist SB366791 in the brain expression of TRPV1 mRNA and in cerebral malaria progression. (a) TRPV1 mRNA expression in brain samples of infected (at stage III/IV) TRPV1 wild type (WT) mice. Disease progression (b) and stage (c); survival rates (d) and body temperature (e) recordings from TRPV1 WT mice infected with *P. berghei*

ANKA. Disease progression, stage and survival rates were registered over 14-days post-infection. Mouse body temperatures were evaluated at baseline and post-malaria induction (at stage III/IV or at day 14^{th} for those that survived the observation period). Mice received the TRPV1 antagonist SB366791 (0.5 mg/kg, s.c., twice a day) or vehicle (10% DMSO in saline), from 24h post-induction of malaria. Results represent the mean ± SEM of all mice per group, obtained from two independent experiments. *n* is indicated on each graph. Data were analysed by repeated measures analysis of variance (ANOVA) followed by the Bonferroni test with FDR correction (panels b and c). Paired and unpaired *t* test were used when appropriate (panels a and e). Survival curves were analysed by the non-parametric Mantel-Cox test (panel d). *$p<0.05$, differs from baseline readings; # $p<0.05$, differs from infected WT mice treated with vehicle.

4 CONSIDERAÇÕES FINAIS

A malária cerebral é uma forma grave da malária causada pelo protozoário *Plasmodium falciparum*. Sem tratamento efetivo, a malária pode evoluir para formas graves que podem acarretar sequelas e morte, como a malária cerebral (MC). Diversos mecanismos contribuem para a MC, especialmente a terapia adequada e a resposta do hospedeiro à infecção. Neste contexto, espécies reativas de oxigênio (EROs) e intermediários reativos de nitrogênio (IRN) possuem papel essencial na evolução da MC. Ambos EROs e IRNs são produzidos como parte de uma resposta inflamatória resultante da infecção por *Plasmodium sp*. na tentativa de erradicar o parasita. Por outro lado, como meio de defesa para sobreviver ao hospedeiro, o plasmódio ativa sua maquinaria anti-oxidante, mecanismo este, explorado no desenvolvimento de fármacos ao longo dos anos. Drogas anti-maláricas atuam promovendo a geração de EROs e óxido nítrico, além de atuarem sobre o potencial de membrana do parasita, promovendo a morte do patógeno. Resistência a fármacos antimaláricos têm sido observada, mesmo quando a terapia combinada é utilizada, o que tem levado à busca por novas terapias que atuem não somente no parasita, mas também na resposta do hospedeiro frente a agressão tecidual.

O TRPV1 é um alvo farmacológico importante no hospedeiro, nesse contexto, uma vez que é conhecidamente um sensor de EROs e também regulador da formação destes produtos e de IRNs. O presente trabalho investigou a importância deste receptor na MC em um modelo murino largamente utilizado experimentalmente para o entendimento dos mecanismos da MC e também de novas terapias para a mesma. Os dados aqui apresentados demonstram que o receptor TRPV1 contribui para a progressão da MC através de mecanismos reguladores da inflamação a nível sistêmico e cerebral. A deleção gênica do TRPV1 ou seu bloqueio farmacológico atenuaram a mortalidade da MC, resposta esta, associada à ativação de vias relacionadas à formação de EROs e IRNs; e a redução na produção de citocinas.

Sendo assim, os achados do presente trabalho contribuem biotecnologicamente para o desenvolvimento de novos alvos terapêuticos capazes de tratar ou prevenir a evolução clínica da malária cerebral, como o uso de antagonistas para o receptor TRPV1, o qual foi demonstrado, aqui, participar da progressão da MC por regular vias da inflamação e dos estresses oxidativo e nitrosativo. Nesse contexto, novos estudos são

necessários, uma vez que a ausência de sinalização via TRPV1 pode agravar coinfecções, como as de origem bacteriana.