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EFEITO DO LICOPENO NA MALÁRIA EXPERIMENTAL

EVERTON LUIZ POMPEU VARELA

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Tese de doutorado defendida ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE, da Universidade Federal do Pará, como requisito para a obtenção do Título de Doutor em Biodiversidade e Biotecnologia.

Orientador: Prof. Livre-Docente Sandro Percário

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Varela, Everton Luiz Pompeu. **Efeito do licopeno na malária experimental.** 2023. 89 f.; Tese (Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE) – Universidade Federal do Pará, Belém-Pará, 2023.

RESUMO

A malária é uma doença potencialmente fatal. Sua letalidade está associada a elevada concentração de radicais livres indutores de estresse oxidativo no organismo hospedeiro. O estresse oxidativo pode ser prevenido pela ação de antioxidantes. Para confirmar esta hipótese, avaliamos os efeitos do fitonutriente licopeno (LYC) sobre as alterações oxidativas induzidas pelo *Plasmodium berghei* (Pb) em camundongos e comparamos aos efeitos da N-acetilcisteína (NAC). Para isso, camundongos Balb/c foram pré-tratados com uma dose de 3,11mg/kg de peso corporal/dia de LYC ou 62mg/kg pc/dia de NAC. Vinte e quatro horas depois, os animais foram infectados pela injeção intraperitoneal de 10^6 hemácias parasitadas. Os tratamentos continuaram diariamente até o dia anterior aos dias 1, 4, 8 ou 12 de infecção. Após esses períodos a parasitemia e a taxa de mortalidade foram avaliadas. Os animais sobreviventes foram submetidos a eutanásia e foram coletados o cérebro e pulmões para análises bioquímica de substâncias reativas ao ácido tiobarbitúrico (TBARS), capacidade antioxidante pela inibição dos radicais ABTS (AC-ABTS) e DPPH (AC-DPPH), ácido úrico (UA) e óxido nítrico (NO). Os resultados demonstraram um aumento progressivo da parasitemia de 0,6%, 5,6%, 15,8% e 40% nos dias 1, 4, 8 e 12, respectivamente, e taxa de mortalidade elevada de 53% e 55% no 8° e 12° dia pós-infecção, respectivamente. As mortes ocorreram devido ao estresse oxidativo, que foi confirmado pelo aumento dos níveis de TBARS, AC-ABTS, AC-DPPH, UA e NO no cérebro e pulmões dos camundongos. O tratamento com LYC diminuiu a progressão da parasitemia para 19% e a taxa de mortalidade para 20% no 12º dia pós-infecção. O LYC também foi capaz de reduzir os níveis de TBARS, UA e NO comparado aos grupos Pb ($p< 0,0001$) e NAC+Pb ($p<0,0001$), atingindo valores semelhantes aos animais Sham. O LYC é um fitonutriente que impede o estresse oxidativo, devido a sua poderosa ação antioxidante. Esta ação pode ser a principal responsável pela redução da parasitemia e da taxa de mortalidade. Portanto, o LYC foi eficaz contra a infecção e alterações oxidativas induzidas por Pb, devido sua ação antioxidante. Assim, o LYC pode representar uma estratégia terapêutica promissora para a diminuição da morbidade e mortalidade causadas pela malária.

Palavras chave: Antioxidante; Estresse oxidativo; Licopeno; Malária; Suplemento.

Varela, Everton Luiz Pompeu. **Effect of lycopene in experimental malaria.** 2023. 89 f.; Thesis (Post-Graduate Program in Biodiversity and Biotechnology - BIONORTE Network) - Federal University of Pará, Belem-Para, 2023.

ABSTRACT

Malaria is a potentially fatal disease. Its lethality is associated with a high concentration of free radicals that induce oxidative stress in the host organism. Oxidative stress can be prevented by the action of antioxidants. To confirm this hypothesis, we evaluated the effects of the phytonutrient lycopene (LYC) on the oxidative changes induced by *Plasmodium berghei* (Pb) in mice and compared it to the effects of N-acetylcysteine (NAC). For this, Balb/c mice were pretreated with a dose of 3.11mg/kg body weight/day of LYC or 62mg/kg bw/day of NAC. Twenty-four hours later, the animals were infected by intraperitoneal injection of 10^6 parasitized red blood cells. Treatments continued daily until the day before days 1, 4, 8, or 12 of infection. After these periods, parasitemia and mortality rate were evaluated. The surviving animals were euthanized, and the brain and lungs were collected for biochemical analysis of thiobarbituric acid reactive substances (TBARS), antioxidant capacity by inhibition of ABTS radicals (AC-ABTS) and DPPH (AC-DPPH), uric acid (UA) and nitric oxide (NO). The results showed a progressive increase in parasitemia of 0.6%, 5.6%, 15.8%, and 40% on days 1, 4, 8, and 12, respectively, and an increased mortality rate of 53% and 55% on the 8th and 12th post-infection days, respectively. The deaths occurred due to oxidative stress, which was confirmed by increased levels of TBARS, AC-ABTS, AC-DPPH, UA, and NO in the brains and lungs of the mice. Treatment with LYC decreased the progression of parasitemia to 19% and the mortality rate to 20% on the 12th day post-infection. LYC was also able to reduce the levels of TBARS, UA, and NO compared to the Pb (p<0.0001) and NAC+Pb (p<0.0001) groups, reaching values similar to those of Sham animals. LYC is a phytonutrient that prevents oxidative stress due to its powerful antioxidant action. This action may be the main responsible for the reduction of parasitemia and mortality rate. Therefore, LYC was effective against infection and Pb-induced oxidative alterations due to its antioxidant action. Thus, LYC may represent a promising therapeutic strategy for reducing morbidity and mortality caused by malaria.

Keywords: Antioxidant; Oxidative stress; Lycopene; Malaria; Supplement.

SUMÁRIO

1 INTRODUÇÃO

A malária continua a ser um problema de saúde pública mundial. Em 2021, foram estimados cerca de 247 milhões de casos e 619 mil óbitos no mundo em decorrência desta doença. Atualmente, a malária é endêmica em 84 países, principalmente em áreas tropicais e subtropicais onde afetam principalmente comunidades pobres, especialmente mulheres e crianças (WHO, 2022). Há décadas, essa doença causa consequências devastadoras na saúde, sociais e econômicas para mais de um bilhão de pessoas. Destaca-se que a falta de estratégias de prevenção eficientes, como medicamentos e/ou vacinas, entre outros, contribui significativamente para este quadro.

Dentre as espécies de *Plasmodium* que causam malária em humanos, o *P. vivax* é o mais incidente no mundo e implicado pelas recaídas da doença (NAIN *et al.,* 2022), enquanto o *P. falciparum*, espécie responsável por promover a forma grave da doença, é o mais letal (HOWES *et al.,* 2016). Estudos sobre a patogênese da malária, tem discutido que as manifestações clínicas agudas ou graves que podem levar a morte, são frequentemente uma consequência do estresse oxidativo sistêmico provocado pelo parasito (PERCÁRIO *et al.,* 2012).

Na malária, o estresse oxidativo se propaga no momento em que o *Plasmodium* invade os eritrócitos e consome a hemoglobina intraeritrocitária, para formar aminoácidos necessários para o seu desenvolvimento (TEKWANI e WALKER, 2005). Contudo, durante o metabolismo da hemoglobina que ocorre no vacúolo digestivo do parasito, a ferroprotoporfirina é danificada, levando a oxidação do seu átomo de ferro ferroso (Fe^{2+}) a ferro férrico (Fe^{3+}), originando a ferriprotoporfirina IX, que por ser altamente reativa, induz uma cascata de formação de espécies reativas de oxigênio e nitrogênio (ERON), incluindo os radicais: superóxido ($O_2^{\bullet-}$), peróxido ($ROO^{\bullet}$), hidroxila ($OH^{\bullet}$), oxido nítrico (NO), peroxinitrito ($ONOO^-$) altamente reativos (BUTZLOFF *et al.,* 2012; NARSARIA *et al.,* 2012).

As ERON, por sua vez, favorecem uma serie de reações oxidativas que podem levar a diminuição do sistema de defesa antioxidante (ASAOLU e IGBAAKIN, 2009). Nesse contexto, foi evidenciado que indivíduos infectados pelo *P. falciparum* e que vivem em áreas endêmicas, são mais susceptíveis às complicações da doença, por apresentarem baixas concentrações plasmáticas de vários micronutrientes, incluindo vitamina A e zinco, e

antioxidantes como, ácido ascórbico (vitamina C), carotenoides e vitamina E (ADELEKAN *et al.,* 1997; NUSSENBLATT *et al.,* 2002). A consequente exposição da célula hospedeira ao estresse oxidativo leva ao dano celular, incluindo redução da deformabilidade dos glóbulos vermelhos, ruptura da membrana e hemólise (HALDAR *et al.,* 2007; SRIVASTAVA *et al.,* 2015; KUMAR *et al.,* 2018), contribuindo significativamente para complicações da doença como acidose metabólica, anemia grave e falência de múltiplos órgãos (BECKER *et al.,* 2004; PERCÁRIO *et al.,* 2012).

Por outro lado, as vitaminas A e E, carotenoides e zinco, têm papéis essenciais no sistema antioxidante e estão implicados na resistência da infecção por malária (SHANKAR e PRASAD, 1998; DAS *et al.,* 1996). Iribhogbe *et al.* (2013) evidenciaram que a vitamina A o zinco e o selênio, podem interferir na progressão das reações oxidativas durante a malária em camundongos infectados pelo *P. berghei* (IRIBHOGBE *et al.,* 2013). Adicionalmente, estudos sugeriram que a suplementação periódica de vitamina A e zinco pode reduzir a incidência de episódios febris e a parasitemia, servindo como uma estratégia eficaz e de baixo custo para diminuir a morbidade por *P. falciparum* em crianças em idade pré-escolar (SHANKAR *et al.,* 1999, 2000). Outros estudos também apoiam a hipótese de que o consumo de carotenoides pelo organismo hospedeiro aumenta durante a infecção da malária, sugerindo que o estado nutricional é um fator modulador importante na malária (DAS *et al.,* 1996; NUSSENBLATT *et al.,* 2002).

Entre os carotenoides, o licopeno (LYC) é o antioxidante com maior potencial para eliminar ERON, superando o precursor de vitamina A β-caroteno ou o α-tocoferol, sendo eficaz na manutenção do equilíbrio oxidativo, devido a sua estrutura molecular possuir 11 duplas ligações conjugadas que aumentam sua capacidade sequestradora e favorecem a estabilização de elétrons desemparelhados dos radicais livres por ressonância (BOHM *et al.,* 2002; BRITO *et al.,* 2019). Também, é reconhecido pela capacidade de induzir a síntese de enzimas antioxidantes, como superóxido dismutase (SOD), glutationa peroxidase (GSH-Px) e catalase (CAT; MILLER *et al.,* 1996; ANGUELOVA e WARTHESEN, 2000).

O LYC é um nutriente encontrado em muitas frutas e vegetais como tomate, mamão, melancia, pimentão vermelho, manga, goiaba, dentre outros, e é responsável pela pigmentação vermelho-alaranjado nesses alimentos. Após seu consumo, o LYC mostrou ser prontamente absorvido pelo corpo, podendo ser encontrado no sangue, leite materno, próstata, testículo e pele (STAHL *et al.,* 1992; AUST *et al.,* 2005; RAO e RAO, 2007), o que pode indicar seu grande significado biológico no sistema de defesa humano.

De fato, sugeriu-se que o aumento da concentração plasmática do LYC está associado a uma eliminação mais rápida da parasitemia em crianças infectadas pelo *P. falciparum* (METZGER *et al.,* 2001). Adicionalmente, um estudo *in vitro* destacou o efeito citotóxico do LYC sobre o *P. falciparum*, através do aumento da produção de radicais livres no citoplasma do *P. falciparum*, levando a perda significativa da funcionalidade mitocondrial e do potencial de membrana e prevenindo a liberação dos merozoítos dos eritrócitos hospedeiros (AGARWAL *et al.,* 2014). Portanto, o efeito *in vitro* observado do LYC no crescimento do parasita pode ter relevância com a eliminação da parasitemia relatada no estudo *in vivo* anterior (METZGER *et al.,* 2001).

Este trabalho, fornece evidências de que o LYC é um antioxidante potente em uma variedade de problemas relacionados a saúde humana, e ainda é eficaz contra a infecção induzida por *P. berghei in vivo*, e reforça o papel importante da suplementação de micronutrientes na prevenção da malária e outras doenças.

1. 2 OBJETIVOS

1.2.1 Objetivo geral

Avaliar os efeitos do antioxidante licopeno sobre a taxa de mortalidade, parasitemia, e alterações oxidativas induzidas pelo *Plasmodium berghei* em camundongos.

1.2.2 Objetivos específicos

Avaliar os efeitos do licopeno sobre a taxa de mortalidade e parasitemia de camundongos infectados pelo *P. berghei*;

Avaliar os efeitos do licopeno em marcadores laboratoriais da peroxidação lipídica em camundongos infectados pelo *P. berghei*;

Avaliar os efeitos do licopeno sobre a atividade antioxidante total de camundongos infectados pelo *P. berghei*;

Verificar a existência de possíveis correlações entre os parâmetros avaliados.

2 CAPITULO I: ARTIGO DE REVISÃO

ARTIGO DE REVISÃO PUBLICADO NA REVISTA NUTRIENTS

Varela ELP, Gomes ARQ, da Silva Barbosa Dos Santos A, de Carvalho EP, Vale VV, Percário S. Potential benefits of lycopene consumption: Rationale for using it as an adjuvant treatment for malaria patients and in several diseases. **Nutrients.** 14;14(24):5303, 2022. doi: 10.3390/nu14245303. PMID: 36558462; PMCID: PMC9787606.

Review

Potential Benefits of Lycopene Consumption: Rationale for Using It as an Adjuvant Treatment for Malaria Patients and in Several Diseases

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Abstract: Malaria is a disease that affects thousands of people around the world every year. Its pathogenesis is associated with the production of reactive oxygen and nitrogen species (RONS) and lower levels of micronutrients and antioxidants. Patients under drug treatment have high levels of oxidative stress biomarkers in the body tissues, which limits the use of these drugs. Therefore, several studies have suggested that RONS inhibition may represent an adjuvant therapeutic strategy in the treatment of these patients by increasing the antioxidant capacity of the host. In this sense, supplementation with antioxidant compounds such as zinc, selenium, and vitamins A, C, and E has been suggested as part of the treatment. Among dietary antioxidants, lycopene is the most powerful antioxidant among the main carotenoids. This review aimed to describe the main mechanisms inducing oxidative stress during malaria, highlighting the production of RONS as a defense mechanism against the infection induced by the ischemia-reperfusion syndrome, the metabolism of the parasite, and the metabolism of antimalarial drugs. Furthermore, the effects of lycopene on several diseases in which oxidative stress is implicated as a cause are outlined, providing information about its mechanism of action, and providing an evidence-based justification for its supplementation in malaria.

Keywords: lycopene; malaria; oxidative stress; carotenoids; supplementation; antioxidants; adjuvant treatment

1. Introduction

Malaria is currently endemic in 85 countries and is found on most continents, but it is mostly confined to tropical and subtropical regions. It is noteworthy that repeated *Plasmodium* infection does not result in complete immunity, so populations in endemic regions are continuously susceptible to infection, transmission, morbidity, and mortality. Moreover, the lack of effective prevention strategies, including medications and/or vaccines, contributes significantly to this scenario. In 2020, there were 241 million cases, and 627,000 people died worldwide from the disease [1]. Almost all malaria-related deaths result from *Plasmodium falciparum* infection.

The pathophysiological mechanisms involved in the disease are complex and multifactorial. Inflammatory molecules are greatly involved and related to several cell signaling pathways. Indeed, after *Plasmodium* infection, an inflammatory reaction may be observed, with a predominance of neutrophils, lymphocytes, and monocytes, which are attracted by the presence of the parasite in the body [2–4]. Furthermore, leukocytes induce the expression of proinflammatory cytokines, including interleukin (IL)-1β, IL-2, IL-6, IL-17, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), that play an important role in the protection against malaria and elimination of parasites, by inducing monocyte phagocytosis, favoring the elimination of parasitized erythrocytes and limiting the progression of uncomplicated malaria to malaria with serious complications [5–7].

Additionally, in the recovery phase, regulatory cytokines, including IL-4, IL-10, chemokines, including IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [8–11] and transforming growth factor-β, neutralize the pro-inflammatory response by inhibiting the production of T helper 1 cytokines, contributing to the elimination of the parasite and reducing the risk of serious clinical complications [12–14].

However, a disturbance in the balance of pro- and anti-inflammatory cytokines and the underlying inflammatory process has been implicated in the pathogenesis of cerebral malaria and is associated with disease severity and death [11,15,16]. Such disturbance may be promoted by oxidative stress, which is known to intensify inflammation through tissue destruction and the release of danger signals by necrotic cells [17,18]. According to Ty et al. [19], reactive oxygen and nitrogen species (RONS) play an important role in triggering inflammation in malaria since these are produced in excess during infection and are potent inducers of inflammatory cytokines, suggesting the important role of oxidative stress in the pathophysiology of the disease [19–21].

Given the tropism of *Plasmodium* species for tissues such as blood [22], important systemic effects, including the induction of cytokines and RONS, which are closely associated with anemia, paroxysms, cerebral malaria, among other symptoms of systemic infection, are marked during the disease [20,23–25].

The oxidative changes occurring during infection that led to oxidative stress are a result of several different mechanisms, including the degradation of hemoglobin by the malaria parasite, producing redox-active by-products, such as free heme and hydrogen peroxide (H_2O_2) [26]. These radicals stimulate a series of oxidative reactions, leading to a decrease in the antioxidant defense system, through the consumption of micronutrients, including vitamin A, zinc, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and carotenoids, among others [27]. In fact, in malaria-endemic areas, *P. falciparum*-infected individuals present lower plasma concentrations of various micronutrients compared to healthy individuals [28].

On the other hand, these micronutrients play essential roles in the antioxidant system and are implicated in resistance to malaria infection [29]. In this sense, it has been shown that vitamin A, zinc, and selenium can interfere with the progression of oxidative reactions during malaria in mice infected with *P. berghei* [30]. Additionally, studies have suggested that the periodic supplementation of vitamin A and zinc can reduce the incidence of febrile episodes and parasitemia, being an effective and low-cost strategy to decrease *P. falciparum* morbidity in preschool children [31,32].

Other studies also support the hypothesis that the use of carotenoids by the host increases during malaria, suggesting that the nutritional status is an important modulating factor in the disease [28,33].

In this regard, it has been suggested that increased plasma lycopene concentration is associated with faster resolution of parasitemia in children infected with *P. falciparum*, being effective in maintaining the oxidative balance [34].

Considering the important involvement of oxidative stress mechanisms in malaria and, therefore, the potential of antioxidant nutrients in preventing it, in the present revision, we intend to demonstrate the beneficial effects of lycopene supplementation in malaria patients and, consequently, in several other diseases mediated by oxidative stress.

2. Oxidative Stress

Oxidative stress occurs when RONS overwhelm cellular defenses, causing damage to proteins, membranes, and deoxyribonucleic acid (DNA) [35]. It is the result of a disturbance in the balance between RONS and antioxidants in favor of RONS [36]. Under physiological conditions, endogenous RONS are generated by enzymatic systems, including nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and nitric oxide synthase (NOS), as a by-product of mitochondrial electron transport chain reactions (Figure 1) or by metal-catalyzed oxidation [37,38].

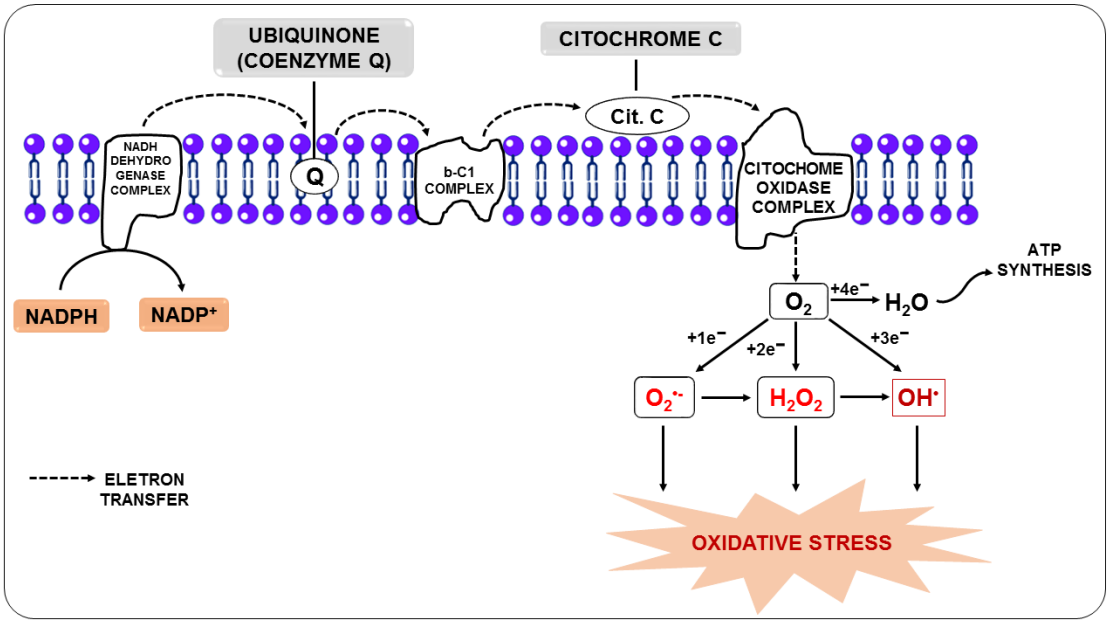

Figure 1. Production of reactive oxygen species from the transfer of electrons from the electron transport chain.

In this regard, the free radical superoxide ($O_2^{\bullet-}$), resulting from the monoelectronic reduction of oxygen, is considered the main precursor of other RONS since, after its formation, it can react with other molecules giving rise to other free radicals, such as hydroxyl ($OH^{\bullet}$), alkoxyl ($RO^{\cdot}$), and peroxyl ($ROO^{\cdot}$), in addition to other molecules that do not meet the definition of free radicals, but take part of oxidative reactions in a meaningful way, such as H_2O_2. Nitric oxide (NO) is among the molecules that can react with $O_2^{\bullet-}$, and the reaction between them generates the free radical peroxynitrite ($ONOO^{-}$). Additionally, $O_2^{\bullet-}$ can be unmuted to form H_2O_2, and it can be broken down through Fenton or Haber-Weiss reactions, leading to the generation of $OH^{\bullet}$ [39,40].

These RONS-generating chain reactions are initially controlled by antioxidant defense systems that act quickly, neutralizing any molecule that can potentially develop into a RONS or any free radical with the ability to induce the production of other pro-oxidants [41]. Three enzymes are critical in this process, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). These enzymes, respectively, unmute $O_2^{\bullet-}$ and break down H_2O_2 or hydroperoxides (ROOH) into harmless molecules such as H_2O, alcohol, and oxygen (O_2) [42]. The class of endogenous antioxidants also includes glutathione reductase, and reduced glutathione (GSH), in addition to small molecules such as coenzyme Q and uric acid (UA), among others [43]. Since they can be synthesized

by the body in response to oxidative aggression, we nominate endogenous antioxidants as *mobilizable antioxidant molecules*.

However, in diseases in which oxidative stress is a pathogenic mediator, including cancer and malaria, mobilizable antioxidants are not sufficient to maintain cell homeostasis due to the decreased synthesis of antioxidant enzymes and increased use of these antioxidants, among other factors [44–46].

In these cases, supplementation with dietary antioxidants is essential to maintain optimal cell function. Vitamins, including vitamins E and C, phenolic substances, such as flavonoids, resveratrol, and carotenoids, including β-carotene and lycopene, and drugs, such as *N*-acetylcysteine (NAC), among others, belong to this category [47,48]. Dietary antioxidants neutralize or eliminate RONS by binding or donating electrons to pro-oxidants, and in the process, they become free radicals but with less harmful effects. These "new radicals" are more easily neutralized and rendered completely harmless by other antioxidants in this group [49]. Thus, this class of antioxidant molecules can also be referred to as *consumable antioxidants*, as they are consumed in the face of oxidative aggression. Thus, consumable and mobilizable antioxidants act synergistically to fight the excessive increase in RONS, which can be a primary cause or a secondary complication of various diseases [50,51], as in malaria [52,53].

3. Oxidative Stress in Malaria

In malaria, oxidative stress is caused by four main mechanisms: a host defense against *Plasmodium* infection; ischemia-reperfusion syndrome; direct production of oxidative species by the parasite; and the metabolism of antimalarial drugs [54].

3.1. Oxidative Stress as a Host Defense Mechanism

RONS are essential for several physiological functions of the body, including cell survival, growth, proliferation, and differentiation, as well as the immune response [55,56].

As for the immune response, RONS are important for phagocytes, including neutrophils and monocytes/macrophages, which are highly activated during malaria, helping these cells phagocytize and destruct parasites [57,58].

In this sense, the body's defense system responds to infection by primarily recruiting neutrophils [59]. When neutrophils engulf the parasites, they induce a respiratory burst (Figure 2), in which O_2 enzymatically reacts with NADPH oxidase present in the plasma and the phagosomal membrane of neutrophils, forming $O_2^{\bullet-}$ [4]. $O_2^{\bullet-}$ and its derivatives H_2O_2 and $OH^{\bullet}$, when released by activated neutrophils in the phagosome, are essential to kill ingested pathogens [60].

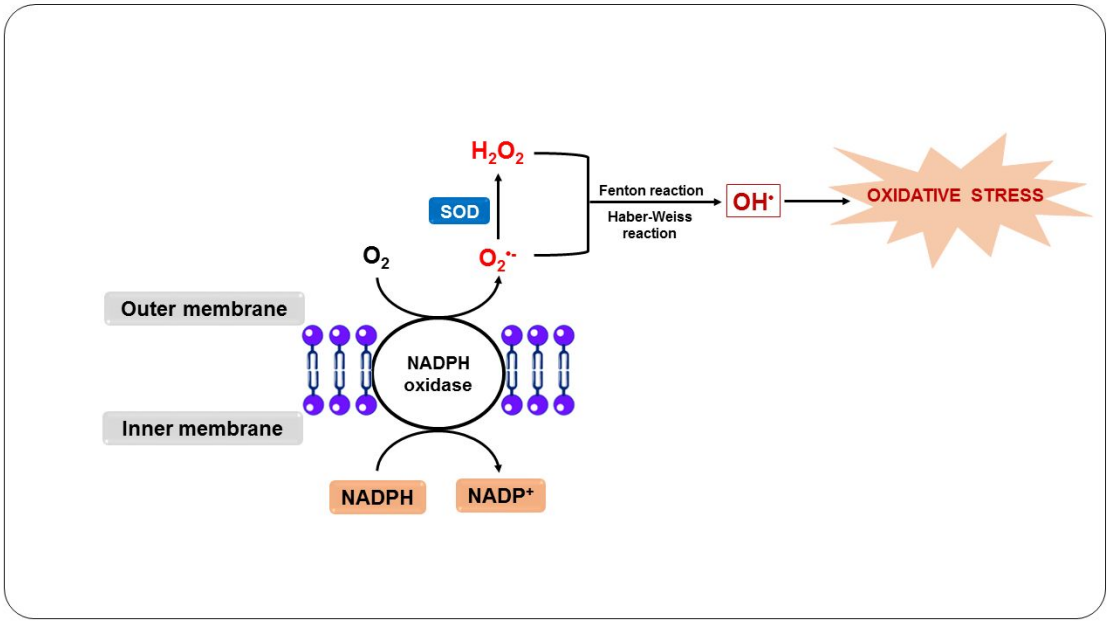

Figure 2. Oxidative stress as a host defense mechanism in response to infection by *Plasmodium* sp.

In addition, activated neutrophils produce cytokines, such as GM-CSF and M-CSF, and chemokines, including MIP-1α and MIP-1β, which attract these cells and are essential for monocyte mobilization [61]. These leukocytes engulf and kill the parasites through the oxidative action of $O_2^{\bullet-}$, which is generated in the same way as in neutrophils, as well as by the action of NO, which is produced by the macrophage from the reaction of NOS with L-arginine [62,63]. Additionally, the NO and $O_2^{\bullet-}$ generated react to form other RONS, such as $ONOO^-$, intensifying the cytotoxicity directed against the parasites [64].

Furthermore, neutrophil and macrophage myeloperoxidase is activated and uses H_2O_2 as a substrate to produce hypochlorous acid, a highly bactericidal compound [65,66]. On the other hand, phagocytosis and the consequent action of RONS, including $O_2^{\bullet-}$ and NO, as well as other toxic products, can exacerbate the condition due to rupture of the parasitized erythrocytes, during which normal uninfected erythrocytes can also be destroyed, stimulating cytoadherence and, consequently, potentially blocking blood flow, causing ischemia and anemia [67,68].

3.2. Oxidative Stress Due to Ischemia-Reperfusion Syndrome

In individuals with malaria, severe anemia induces microvascular dysfunction, leading to recurrent episodes of initial restriction of blood supply to organs, which can lead to ischemia and nutrient and oxygen deprivation, followed by subsequent restoration of concurrent perfusion and reoxygenation [54,69]. This process is called ischemia-reperfusion syndrome and can occur in malaria due to the sequestration of parasitized erythrocytes, as a result of the destruction of erythrocytes caused by the parasites and RONS during the paroxysm of malaria, and due to cytoadherence of erythrocytes to blood vessels [70].

Furthermore, this syndrome can trigger anaerobic metabolism, the production of lactic acid, and the consequent depletion of adenosine triphosphate (ATP). As ATP availability is reduced, ATP-dependent ion channels begin to fail. At the same time, calcium overload and excessive RONS production open the mitochondrial permeability transition pore, further reducing ATP levels [71,72]. During the ischemic process, the degradation of ATP causes the accumulation of xanthine oxidase (XO) and hypoxanthine due to the lack of oxygen. When the blood supply is resumed, XO acts on hypoxanthine resulting in the production of $O_2^{\bullet-}$ (Figure 3), which can later be converted into $OH^{\bullet}$ in the presence of transition metals and, consequently, trigger oxidative stress [73,74].

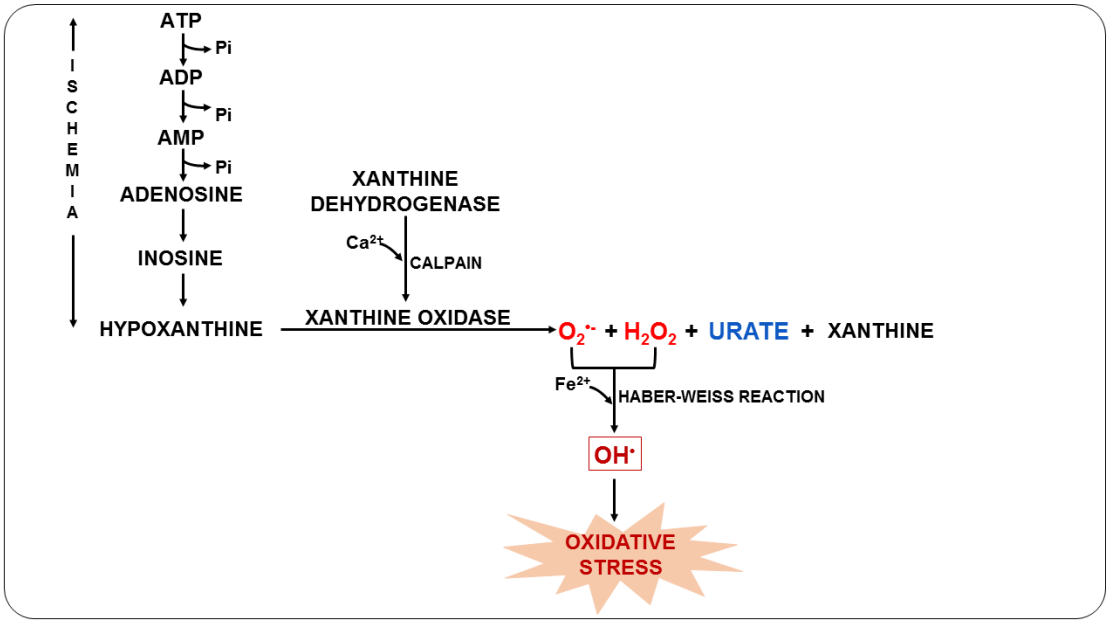

Figure 3. Oxidative stress due to ischemia-reperfusion syndrome during malaria.

During this process, UA is also formed, which is a weak organic acid present mainly as monosodium urate at physiological pH [75]. UA can be found in the host organism during malarial infection and can act by eliminating RONS and chelating transition metal ions or even by reducing NOS expression, impairing NO release [76,77]. Previous studies have shown that plasma UA levels in *P. falciparum*-infected children increase during acute episodes and with disease severity, suggesting that UA is an important mediator in the pathophysiology of malaria [78,79].

In the ischemia-reperfusion syndrome, RONS can be produced during ischemia but is massively increased during reperfusion, amplifying and propagating oxidative damage and destroying the integrity of proteins, membranes, and microvascular endothelium [80].

3.3. Oxidative Stress Due to the Metabolism of the Parasite

Another important oxidative mechanism in malaria is mainly triggered by the metabolism of the parasite, as well as by the potentially oxidative by-products generated and released from red blood cells destroyed by the action of the parasite [81]. Inside the erythrocyte, the parasite digests hemoglobin in its acidic digestive vacuole, forming essential amino acids for parasite development and proliferation [82]. However, in this process, ferroprotoporphyrin IX or heme complex (FPIX) is released, which is toxic to the parasite. On the other hand, this complex can still be detoxified within the parasite by polymerization [83].

Although the parasite manages to polymerize FPIX, resulting in a nontoxic derivative, hemozoin, also known as a malarial pigment, a significant amount escapes polymerization [84]. Thus, the ferrous iron (Fe^{2+}) from FPIX is oxidized to the ferric state (Fe^{3+}), with the consequent production of superoxide, which dismutates to H_2O_2 (Figure 4). This oxidative reaction chain leads to the production of $OH^{\bullet}$ from reactions involving H_2O_2 and Fe^{3+}, such as the Fenton and Haber–Weiss reactions [85].

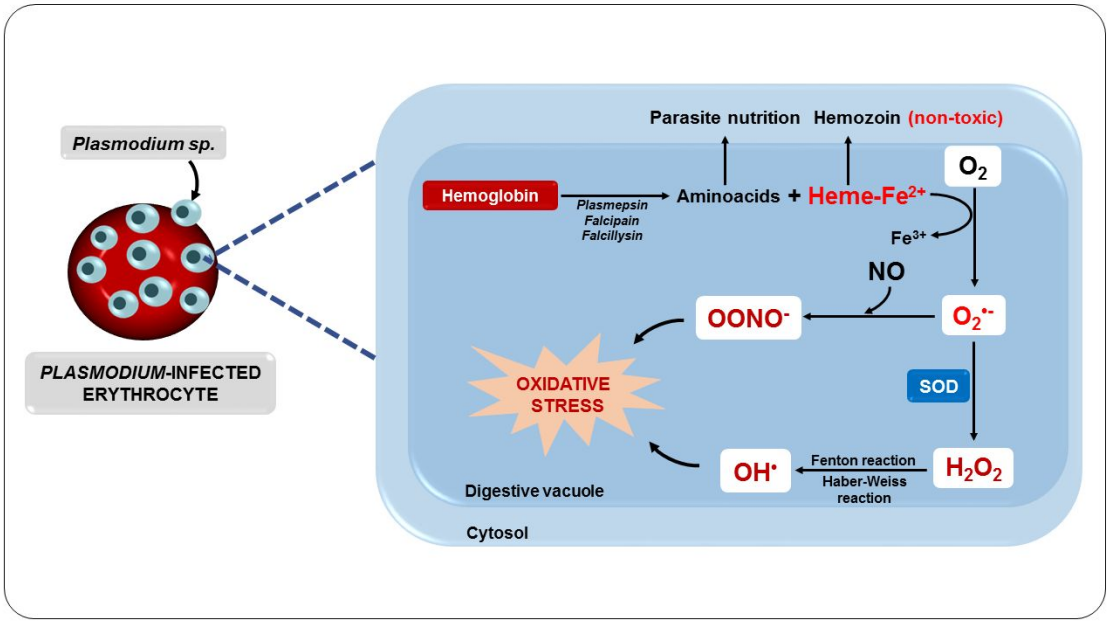

Figure 4. Oxidative stress as a consequence of parasite metabolism.

These free radicals can cause damage to the parasite's digestive vacuole membrane, eventually killing it [81]. However, the rapid development and proliferation of the parasite, associated with the RONS generated and released inside the erythrocytes, cause structural damage to the erythrocytes [86]. This results in increased membrane permeability for ions, increased cell volume, oxidation of sulfhydryl groups, and reduced deformability, contributing to the loss of erythrocyte function and cell lysis [67,87].

Consequently, all intra-erythrocyte content, including RONS, will be released to the extracellular environment, resulting in damage to several biomolecules, such as lipids, proteins, and DNA, as well

as enzyme inactivation, apoptosis induction, modification of surface adhesion molecule expression of leukocytes and endothelial cells, and alteration in the bioavailability of NO, compromising homeostasis and, ultimately, its survival [88,89]. These changes expose the host organism to a highly oxidative environment (Figure 5), implying the development of systemic complications such as reduced blood flow and severe anemia and also facilitating the entry of parasites into tissues such as the lung and brain, which can lead to organ failure [83,90–92].

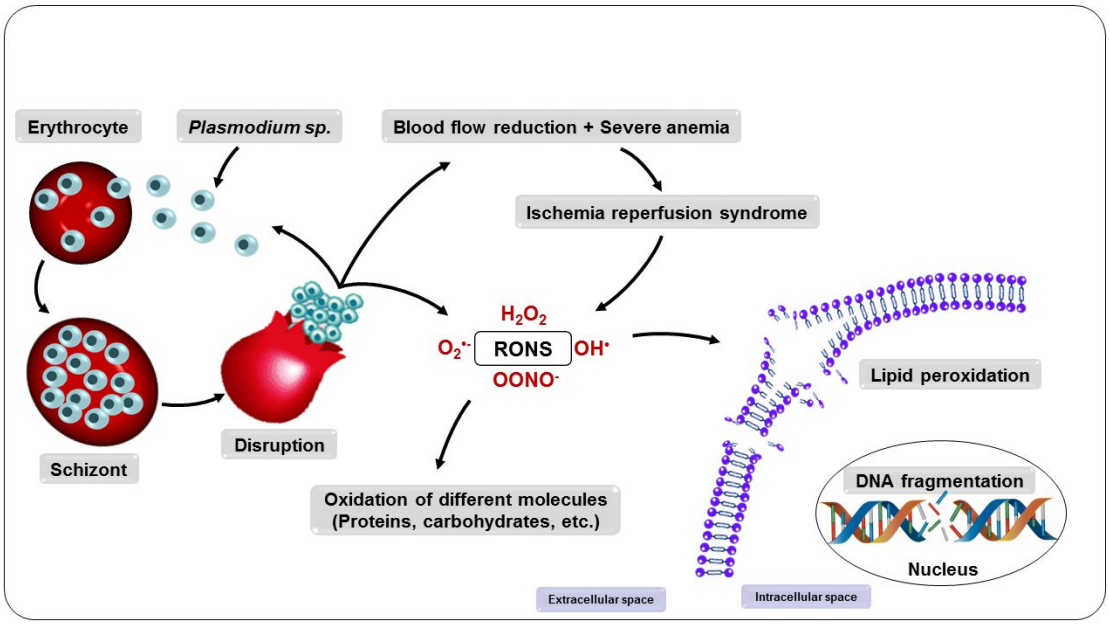

Figure 5. Consequences of the multiplication of parasites in the erythrocyte.

3.4. Oxidative Stress as a Consequence of the Metabolization of Antimalarial Drugs

The drug treatment of malaria is specially designed to interrupt parasite proliferation, responsible for the pathogenesis and clinical manifestations of the infection, to destroy the latent forms of the parasite (hypnozoites) to prevent late relapses, and to prevent the transmission of the parasite, through the use of drugs that prevent the development of sexual forms of the parasites [93,94].

In this context, one of the main targets of antimalarial drugs is the intracellular pathway of heme metabolism, which is implicated in the production of RONS and the consequent death of the parasite [95,96]. Therefore, chloroquine, a quinoline blood schizonticidal drug used to treat severe and uncomplicated cases of malaria, can act by preventing FPIX polymerization, causing the accumulation of FPIX in the parasite's digestive vacuole and consequent lethal oxidative stress in the parasite [97–99]. However, there are increasing reports of *P. falciparum* resistance to quinoline antimalarials, highlighting the importance of the *P. falciparum* chloroquine resistance transporter, a member of the drug/metabolite transporter superfamily located in the parasite's digestive vacuole, as the main responsible for chloroquine resistance [100–102].

Other studies indicate that, in addition to showing chemical similarity with chloroquine and a similar mechanism of action, other quinolines, such as quinine, amodiaquine, lumefantrine, and mefloquine are effective against many strains of parasites resistant to chloroquine [103–105]. In addition, some of these drugs are widely used in combination therapies with artemisinin derivatives, including artemether plus lumefantrine and artesunate plus amodiaquine, and provide synergistic antimalarial activity along with preventing the development of antimalarial drug resistance [106–108].

The site of action of artemisinin and its derivatives dihydroartemisinin, artemether, arteether, and artesunate is believed to be the parasite's digestive vacuole, where these drugs can interfere with the FPIX complex, giving rise to RONS, leading to damage to nearby proteins, and still interacting with

the mitochondrial electron transport chain of the parasite, enhancing RONS production, impairing mitochondrial functions, and killing the parasite [109–111].

Artemisinins act quickly and are very potent against blood-stage parasites. They are active against the sex stages of the parasite, which is important for blocking transmission [112,113]. However, due to their short half-life, these drugs are used in conjunction with other long-acting drugs that remain in the body for longer to fight potential remaining parasites [114,115].

Accordingly, studies show that primaquine increases the effect of combination therapy with artemisinin derivatives in eliminating malaria and reduces the risk of artemisinin-resistant infections [116].

Furthermore, only primaquine is recognized for completely eliminating *P. vivax* and *P. ovale* that form hypnozoites—the latent form of the parasite that remains in the liver and is responsible for disease relapse in individuals infected by these parasites—refractory to most drugs and for providing a radical cure [93,117]. Primaquine, an 8-aminoquinoline, can act directly on erythrocytes leading to massive production of RONS and consequent lipid peroxidation of the cytoskeleton and membrane, as well as hemolysis [118]. However, the use of primaquine in individuals with glucose-6-phosphate dehydrogenase deficiency can result in clinical manifestations of hemolysis, such as severe anemia, fatigue, jaundice, and acute renal failure, thereby limiting its use [119,120].

In this scenario, as a product of the normal host's metabolism or from the metabolism of the parasite, or as an effect of pharmacological treatment, intensely produced RONS cause damage to lipids, proteins, and DNA, leading to oxidative stress that impairs the normal functioning of the infected organism [98]. Therefore, the search for adjuvant therapies that can improve the clinical outcomes of malaria continues because, despite their benefits, treatments eventually cause oxidative damage, which limits their use [121].

3.5. Nitric Oxide in Malaria

Scientific evidence demonstrates that a specific RONS is particularly involved in the pathophysiology of this disease: NO [122,123]. It has been suggested that the low bioavailability of NO promotes oxidative stress in tissues such as the brain and lungs [124]. On the other hand, it has been shown that NO at high concentrations can kill *Plasmodium* [125,126]. NO is an important mediator of biological processes such as vascular homeostasis, neurotransmission, immunity, and inflammation [127–129]. Furthermore, it is a free radical produced by three different nitric oxide synthase enzymes, neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), which are constitutively expressed, and the inducible NOS (iNOS or NOS2), which is induced by inflammatory stimuli [130–133]. NO is very reactive and has a very short half-life. For this reason, nitrite and nitrate measurements, which are the final metabolites of NO, have been used to measure the concentration of NO indirectly [134,135].

Experimental evidence indicates that NO plays an important role in the defense against plasmodia in vitro and in vivo [136,137]. In this context, studies have shown that circulating levels of nitrite and nitrate were higher in anopheline mosquitoes—a natural vector of malaria in humans—infected with *Plasmodium* and that increased NO concentrations at the beginning of the sporozoite stage induced the formation of toxic metabolites, limiting parasite development [138].

In children and adults with malaria, elevated plasma levels of nitrites and nitrates have been associated with more rapid parasite clearance [139]. Indeed, previous studies have shown that children infected with *P. falciparum* had elevated levels of NO and iNOS activity, suggesting the protective role of NO in children with malaria [140]. Protection against severe malaria in this population of children appears to be associated, at least in part, with a polymorphism in the iNOS gene, which produces high levels of NO during an inflammatory event [141]. These studies suggest that NO production during malaria depends on the severity of the disease and the degree of patient immunity [142].

In an animal model of experimental cerebral malaria (ECM), Serghides et al. [143] demonstrated that pretreatment with inhaled NO reduced the accumulation of parasitized erythrocytes in the brain, decreased endothelial cell expression, and preserved vascular integrity. From these results, the

authors suggested that prophylaxis with NO inhalation can reduce systemic inflammation and endothelial activation during ECM. In a similar model, Ong et al. [144] showed that cerebrovascular dysfunction is characterized by vascular constriction, occlusion, and cell damage, resulting in impaired perfusion and reduced cerebral blood flow and oxygenation, and was associated with low NO bioavailability.

Given the critical importance of NO-derived and -non-derived oxidative stress in the underlying pathophysiological mechanisms of the disease, studies have shown that natural or synthetic exogenous antioxidants, including vitamin A, E, zinc, selenium, NAC, curcumin, *Agaricus sylvaticus* mushroom, and carotenoids, can benefit the treatment of malaria [145–148]. Several studies have indicated an association between the use of carotenoids and a decrease in oxidative changes, suggesting that the antioxidant properties of these compounds are an important factor against malaria-induced oxidative stress [149,150]. The recent interest in carotenoids has focused on the role of lycopene in human health [151,152].

4. Lycopene

Lycopene is a natural constituent synthesized by plants and microorganisms [153]. It is a red pigment found in some fruits and vegetables, such as guava, watermelon, papaya, pitanga *(Eugenia uniflora—Myrtaceae)*, tomatoes, and their derivatives [154–157] and can be extracted from these vegetables by chemical reactions using organic solvents, such as ethanol and ethyl acetate and/or using a supercritical fluid such as supercritical carbon dioxide, or by heat treatment at different temperatures ranging from 60 to 140 °C [158–163]. It is widely used as a supplement in functional foods, nutraceuticals, and pharmaceuticals, as well as an additive in cosmetics [164,165].

Lycopene is an intermediate product of the β-carotene biosynthetic pathway that does not have provitamin A activity, as it does not have the β-ionone ring in its structure, which is responsible for this characteristic [166]. This compound is a noncyclic, fat-soluble hydrocarbon that contains 11 conjugated double bonds and 2 unconjugated double bonds, thereby offering it greater reactivity. This polyene can also exist in all-*trans* and *cis*-lycopene isomeric forms (Figure 6). Conversion from all-*trans*- to *cis*-lycopene forms can occur by geometric isomerism induced by light, thermal energy, or chemical reactions [167,168].

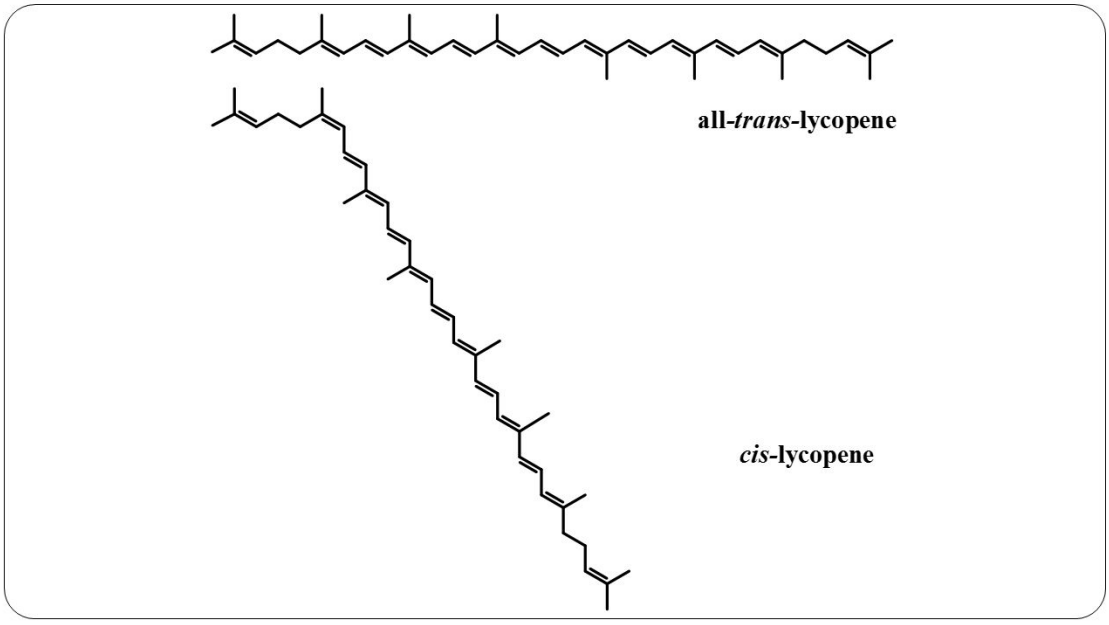

Figure 6. All-*trans*-lycopene and *cis*-lycopene structures.

4.1. Sources

Tomatoes and tomato products are the main source of lycopene and are considered an important source of carotenoids in the human diet. In raw or fresh tomatoes, lycopene occurs mainly as a *trans* isomer [169]. However, *cis* isomers are better absorbed by the human body than *trans* isomers. *Cis* isomers form during cooking, food processing, and storage, which do not affect the total lycopene content [170].

Studies have shown higher plasma lycopene concentrations after ingestion of processed tomatoes compared to raw tomatoes [171,172]. In fact, processing and homogenization induce the disruption of the food membrane, converting lycopene from the *trans* to *cis* form, increasing its solubility and, consequently, its availability [173]. In addition, the acidic pH of the stomach also appears to secondarily contribute to such conversion, as it can lead to the transformation of the *trans* to *cis* form [174]. Thus, lycopene can be rapidly and completely absorbed without energy expenditure in the intestinal wall after oral administration in animals and humans [175].

4.2. Absorption

After absorption, lycopene can be found in high concentrations in human body fluids and tissues, such as breast milk, prostate, testis, and skin [176]. Furthermore, it is the predominant carotenoid in human plasma, naturally present in a higher concentration than β-carotene and other dietary carotenoids, which may indicate its greater biological significance for the human defense system [177].

Studies suggest that lycopene is transported between cells to target organs by specific proteins or migrates aggregated to chylomicrons, with the isometric form of lycopene being decisive for this process [178]. This is because, after passing through the stomach, trans isomers can readily aggregate within the intestine and form crystals, greatly reducing their absorption by micelles, while the cis form allows lycopene to be more efficiently incorporated into mixed micelles [179]. The lycopene-loaded micelles are then absorbed into enterocytes, from where they are released in chylomicrons, which exit to the lymph, passing from there to the systemic circulation to the liver. The liver stores and secretes carotenoids as very low-density lipoproteins (VLDL), which are subsequently absorbed by various tissues, including adrenal, kidney, adipose, splenic, lung, and reproductive organ tissues, and are subsequently recovered as other low-density (LDL) and high-density (HDL) lipoproteins [178,180]. During absorption, lycopene taken up by the enterocyte can also be cleaved by β-carotene 9′,10′-oxygenase (BCO2) to form apo-10′-carotenoid metabolites, including lycopene apo-10′-lycopenols [181–183].

4.3. Metabolism

Lycopene can be metabolized through isomerization, followed by oxidation to produce epoxides, or undergo eccentric cleavage by BCO2 to form apolycopenols [181,182,184,185]. Additionally, lycopene cleavage products can be generated through autoxidation, via reaction with free radicals [186], by processes that simulate biological tissues [187], or even by chemical reactions that cause the interruption of the polyene chain, affecting the carbon-carbon double bond system, and by addition or cleavage, resulting in several isomers and apolycopenols [159,188].

Among the most interesting lycopene metabolites formed by oxidative degradation of the hydrocarbon chain are the apolycopenols [189]. Apolycopenols have already been detected in animal tissues, such as ferret lungs [182] and the liver of rats [190], after treatment with lycopene. In addition, several apolycopenols have been isolated from fruits, vegetables, and human plasma [191].

Increasing evidence suggests that many of lycopene's biological actions may be mediated, at least in part, by its metabolites and/or oxidation products [192–194]. In this regard, lycopenols were shown to reduce the proliferation of cancer cells, induce apoptosis, regulate the cell cycle, induce the expression of nuclear transcription factors, and enhance cell-to-cell communication [189,195–198]. Furthermore, the study by Böhm et al. [199] showed that the cis isomers obtained from processed foods had an antioxidant potential twice as intense as β-carotene. Additionally, studies by Lian and Wang [200] showed that treatment of human bronchial epithelial cells (BEAS-2B) with apo-10′-lycopenoic acid (10 μM) increased GSH levels and suppressed RONS production and oxidative damage induced by H_2O_2 in vitro. In addition, it was reported that apo-10′-lycopenoic acid induced

the expression of phase II antioxidant enzymes mediated by factor 2-related nuclear erythroid factor 2 (Nrf2), including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione S-transferases (GST), GR, and γ-glutamylcysteine synthetase (γ-GCS) [200].

Apolycopenol treatment also inhibited methemoglobin-induced lipid peroxidation in a chemical model of postprandial oxidative stress in the gastric compartment [201].

In another in vitro study, both apo-10′-lycopenoic acid and apo-14′-lycopenoic acid inhibited RONS production and oxidative DNA damage induced by H_2O_2 and cigarette smoke. This effect was accompanied by the inhibition of mitogen-activated protein kinase (MAPK) phosphorylation, the expression of heat shock proteins (hsp)70 and hsp90, and the inactivation of NF-κB, molecules that are activated in situations of oxidative stress and which have also been implicated in the modulation of various intracellular redox functions [202].

5. Antioxidant Effects of Lycopene

Among the carotenoids, lycopene is the most effective antioxidant against RONS and may contribute to preventing or reducing oxidative damage to cells and tissues in vivo and in vitro [203]. Evidence supports the role of lycopene as a potent antioxidant, capable of scavenging singlet oxygen ($^1O_2$) and other free radicals, such as ROO^-, with a potential twice as high as β-carotene, and ten times as efficient as α -tocopherol, although lycopene circulates at much lower concentrations than vitamin E [204,205]. During the elimination of $^1O_2$, energy is transferred from this radical to the lycopene molecule and, as it has an open chain with 11 conjugated double bonds in its structure, this favors stabilization of the unpaired electron of the radical by resonance [206,207]. Additionally, it was observed that lycopene effectively eliminates other RONS, such as $OH^•$, $O_2^{•-}$, and $ONOO^-$ [208].

Furthermore, the lipophilic characteristic of lycopene favors its interaction with the lipid bilayer of the cell membrane, thereby preventing the breakdown of fatty acids and the oxidation of lipids, proteins, and DNA [180]. In this sense, Suwannalert et al. [209], investigating serum levels of lycopene and malondialdehyde (MDA) in elderly susceptible to oxidative stress, demonstrated that lycopene levels were inversely related to MDA levels. Additionally, Yonar and Sakin [206] demonstrated that lycopene treatment prevented deltamethrin-induced oxidative stress by decreasing MDA levels in fish (*Cyprinus carpio*) and significantly increasing SOD, CAT, and GSH-Px activities and the level of GSH. Similar results were found by Kujawska et al. [210], who reported that treatment with tomato extract enriched with lycopene was able to suppress the oxidative stress induced by *N*-nitrosodiethylamine in rats and increase the enzymatic antioxidant activity in these animals.

5.1. Cardioprotective Effect of Lycopene

Oxidative stress produced by RONS is implicated in the development of several diseases, including atherosclerosis and several heart diseases [50,211], but studies suggest that lycopene supplementation or consumption of tomato and its derivatives can improve endothelial function and lead to reduced blood pressure [187]. In this sense, Mohamadin et al. [212] investigated the cardioprotective potential of lycopene against isoproterenol-induced oxidative stress and cardiac lysosomal damage in rats. According to the authors, lycopene supplementation (4 mg/kg/day) significantly improved lysosomal membrane damage, as well as changes in cardiac enzymes, including aspartate aminotransferase, creatine kinase isoenzyme MB, and troponin T, as well as oxidative stress markers such as MDA, GSH, GSH-Px, SOD, and CAT.

Previously, Bose and Agrawal [213] had already observed, in a clinical study with grade I hypertensive patients, that tomato supplementation for 60 days improved the levels of antioxidant capacity and reduced lipid peroxidation in these patients. Ferreira-Santos et al. [151] reported that a lycopene-supplemented diet prevented angiotensin II-induced hypertension with no effect in normotensive rats. The authors suggested that the infusion of angiotensin II caused a decrease in the activity of antioxidant enzymes, and the treatment with lycopene improved the antioxidant balance, increasing the activity of GSH-Px and SOD, reducing oxidative stress, and improving cardiovascular remodeling. These results confirm the antihypertensive potential of lycopene without the risk of causing hypotension in normotensive individuals.

5.2. Anti-Atherosclerotic Effect of Lycopene

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipids and inflammatory cells in the walls of medium and large-caliber arteries, which is the main cause of heart disease and mortality in Western societies. The pathogenesis of atherosclerosis involves the activation of inflammatory mediators, cytokines, and increased oxidative stress [214,215].

In evaluating the effect of lycopene in an animal model of atherosclerosis, Renju et al. [216] demonstrated that CAT, SOD, and GSH-Px activities and GSH levels were increased, while the levels of thiobarbituric acid reactive substances (TBARS), total cholesterol, triglyceride, low-density lipoprotein (LDL), very-low-density lipoprotein, and inflammatory mediators, including cyclooxygenase-2 (COX-2) and 15-lipoxygenase, decreased after treatment with lycopene isolated from the alga *Chlorella marina*. Additionally, Martín-Pozuelo et al. [217] showed that tomato consumption improved the expression of genes such as fatty acid-binding protein 2, which encodes enzymes involved in lipid metabolism, thus reducing the synthesis of fatty acids, triglycerides, and cholesterol, preventing their accumulation and modulating the progression of steatosis induced in rats. Moreover, according to Navarro-González et al. [218], lycopene competes with hydroxymethylglutaryl coenzyme A in the liver, thus preventing the formation of mevalonate, and consequently reducing cholesterol synthesis by reducing the activity of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase. For this reason, the consumption of tomato juice and the accumulation of lycopene in the liver were able to improve plasma cholesterol levels in steatosis induced in animals [218].

In this sense, Kumar et al. [219] observed that treatment with lycopene induced an increase in high-density lipoprotein and reduced levels of total cholesterol, LDL, triglycerides, and TBARS in rats fed a high-cholesterol diet. Brito et al. [220] also demonstrated that lycopene extracted from guava (*Psidium guajava* L.) reduced MDA and triglyceride levels, as well as reduced plasma activity of myeloperoxidase and hepatic steatosis in an animal model of dyslipidemia. The results indicated that lycopene has hypolipidemic and anti-atherogenic potential.

5.3. Hepatoprotective Effect of Lycopene

Oxidative stress is believed to be an important contributor to the pathogenesis of liver diseases, ranging from simple steatosis to its more severe form or even the genesis of hepatocellular carcinoma [221]. When investigating the role of lycopene in an animal model of hepatotoxicity, studies demonstrated that lycopene improved biochemical indices, both in the blood and in the liver of animals. Furthermore, lycopene restored the antioxidant capacity and increased the levels of GSH, GSH-Px, glutathione S-transferase (GST), CAT, and SOD, which, together with lycopene, could limit the production of oxidants [222–224]. Similar results were observed in the study by Abdel-Daim et al. [225], where zinc oxide poisoning in fish caused severe lipid peroxidation with a significant increase in the level of MDA in the liver, kidney, and gill tissues, and treatment with lycopene significantly reduced the production of this oxidative stress biomarker

Recently, Ni et al. [152] demonstrated that lycopene inhibited and reversed lipotoxicity-induced insulin resistance, preventing nonalcoholic steatohepatitis in mice, attenuating hepatic lipid accumulation, and increasing lipolysis. The beneficial effects of lycopene were attributed in part to decreased hepatic recruitment of T cells and macrophages, and to a reduction in macrophage M1/Kupffer cells, which attenuated insulin resistance, as well as liver inflammation and fibrosis, in preexisting steatohepatitis. These effects have been associated with a decrease in oxidative stress in cells.

5.4. Anti-Diabetic Effect of Lycopene

Lycopene appears to have beneficial effects in improving factors related to diabetes progression, including oxidative stress, inflammation, and endothelial dysfunction [226]. It was observed that the administration of lycopene in rats decreased glucose levels, increased insulin concentration, reduced H_2O_2, TBARS, and iNOS levels, increased cNOS activity and NO levels, as well as increased total antioxidant capacity with increased CAT, SOD, and GSH-Px activity [227–229].

In humans, in a placebo-controlled clinical trial with patients with type 2 diabetes mellitus, Neyestani et al. [230] found a negative correlation between total antioxidant capacity and MDA in the lycopene-treated group, indicating that lycopene supplementation attenuates oxidative stress in these patients. According to Yin et al. [231], lycopene strengthens the antioxidant defense system against oxidative stress, attenuating insulin signaling deficits, inhibiting neuroinflammation, and improving cognitive function. These studies suggest that lycopene may help improve the progression of diabetes in humans.

5.5. Anti-Cataract Effect of Lycopene

The ocular environment is rich in endogenous sources of RONS. Although there are several physiological defenses to protect ocular lenses from the toxic effects of light and oxidative damage, evidence suggests that long-term chronic exposure to oxidation can damage the lens and predispose it to the development of cataracts [232]. In this sense, Gupta et al. [233] showed that lycopene supplementation in rats restored GSH, SOD, CAT, and GST levels and, consequently, prevented sodium selenite-induced cataracts. According to the authors, lycopene protects against the experimental development of cataracts due to its antioxidant properties and may be useful for cataract prophylaxis or therapy [234]. Also, Göncü et al. [235] demonstrated the anti-inflammatory effect of lycopene on lipopolysaccharide-induced uveitis in rats. According to the authors, the anti-inflammatory activity of lycopene was mediated by the inhibition of TNF-α, NO, and IL-6 production, resulting in reduced inflammation and uveal oxidative stress.

5.6. Anti-Cancer Effects of Lycopene

Studies have shown that lycopene can reduce the risk of cancer by inducing antioxidant enzymes and phase II detoxifying enzymes such as NAD(P)H quinone oxidoreductase 1 and γ-glutamylcysteine synthetase [236]. These enzymes eliminate many harmful substances, converting them into hydrophilic metabolites that can be readily excreted from the body. In fact, lycopene administration significantly suppressed gastric cancer in vivo, reducing lipid peroxidation, increasing the levels of vitamin C, vitamin E, and GSH, and increasing circulating activity dependent on enzymes such as GSH-Px and GST [237]. Lycopene also prevented experimental oral carcinogenesis by inhibiting oxidative stress through the upregulation of detoxification pathways [238]. Recently, Cheng et al. [239] demonstrated the efficacy of lycopene in inhibiting the oxidative stress induced by cigarette smoke in lung cancer epithelial cells.

Other potentially beneficial effects of lycopene include inhibition of carcinogenic activation, proliferation, angiogenesis, invasion, and metastasis, blocking tumor cell cycle progression, and induction of apoptosis through its antioxidant activity and changes in various signaling pathways [240–244]. In addition, lycopene improved communication between cells by stimulating gap junctions, which is believed to be one of the protective mechanisms related to the cancer-preventive activities attributed to lycopene [195].

In in vitro studies, lycopene treatment selectively interfered with cell growth and induced apoptosis in cancer cells without affecting normal cells [200,245]. In vivo studies have shown the protective effects of lycopene against liver cell carcinoma and prostate cancer [197,237,246–253]. In addition to the correlation between lycopene and prostate cancer demonstrated in clinical studies, increasing evidence suggests that lycopene plays an important role in preventing cancer in other organs such as the breast, lung, gastrointestinal tract, pancreas, cervix, and ovaries [254–257].

6. Effects of Lycopene on Malaria

The use of antioxidant compounds in the treatment of tropical diseases has increased, including Chagas disease, dengue, and malaria, as several studies have suggested the involvement of oxidative stress in the pathogenesis and progression of these diseases [54,258,259]. In this context, studies show that the discovery of new antimalarial drugs is necessary, and natural antioxidant products are important sources for obtaining new antimalarial compounds or even as adjuvant therapy, enhancing the activity of antimalarial drugs [260–264].

A study by Metzger et al. [34] demonstrated that natural products can be used in malaria chemotherapy. According to this study, increased plasma lycopene concentration was associated with faster clearance of parasites in children. In a related study, Caulfield et al. [265] demonstrated that the nutritional deficiency of the host is associated with the morbidity and mortality of children with severe malaria. In this sense, previous studies suggest that changes in plasma concentrations of micronutrients, including vitamins A and C, retinol, β-carotene, α-carotene, β-cryptoxanthin, lutein, and lycopene, occur due to increased use of these antioxidants in patients with malaria, suggesting that there may be a need for vitamin supplementation in patients with malaria [266]. In corroborating this suggestion, the nutritional deficit seems to be associated with a redirection of these antioxidants to the liver to aid in the synthesis of acute-phase proteins in other organs, repair tissue damage caused by the infectious organism, and aid in the host's oxidative defense mechanisms [33].

In fact, Sondo et al. [32] had already reported that periodic supplementation of high doses of vitamin A and zinc could reduce the morbidity caused by malaria. In this sense, Agarwal et al. [147] investigated the effect of lycopene on the growth of *P. falciparum* in vitro, monitoring the progression at different stages. These authors showed that lycopene treatment induced an increased production of RONS in the cytoplasm of the parasite, which caused the parasite to lose its mitochondrial membrane potential and cytotoxicity, resulting in merozoites not being released from the erythrocytes of the host, suggesting that the inclusion of lycopene in the diet may be useful in changing the clinical outcomes of malaria.

Preliminary results from our research group demonstrated that lycopene supplementation in mice (BALB/c; 3.11 mg/kg) infected with the *P. berghei* strain showed a delay in the induction and a decreased progression of parasitemia. Also, the animals supplemented with lycopene showed a higher rate of survival compared to the positive control [267], suggesting lycopene prophylactic and antiparasitic activity, which may be due to the cytotoxic effect of lycopene against the parasite [147], suggesting an important role of lycopene supplementation in preventing malaria [268].

6.1. Neuroprotective Effect of Lycopene

Individuals infected with *P. falciparum* can rapidly progress to severe anemia, respiratory distress, and cerebral malaria [269]. Cerebral malaria is associated with debilitating neurological impairments in survivors, as well as higher number of malaria deaths [270]. Although there is no complete understanding of the exact mechanisms and processes that lead to neuronal cell death in cerebral malaria, studies demonstrate that elevated levels of the inflammatory cytokines and RONS contribute to neuronal cell death in cerebral malaria [271,272].

Furthermore, considerable evidence suggests that microvascular dysfunction, sequestration of parasitized blood cells in the microcirculation, an abrupt reduction in blood flow, and cerebral hypoxia are essential for ischemic stroke, characterized by the presence of both ischemic and reperfusion-induced injuries in the brain, leading to neuronal dysfunction and death [273]. In this context, studies by Paul et al. [274] and Farouk et al. [275] point out that lycopene is a powerful antioxidant, permeable to the blood-brain barrier, with neuroprotective activity. Previously, Hsiao et al. [276] showed that treatment with lycopene in rats (4 mg/kg) prevented ischemic brain injury induced by middle cerebral artery occlusion by inhibiting microglia activation and NO production, resulting in reduced infarction volume in brain injury by the ischemia-reperfusion syndrome. Additionally, lycopene has been shown to protect the brain from ischemic damage by its ability to increase GSH production and decrease RONS production. Furthermore, lycopene activates the expression of nuclear factor erythroid 2 related factor 2 and heme oxygenase-1, one of the antioxidant pathways involved in the attenuation of oxidative stress and the maintenance of the redox state in various tissues and organs, such as the brain tissue [277].

Oxidative stress is also strongly implicated in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD) [278] and Parkinson's disease (PD) [279]. In this sense, Kaur et al. [280] demonstrated that lycopene supplementation in rats (10 mg/kg) for 30 days was able to reduce oxidative stress in rotenone-induced PD, restoring GSH and SOD levels and reversing complex I inhibition of the electron transport chain, exerting a protective effect on motor and cognitive deficits.

Furthermore, according to Prema et al. [281], lycopene induces increased expression of the antiapoptotic protein B-cell lymphoma 2 protein (BCL-2) and decreased release of the proapoptotic proteins cytochrome c, protein x associated with BCL-2 (BAX), and caspases-3, 8, and 9, preventing apoptosis in mice with PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

Previously, studies have shown the activation of inositol-requiring enzyme 1, induction of X box-binding protein 1, upregulation of BAX, downregulation of BCL-2, and cleavage of caspase-3 indicating the endoplasmic reticulum stress-mediated apoptotic pathway in PbA-infected mouse brains involved in neuronal cell death in severe/cerebral malaria [271]. Thus, lycopene reverses neurochemical deficits, oxidative stress, apoptosis, and physiological abnormalities in malaria and PD-induced mice.

Other studies reinforce the importance of lycopene in neuronal mitochondrial function. In a rat cortical neuron culture model using an established paradigm of β-amyloid (Aβ) peptide-induced cell injury, Qu et al. [282] found that lycopene significantly inhibited intracellular RONS and prevented Aβ-induced mitochondrial fragmentation. Furthermore, it inhibited the opening of mitochondrial permeability transition pores as well as the release of cytochrome c. Lycopene also prevented a decrease in the enzymatic activity of the mitochondrial complex and a reduction in the generation of ATP, besides preventing the occurrence of damage to the mitochondrial DNA and improving the level of the mitochondrial transcription factor A in the mitochondria. These results suggest that the ability of lycopene to prevent Aβ-induced neurotoxicity is closely related to the inhibition of mitochondrial oxidative stress and improvement of mitochondrial function [282,283].

Behavioral experiments confirmed that lycopene consistently reduced Aβ accumulation in elderly CD-1 mice [284]. Lycopene also attenuated age-associated cognitive impairments, including those involving locomotor activity, working memory, and spatial cognitive memory. Lycopene administration reversed the systemic and oxidative stress responses of the central nervous system induced by aging. Furthermore, lycopene downregulated the expression of inflammatory mediators and prevented synaptic dysfunction in aged mouse brains [285]. Huang et al. [286] also showed the antagonistic effect of lycopene on neuronal oxidative damage induced by tert-butyl hydroperoxide in vitro. Moreover, lycopene increased cell viability, improved neuron morphology, increased GSH levels, and decreased the production of RONS. Lycopene also reduced the expression of BAX, cytochrome c, and caspase-3 and increased the expression of BCL-2 and phosphoinositide 3-kinase/Akt (PI3K/Akt) [286]. Recent studies confirm that lycopene prevents neuronal apoptosis through the activation of the PI3K/Akt signaling pathway, important regulators for preventing mitochondrial damage and apoptosis induced by oxidative stress, ischemia-reperfusion syndrome, that play an important role in severe/cerebral malaria [287–289].

6.2. Effects of Lycopene as an Immunomodulator

Other factors related to neuronal injury and death in severe/cerebral malaria include the release of RONS, mitochondrial dysfunction, induction of programmed cell death, microglia activation, and release of inflammatory mediators [290,291].

Studies indicate that in malaria infection, increased expression of high mobility group box-1 is observed, which interacts with cell surface receptors such as toll-like receptor-4 (TLR-4), leading to the overproduction of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α, and IFN-γ) and anti-inflammatory cytokines (IL-4, IL-10, and IL-13) [289,291–293]. The action of these cytokines in conjunction with disturbances present in the microcirculation can affect both the integrity and functions of the blood-brain barrier, leading to vascular congestion, disruption of the blood-brain barrier, cerebral edema, impaired perfusion, and neuronal damage [294,295].

Several studies have highlighted the ability of carotenoids and their metabolites to regulate intracellular signaling cascades, modulating gene expression and protein translation in metabolic pathways associated with inflammatory and oxidative stress [296,297]. In this sense, studies indicate that lycopene can modulate the production of IL-1β, TNF-α, IL-2, IL-10, and IFN-γ, exerting an immunomodulatory effect on the peripheral blood mononuclear cells of healthy individuals [298], as well as suppressing the production of NO, IL-6, and TNF-α [299,300]. According to Feng et al. [299]

and Vasconcelos et al. [301], lycopene interferes with the phosphorylation of the inhibitory protein kappa B, protecting it from degradation and preventing the release and translocation of NF-κB, a transcription factor that plays an important role in regulating the expression of genes responsible for inflammation, such as TNF-α, IL-1β, iNOS, and COX-2, proliferation, and apoptosis (Figure 7).

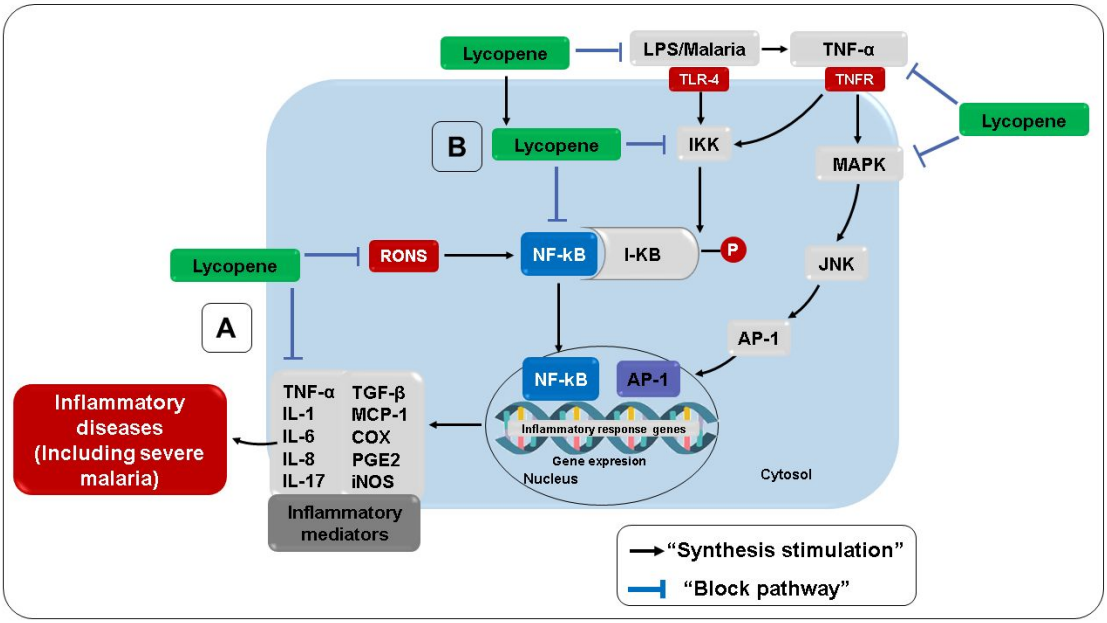

Figure 7. Anti-inflammatory effects of lycopene. (**A**) Direct anti-inflammatory activity. (**B**) Indirect anti-inflammatory activity. TGF-β, Transforming growth factor-beta; AP-1, activator protein-1; JNK, c-jun *N*-terminal kinase; MAPK, mitogen-activated protein kinases; I-κB, kappa B inhibitory protein; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha; IL-1, interleukin 1; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1.

Other studies reinforce that the blockade of NF-κB activation by lycopene appears not to be tissue- or cell-type specific and may represent a way in which lycopene can inhibit the production of other inflammatory mediators, including TNF-α, NO, and IL-6, resulting in reduced inflammation [302,303]. In this sense, Gouranton et al. [304] showed that lycopene reduced TNF-α-induced activation of the NF-κB signaling pathway in adipocytes. According to the authors, this effect was fundamental for the TNF-α-mediated decrease in the expression of proinflammatory cytokines and chemokines in adipocytes and pre-adipocyte 3T3-L1 cells. The same effect was observed in human adipocytes, where lycopene decreased the expression of IL-6, monocyte chemotactic protein 1, and IL-1β induced by TNF-α [304].

The reduced production of the proinflammatory cytokines IL-1β and TNF-α, as well as the increased secretion of the anti-inflammatory cytokine IL-10, indicate that lycopene can boost anti-inflammatory responses [305]. In addition, lycopene can increase IL-12 and IFN-γ secretion in human peripheral blood mononuclear cells, indicating that lycopene enhances the immune response of the host [306]. In this sense, evidence from an ex vivo study indicates the stimulatory effect of lycopene on cytokine production by T-helper 1 lymphocytes, resulting in a cell-mediated immune response. Yamaguchi et al. [307] observed that the oral administration of lycopene in mice (5 mg/kg/day) significantly suppressed capsaicin-induced production of IL-2, IFN-γ, and IL-4 in lymphoid tissue cells in the small intestine wall, cytokines that are involved in the development of immunity to the antigens present there. Furthermore, lycopene did not alter the T lymphocyte population, indicating that lycopene accelerates and/or suppresses T-helper cytokines in these cells, acting to modulate the immune response.

Other studies have also verified the potential anti-inflammatory effect of lycopene combined with other substances, such as lutein, omega-3, and carnosic acid [308,309]. In this sense, Phan et al. [310]

showed a reduction in IL-8 secretion by human colorectal Caco-2 cells in the presence of lycopene and anthocyanin mixtures. Previous studies have shown that the association with substances such as lutein, selenium, and β-carotene promotes a synergistic effect, intensifying NO, TNF-α, SOD, and prostaglandin E2 production inhibition, as well as MDA derived from the down-regulation of iNOS, COX-2, NADPH oxidase, or 5-lipoxygenase expression, and inhibition of TNF-α secretion [311,312].

Together, these data support the anti-inflammatory and immunomodulatory effect of lycopene on major cell subtypes, namely, adipocytes, pre-adipocytes, and macrophages, cells that are involved in the production of inflammatory cytokines and chemokines in malaria.

7. Future Trends and Conclusions

The benefits provided by lycopene can be attributed mainly to its direct antioxidant activity [229]. This activity is generally responsible for protecting the cellular system from a variety of RONS, including $^1O_2$, $O_2^{\bullet-}$, NO, and $OONO^-$, as well as having an indirect action through the upregulation of antioxidant substances, in addition to preventing other diseases [313]. Finally, Figure 8 summarizes the mechanisms of action considered in the present review.

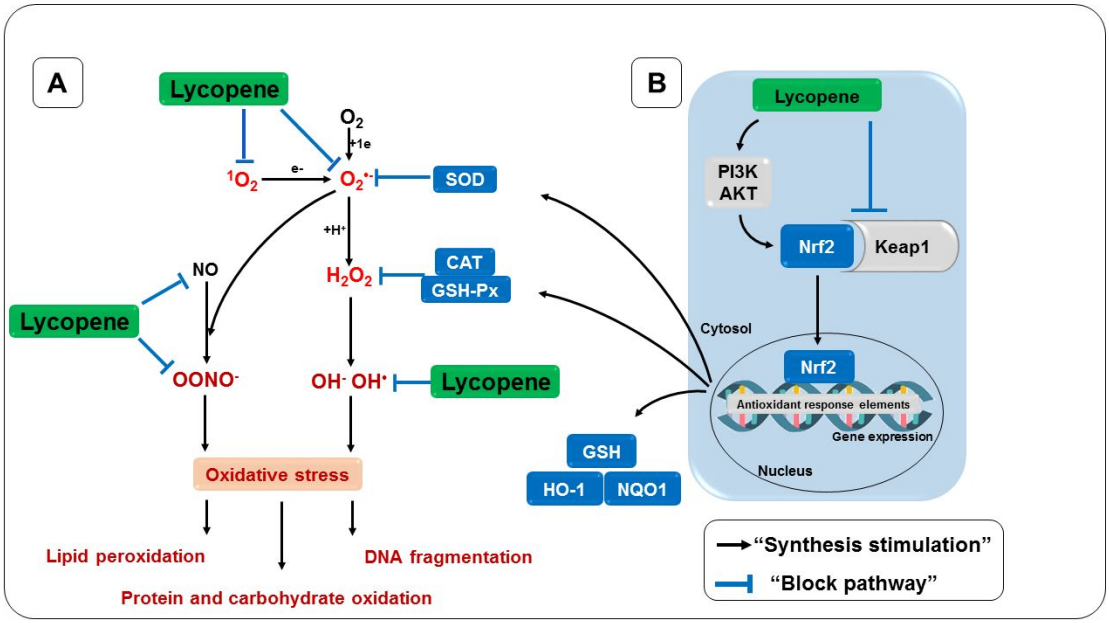

Figure 8. Antioxidant effect of lycopene. (**A**) Direct antioxidant activity. (**B**) Indirect antioxidant activity. Keap1, Kelch-like inhibitory protein 1; Nrf2, erythroid nuclear factor 2; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; PI3K/AKT, phosphoinositide 3-kinase/AKT.

Furthermore, the antioxidant status of lycopene, similar to other carotenoids, has also been implicated in the pathogenesis of malaria in vitro and in vivo [33]. In a previous study, Agarwal et al. [147] showed the cytotoxic effects of lycopene against *P. falciparum* in vitro, suggesting an important role of lycopene in preventing malaria [268]. Although treatment regimens with various antimalarials are used in clinical practice, there are still no substances that can prevent the disease. Thus, it can be suggested that dietary lycopene may be useful in changing the clinical outcomes of malaria. This review provides evidence of the antioxidant and anti-inflammatory benefits of lycopene supplementation, therefore suggesting it be included when formulating new prevention strategies to fight malaria and several other diseases.

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Abbreviations

$^1O_2$	singlet oxygen
ATP	Adenosine triphosphate
UA	Uric acid
Aβ	β-amyloid
BAX	Protein x associated with BCL-2
BCL-2	B-cell lymphoma protein 2
CAT	Catalase
COX-2	Cyclooxygenase-2
AD	Alzheimer's disease
DNA	deoxyribonucleic acid
PD	Parkinson's disease
eNOS or NOS3	endothelial nitric oxide synthase
Fe	Iron
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FPIX	Ferroprotoporphyrin IX or heme complex
GM-CSF	Granulocyte and macrophage colony-stimulating factor
GSH	reduced glutathione
GSH-Px	Glutathione peroxidase
GST	Glutathione S-transferases
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon-gamma
IL	interleukin
iNOS or NOS2	inducible nitric oxide synthase
Keap1	Kelch-like inhibitory protein 1
LDL	Low-density lipoprotein
MAPK	mitogen-activated protein kinase
ECM	experimental cerebral malaria
M-CSF	macrophage colony-stimulating factor
MDA	malondialdehyde
MIP-1α	macrophage-1α inflammatory protein
MIP-1β	macrophage-1β inflammatory protein
NAC	*N*-acetylcysteine
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	nuclear factor kappa B
nNOS or NOS1	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2	Oxygen
$O_2^{\bullet-}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite radical
PI3K/Akt	phosphoinositide 3-kinase/Akt
RO^-	alkoxy radical
RONS	reactive oxygen and nitrogen species
ROO^-	peroxyl radical
ROOH	hydroperoxide
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TNF-α	tumor necrosis factor-alpha

XO	xanthine oxidase

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3 CAPÍTULO II: ARTIGO ORIGINAL

ARTIGO ORIGINAL PUBLICADO NA REVISTA ANAIS DA ACADEMIA BRASILEIRA DE CIÊNCIAS

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BIOMEDICAL SCIENCES

Lycopene supplementation promoted increased survival and decreased parasitemia in mice with severe malaria: comparison with N-cetylcysteine

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Abstract: Oxidative stress is involved in the pathogenesis of malaria, causing anemia, respiratory complications, and cerebral malaria. To mitigate oxidative stress, we investigated the effect of nutritional supplementation whit lycopene (LYC) on the evolution of parasitemia and survival rate in mice infected with *Plasmodium berghei* ANKA (Pb), comparing to the effects promoted by N-acetylcysteine (NAC). Therefore, 175 mice were randomly distributed into 4 groups; ***Sham*:** untreated and uninfected animals; **Pb**: animals infected with Pb; **LYC+Pb:** animals treated with LYC and infected with Pb; **NAC+Pb**: animals treated with NAC and infected with Pb. The animals were followed for 12 days after infection, and survival and parasitemia rates were evaluated. There was a 40.1% increase in parasitemia in the animals of the Pb group on the 12th day, and a survival rate of 45%. LYC supplementation slowed the development of parasitemia to 19% and promoted a significative increase in the survival rate of 80% on the 12th day after infection, compared to the Pb group, effects superior to those promoted by NAC, providing strong evidence of the beneficial effect of LYC on *in vivo* malaria and stressing the importance of antioxidant supplementation in the treatment of this disease. **Key words:** Antioxidants, oxidative stress, Lycopene, malaria, N-acetilcysteine.

INTRODUCTION

Malaria is a serious global public health problem with a significant number of cases in 2020, when some 241 million cases occurred and 627,000 people died as a result of this disease. Currently, malaria is endemic in 85 countries, mainly in tropical and subtropical areas, where it mainly affect poor communities, especially pregnant women and children, and causing devastating social and economic consequences (WHO 2021).

Plasmodium vivax is the most commonly malaria-causing *Plasmodium* species in the world and is implicated in relapses of the disease (Angrisano & Robinson 2022, Rougeron et al. 2022). *P. falciparum* is recognized as the main cause of severe forms of the disease, being the most lethal species (Howes et al. 2016, Pais et al. 2022).

Some factors have been implicated in the pathogenesis of malaria, but one of the key processes contributing to the severity of the disease is excessive production of reactive oxygen and nitrogen species (RONS) in the host organism (Moreira et al. 2021, Gomes et al. 2022), Indeed, RONS can impact antioxidant defenses, promoting important cellular damage, including the reduction of red blood cell deformability causing consequent hemolysis, metabolic acidosis, severe anemia, and cerebral malaria (Haldar et al. 2007, Srivastava et al. 2015, Kumar et al. 2018). Ultimately, it may lead to the death of the host (Quadros Gomes et al. 2015, Barbosa et al. 2021).

Studies have found that populations in malaria endemic areas are more susceptible to complications of the disease, especially those caused by *P. falciparum*, because they have low plasma concentrations of several micronutrients important for host-defense mechanisms, including vitamin A and zinc, in addition to antioxidants such as ascorbic acid (vitamin C), vitamin E (α-tocopherol) and carotenoids such as lycopene (LYC) and β-carotene (Adelekan et al. 1997, Nussenblatt et al. 2002).

Among carotenoids, LYC stands out a potent mobilized antioxidant, which has been shown to reduce oxidative stress and prevent excessive production of RONS, especially those involved in malaria (Miller et al. 1996, Anguelova & Warthesen 2000). LYC is an essential micronutrient for living organisms, and its primary source is photosynthetic organisms, including green plants, algae, and cyanobacteria, being found in greater quantity in tomatoes and derivatives (Cohn et al. 2004, Wang et al. 2020).

LYC has analogues, including *cis* and *trans* isomers and apo-lycopenols, such as apo-10'lycopenoic acid (Lian & Wang 2008, Rodriguez & Rodriguez-Amaya 2009, Reynaud et al. 2011). Both isomers are non-cyclic liposoluble hydrocarbons with saturated and unsaturated lateral chains, which offer greater reactivity with RONS (Novikov et al. 2022). These carotenoids have potent activities, including antioxidant (Sy et al. 2012, Catalano et al. 2013), anti-inflammatory (Feng et al. 2010, ElAshmawy et al. 2018), anticancer (Aust et al. 2003, Cheng et al. 2020), cardioprotector (Ferreira-Santos et al. 2018), hepatoprotector (Ni et al. 2020), nephroprotector (Karahan et al. 2005), neuroprotector (Yin et al. 2014, Paul et al. 2020), antidiabetic (Guo et al. 2015), anticataract (Mohanty et al. 2002), and cholesterol reduction (Renju et al. 2014), being more potent than β-carotene or α-tocopherol (Liu et al. 2008, Erdman et al. 2009).

Additionally, a significant antiparasitic effect of LYC has been reported in experimental infection with *P. falciparum in vitro* (Agarwal et al. 2014). Other studies suggest that treatment with antioxidants may improve antiparasitic immune response (Val et al. 2015, Dkhil et al. 2019). However, it is unclear whether LYC stimulating actions demonstrated in *in vitro* studies, such as RONS inhibition and apoptosis induction, may occur in *in vivo* malaria.

Thus, this is the first study to clarify whether LYC is an appropriate candidate to antimalarial adjuvant, capable of reducing oxidative stress in male Balb/c mice infected with *P. berghei* ANKA, a murine malaria strain, responsible for inducing in mice a syndrome similar to that caused by *P. falciparum* in humans, and that it is well characterized in regards of the involvement of oxidative mechanisms in its pathophysiology.

MATERIALS AND METHODS

We used 175 male mice of the species *Mus musculus* and Balb/c breed, adults, 7-10 weeks old, weighing between 25 and 40g, from the Vivarium of the Evandro Chagas Institute (Ananindeua, ParáBrazil). The animals were housed in the Experimental Vivarium of the Oxidative Stress Research Laboratory (LAPEO) of the Institute of Biological Sciences (ICB) of the Universidade Fededral do Pará (UFPA), at room temperature of 24±2°C, light/dark cycle of 12 hours (lights from 7:00h to 19:00h), and free access to food and water. Before any experimental procedure, the animals were acclimated to laboratory conditions for 15 days.

The project was approved by the Ethics Committee on the Use of Experimental Animals of UFPA (CEUA/UFPA; protocol 3235130919), and the animals were manipulated and cared for in accordance with the ethical standards of animal experimentation set forth by the Brazilian Society of Laboratory Animal Science.

Preparation and administration of lycopene and N-acetylcysteine

The LYC administration protocol was chosen based on a dose-response study on the effects of LYC supplementation on oxidative stress biomarkers (Devaraj et al. 2008), and the dose was calculated by allometric extrapolation (Nair & Jacob 2016). The animals received 3.11mg/kg b.w./day of LYC *via* gavage (Table I).

The N-acetylcysteine (NAC) administration protocol was chosen based on a randomized, doubleblind, placebo-controlled study of chronic obstructive pulmonary disease (Zheng et al. 2014) and the dose was calculated by allometric extrapolation (Nair & Jacob 2016). The animals received 62mg/kg b.w./day of NAC *via* gavage (Table I).

The antioxidant drug NAC has been proposed as adjunctive treatment in severe falciparum malaria both *in vitro* and *in vivo* studies (Watt et al. 2002, Treeprasertsuk et al. 2003, Arreesrisom et al. 2007, Quadros Gomes et al. 2015) and, therefore, was employed as standard in this study.

Treatment with both substances was started 24 hours before infection of the animals with *Plasmodium berghei*, being repeated every 24 hours, until the day before the euthanasia of the animals.

Table I. Method for the calculation of allometric extrapolation of doses to be administered to mice (Balb/c, body weight of 0.025 kg).

A: dose calculation by allometric extrapolation – Lycopene	**B: dose calculation by allometric extrapolation – N-acetylcysteine**
Basal Metabolic Rate of reference animal (BMR man): BMR man = k x $m^{0.75}$ = 70 x $70^{0.75}$ = 70 x 24.20 BMR man = 1.694 kcal **BMR of target animal (BMR mice):** BMR mice = k x $m^{0.75}$ = 70 x $0.025^{0.75}$ = 70 x 0.063 BMR mice = 4.4 kcal **Total Dose indicated in the literature (TD):** TD = DOSE man ÷ BMR man = 30 ÷ 1.694 TD = 0.0177 mg/kcal **TD of the target animal (TD mice):** TD mice = TD x BMR mice = 0.0177 x 4.4 TD mice = 0.077 mg of Lycopene Therefore, the total dose of lycopene indicated to one mouse of 0.025 kg is 0.077 mg (or 3.11 mg/kg), which	BMR man: BMR man = k x $m^{0.75}$ = 70 x $70^{0.75}$ = 70 x 24.20 BMR man = 1.694 kcal **BMR mice:** BMR mice = k x $m^{0.75}$ = 70 x $0.025^{0.75}$ = 70 x 0.063 BMR mice = 4.4 kcal **TD:** TD = DOSE man ÷ BMR man = 600 ÷ 1.694 TD = 0.354 mg/kcal **TD mice:** TD mice = TD x BMR mice = 0.354 x 4.4 TD mice = 1.55 mg of N-acetylcysteine Therefore, the total dose of N-acetylcysteine indicated to one mouse of 0.025 kg is 1.55 mg (or 62 mg/kg),

was administered every 24 h.	which was administered every 24 h.

A: Taking as reference the adult man (*Homo sapiens*; average weight of 70 kg; proposed lycopene dose = 30 mg) is taken as reference. B: Taking as reference the adult man (*Homo sapiens*; average weight of 70 kg; dose of N-acetylcysteine proposed = 600 mg). k = constant of large taxonomic groups (placental mammals = 70); m = body mass.

Malaria induction

The *Plasmodium berghei ANKA* (Pb) strain was originally supplied by the Evandro Chagas Institute (Ananindeua, Pará-Brazil). For the infection, $1x10^6$ red blood cells infected *by P. berghei* ANKA were injected intraperitoneally (i.p.) into mice, and their survival and parasitemia rates were monitored. The day of infection was defined as day 0.

Animals and experimental groups

175 mice were randomly distributed into 4 groups (Figure 1), including: ***Sham*** (n=28): mice that received just the vehicle (water; gavage) and non-parasitized red blood cells (i.p.); **Pb** (n=49): mice that received just the vehicle (water; gavage) and Pb-infected red blood cells (i.p.); **LYC+Pb** (n=49): mice treated with 3.11mg/kg b.w./day of LYC (gavage) and infected with Pb (i.p.); **NAC+Pb** (n=49): mice treated with 62mg/kg b.w./day of NAC (gavage) and infected with Pb (i.p.). Each group was subdivided into 4 subgroups, depending on the number of days of follow-up of the group, and the animals of these subgroups underwent euthanasia after 1, 4, 8, or 12 days after infection.

Due to the high mortality expected for the subgroups of animals with longer infection periods (groups Pb, LYC+Pb, and NAC+Pb), their subgroups 1 and 4 days were composed of 7 animals each. Subgroups 8 and 12 days consisted of 15 and 20 animals, respectively. Since they would not undergo infection, all subgroups of the ***Sham*** group consisted of 7 animals.

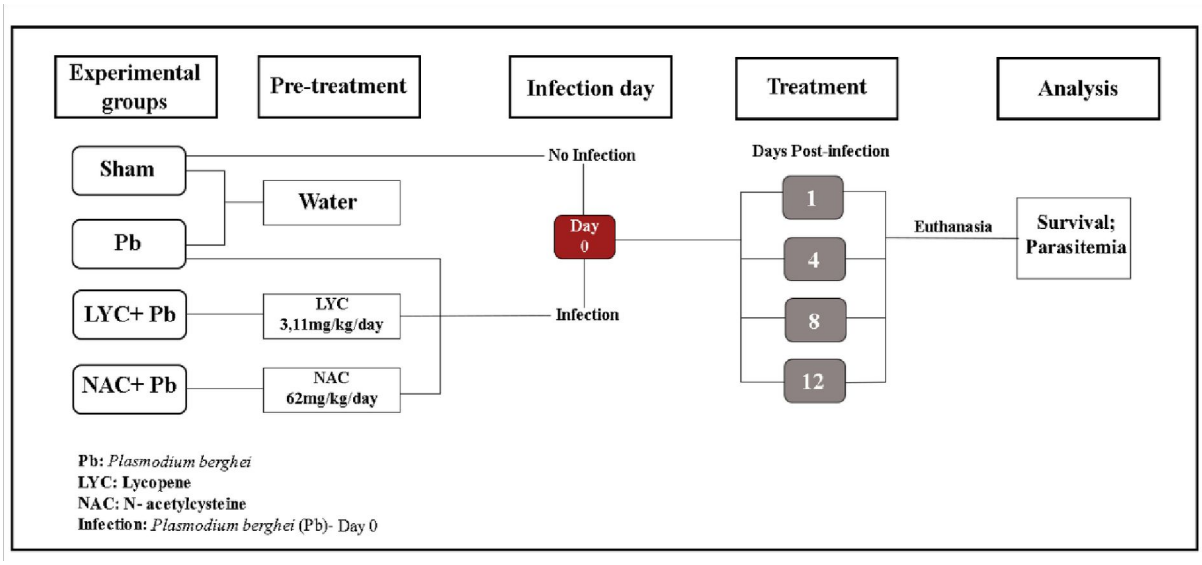

Figure 1. Schematic representation of the experimental design. LYC: Treatment with lycopene; NAC: Treatment with N-acetylcysteine.

Determination of survival rate

At the end of the period of 1, 4, 8 and 12 days, the survival rate was calculated by equation 1:

$$\text{Survival rate (\%)} = \frac{number\ of\ infected\ animals\ alive\ at\ the\ end\ of\ the\ study}{total\ number\ of\ infected\ animals\ alive\ at\ the\ begining\ of\ the\ study} \times 100 \quad (1)$$

Determination of parasitemia

At the end of the period of 1, 4, 8, or 12 days, 30μL of blood was collected by puncture of the caudal vein to produce blood smears, which were fixed with methanol (Dynamics, Cat # 1230) and stained with Giemsa (10%; Merck, Cat #1092041022). Parasitemia was determined by cell counting using the optical microscope (1000X), allowing to evidence the presence of the parasite within red blood cells. After counting, the percentage of parasitemia was calculated using equation 2:

$$\text{Parasitemia (\%)} = \frac{number\ of\ infected\ erythrocytes}{total\ number\ of\ erythrocytes} \times 100 \quad (2)$$

Statistical analysis

Data were expressed as mean ± standard deviation. All data were compared and analyzed using the one-way Variance Analysis test (ANOVA). Significant differences were compared between the groups, through Tukey's *post-hoc* test. In all tests, a significance level of 5% was considered ($p \leq 0.05$).

RESULTS

Effect of lycopene on survival rate

The survival rate of Pb group animals decreased from 100% on the 4th day to 46.7% on the 8th day after infection, and on the 12th day after infection it further decreased to 45%. On the other hand, animals treated with NAC presented a survival rate of 93.3% and 70% on days 8

and 12 post-infection, respectively, higher than the animals of the Pb group on the same days (p<0.0001). Additionally, animals treated with LYC exhibited a survival rate of 80% on both days 8 and 12 post-infection. In addition, LYC increased the survival of the animals significantly (p<0.0001) in relation to Pb group on days 8 and 12 post-infection and NAC+Pb group, on day 12 post-infection (Figure 2).

Effect of lycopene on the progression of parasitemia

Figure 3 shows the evolution of parasitemia in the Pb, LYC+Pb, and NAC+Pb groups. Parasitemia progressively evolved in all groups, but the rate of progression was significantly lower in animals treated with LYC during the study period (p<0.0001). In addition, the animals treated with LYC showed a significant reduction in parasitemia (p<0.0001), in relation to the Pb group on days 4, 8, and 12 postinfection and NAC+Pb group on day 12 post-infection (Figure 3).

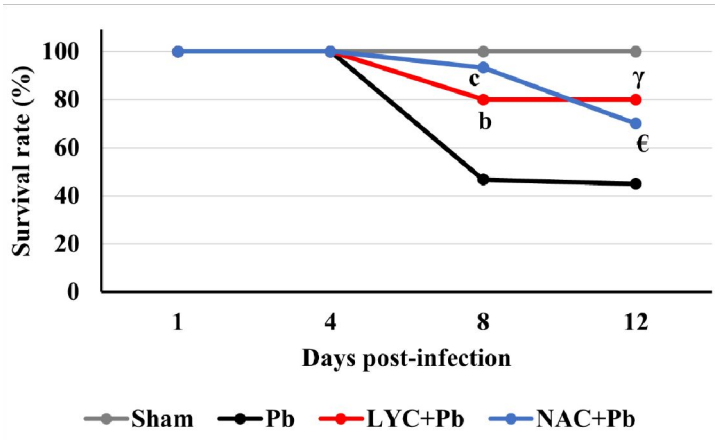

Figure 2. Survival rate of Balb/c mice infected with *Plasmodium berghei* ANKA treated with lycopene (LYC) or N-acetylcysteine (NAC). The ANOVA test, followed by Tukey's *post-hoc* test, was used to compare the Shan, Pb, LYC+Pb and NAC+Pb groups. ^{b}p<0.0001 *versus* Pb group and NAC+Pb; ^{c}p<0.0001 *versus* Pb group; $^{\gamma}p$<0.0001 *versus* Pb group and NAC+Pb; $^{€}p$<0.0001 *versus* Pb. Sham group: untreated and uninfected animals; Pb: animals injected (i.p.) with 10^6 red blood cells infected with Pb; LYC+Pb: animals treated with 3.11 mg/kg b.w./day of lycopene and infected with Pb; NAC: animals treated with 62 mg/kg b.w./day of NAC and infected with Pb.

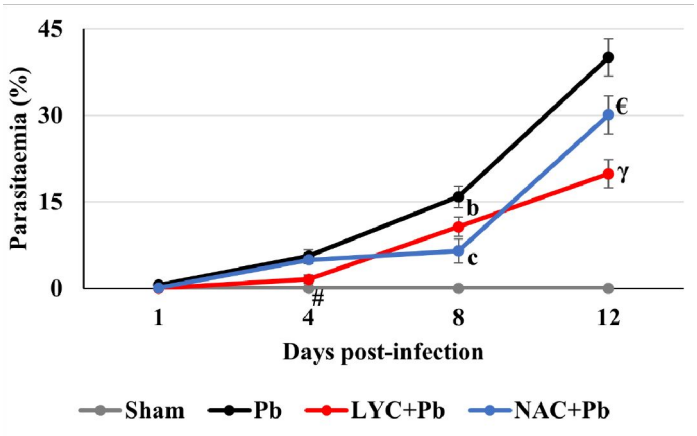

Figure 3. Temporal evolution of parasitemia of Balb/c mice infected with *Plasmodium berghei* ANKA (Pb) and treated with lycopene (LYC) or N-acetylcysteine (NAC). The ANOVA test, followed by Tukey's *post-hoc* test, was used to compare the Pb, LYC, and NAC groups. $^{\#}p$=0.0090 *versus* Pb group; ^{b}p<0.0001 *versus* Pb group; ^{c}p<0.0001 *versus* Pb and LYC+Pb group; $^{\gamma}p$<0.0001 *versus* Pb and NAC+Pb group; $^{€}p$<0.0001 *versus* Pb. Sham group: untreated and uninfected animals; Pb: animals injected (i.p.) with 10^6 red blood cells infected with Pb; LYC+Pb: animals treated with 3.11 mg/kg b.w./day of LYC and infected with Pb; NAC: animals treated with 62 mg/ kg b.w./day of NAC and infected with Pb.

DISCUSSION

Many of LYC's reported health benefits are attributed to its potent antioxidant activity, which includes effects such as cardioprotection, hepatoprotection, antidiabetc, anti-atherogenic, neuroprotective and anticancer (Duzen et al. 2019, Yin et al. 2019, Fu et al. 2020, Xue et al. 2021, Alhoshani et al. 2022, Mannino et al. 2022).

The antioxidant activity of LYC was also demonstrated in the pathogenesis of malaria in children (Das et al. 1996). Additionally, studies conducted by Agarwal et al. (2014), evidenced the *in vitro* cytotoxic effect of LYC against *P. falciparum.*

In the present study we used Balb/c mice as the vertebrate host for Pb to evaluate the effect of LYC supplementation on the evolution of parasitemia and survival in these animals.

The concentration used to evaluate the effect of LYC supplementation was chosen based on a dose-response study, which demonstrated the beneficial effects of LYC on oxidative stress biomarkers after daily intake of 6.5mg, 15mg, or 30mg of LYC (Devaraj et al. 2008). Since the daily intake of 30mg of LYC presented maximum antioxidant effect against oxidative stress, this concentration was used as a parameter for the calculation of allometric extrapolation, leading to the establishment of the LYC dose of 3.11mg/kg body weight, which was given daily until the day before euthanasia of the animals.

It was demonstrated a progressive increase in parasitemia in the Pb group during the period of 12 days after infection. In addition, a high degree of parasitemia was observed on the 12th day, reaching percentages of 40.1%. Notwithstanding, it was observed that on days 8 and 12 post-infection, 53.3% and 55% of the animals in this group died, respectively.

Additionally, the parasite count in peripheral blood may have underestimated the actual picture of parasitemia, since parasite populations may have been trapped inside microvessels of the spleen, kidneys, liver, lungs, and brain (Zaid et al. 2020), leading to lower availability of infected cells within the blood stream.

Previous studies have shown that children and adults with malaria generally have a high prevalence of malnutrition and micronutrient deficiencies, including vitamin A, β-carotene, LYC and zinc (Thurnham & Singkamani 1991, Zeba et al. 2008), and this situation creates a complexity of interactions with serious consequences for the health of the host.

According to Nacer et al. (2012), in addition to pallor, biliverdine secretion in the urine, arched posture, and lethargy, hyperparasitemia also leads to brain complications and death.

Another important factor is the exaggerated production of RONS during the disease. Pathophysiological changes in malaria escalate during the erythrocytic cycle. At this stage, parasites invade erythrocytes, consume and hydrolyse intraerythrocyte hemoglobin, seeking the amino acids for its own development (Tekwani & Walker 2005).

After the breakdown of the protein, ferrous iron (Fe^{2+}) from the released ferroprotoporphyrin can be rapidly oxidized to ferric iron (Fe^{3+}), giving rise to ferriprotoporphyrin IX, which undergoes oxidation and reduction reactions, producing RONS, such as superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), nitric oxide (NO), peroxynitrite ($ONOO^-$), free radicals of highly reactivity (Müller 2004, Klonis et al. 2013).

Antioxidants can antagonize the deleterious effects of RONS and restore redox balance, but in malaria infection this defense is totally tampered due to the high metabolic rate of the parasite, which grows and multiplies rapidly, generating large amounts of RONS, leading to the consumption and decrease of the host's antioxidant defense system (Delhaye et al. 2016).

As a consequence of this intracellular process, there is a reduced erythrocyte deformability, which cause erythrocyte hemolysis, and additional release of RONS to extracellular medium, causing damage to other cellular structures, including membrane lipids, proteins, and DNA (Cadet et al. 2010, Rahal et al. 2014).

In the present study, animals treated with LYC showed a survival rate higher than the Pb and NAC+Pb groups on days 8 and 12 post-infection. We believe that this prophylactic activity of LYC is due to the elimination of RONS, which has been cited as a crucial factor in this stage of malaria development (Quadros Gomes et al. 2015, Al-Shaebi et al. 2018).

According to the present results, animals supplemented with LYC up to the 12^{th} day presented the development of parasitemia at a slower rate compared to that observed in the Pb group. Moreover, LYC displayed antiparasitic potential higher than those of groups Pb and NAC+Pb in the 12^{th} day post-infection.

Indeed, the delay in the induction and progression of parasitemia caused by treatment with LYC suggests its prophylactic and antiparasitic activity, which may be due to a cytotoxic effect of LYC against malaria parasites (Agarwal et al. 2014), as the reduction of parasitemia may be associated with increased plasma LYC concentration in these animals (Metzger et al.

2001). In fact, the lipophilic characteristic of LYC can also favor its interaction with the lipid bilayer of the cell membrane (Sy et al. 2012) facilitating its absorption in tissues such as brain, heart, liver, spleen, lung, and kidneys, preventing them from the damage caused by parasite and/or RONS (Guo et al. 2019). Therefore, antioxidants such as LYC can block the damage triggered by RONS by sharing electrons with RONS, subsequently neutralizing them (Jain et al. 2018).

Corroborating to the present results, previous studies have shown the preventive effect of LYC (10mg/kg, orally) on lipid peroxidation, oxidative damage to DNA, and on the histopathological changes in liver of animals submitted to treatment with ferric nitrilotriacetate (Matos et al. 2001). Ateşşahin et al. (2006), also stated that 10 days of treatment with LYC (4mg/kg/day) prevented cisplatin-induced lipid peroxidation in rat testicles. Moreover, data from our laboratory demonstrated that NAC supplementation to Pb-infected mice prevented the oxidative changes imposed by the infection, suggesting that NAC may display antioxidant properties or that it is involved in redox signaling processes (Varela & Percário 2022).

In face of these results, it is possible to suggest that LYC can act in both ways: protecting against infection-induced damage, creating an antioxidant defense line in the host organism, inducing improvement of clinical parameters observed in supplemented animals, and by a direct antiparasitic effect of LYC against the parasites themselves, as suggested by Agarwal et al. (2014). Therefore, we suggest an important role of LYC supplementation both in malaria prevention and treatment.

The data obtained in the present study provide strong evidence that lycopene is effective against *P. berghei* infection and suggest that lycopene may become an important, viable, and safe strategy for the development of a biotechnological product with effective action in the prevention and auxiliary treatment of malaria and other diseases, but more studies are needed to prove these potential benefits.

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4 CAPÍTULO III: ARTIGO ORIGINAL

ARTIGO ORIGINAL SUBMETIDO À REVISTA NUTRIENTS

Type of the Paper (Article)

Lycopene mitigates malaria-induced formation of reactive oxygen nitrogen species and oxidative damage in mice brain and lungs

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Abstract: (1) Background: The severity of malaria is associated with low bioavailability of antioxidants and high concentration of free radicals that induce oxidative damage in cerebral and pulmonary microcirculation. This can be prevented by the action of consumable antioxidants present in foods. Therefore, we investigated the protective role of lycopene (LYC) on the oxidative changes induced by *Plasmodium berghei* (Pb); (2) Methods: Mice were infected by intraperitoneal injection of 10^6 parasitized red blood cells and treated via gavage with LYC (3.11mg/kg bw/day) or NAC (62mg/kg bw/day). They were then evaluated for 1, 4, 8 or 12 days after infection. Levels of thiobarbituric acid reactive substances (TBARS), antioxidant capacity by inhibition of ABTS radicals (AC-ABTS) and DPPH (AC-DPPH), uric acid (UA), and nitric oxide (NO) were measured in brain and lung tissues; (3) Results: The infection caused oxidative stress confirmed by increased levels of TBARS, AC-ABTS, AC-DPPH, UA, and NO in the tissues leading to the death of the animals. LYC prevented the increase in TBARS, UA, and NO levels compared to Pb ($p<0.0001$) and NAC+Pb groups ($p<0.0001$), reaching values similar to those of Sham animals; (4) Conclusions: These results are striking evidence of the beneficial effect of lycopene supplementation on oxidative stress in experimental malaria in vivo and emphasize the importance of antioxidant supplementation in the treatment of the disease.

Keywords: Lycopene; N-Acetylcysteine; Malaria; Oxidative stress; Antioxidant.

1. Introduction

Free radicals are intermediate species with unpaired electrons. The production of these molecules in the human body is inevitable, since many of these molecules originate from the process of physicochemical oxidation of molecular oxygen (O_2) and/or molecular nitrogen (N_2), leading to the production of reactive oxygen and nitrogen species (RONS) [1]. RONS are highly oxidizing intermediates produced continuously in biological systems from biochemical reactions, including nitric oxide (NO) synthesis and mitochondrial electron transport chain reactions, as well as by metal-catalyzed oxidation and reduction, among others [2].

The main RONS generated in the human body are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), NO, nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) [3]. There are other biologically important free radicals such as lipid hydroperoxide (ROOH), lipid peroxyl radical ($ROO^{\bullet}$), and lipid alkoxyl radical ($RO^{\bullet}$).

The unpaired electrons give RONS high instability and reactivity. As a result, they have a short half-life of milliseconds or less, but enough time to trigger intermolecular interactions, as well as responses in nearby target cells, leading to oxide-reduction reactions, altering and/or damaging biomolecules, cells and tissues [4].

In response to the production of RONS, the human body induces the synthesis of mobilizable antioxidants that act by preventing, neutralizing or reducing the oxide-reduction reactions of RONS in the body [5,6]. Three enzymes are critical in this process, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). These mobilizable antioxidants, respectively, neutralize $O_2^{\bullet -}$ and break down H_2O_2 or ROOH into harmless molecules such as H_2O, alcohol, and O_2 [7].

However, when these antioxidants are not at adequate and sufficient levels to compensate for the harmful effects of RONS, oxidative stress is installed in the body. Oxidative stress is therefore an imbalance between RONS and antioxidants in favor of RONS [8]. Oxidative stress is involved in: 1- Beneficial effects, such as the body's immune defense system through toxic action on invading pathogens; 2- Harmful effects, leading to an interruption of redox signaling and control and/or molecular damage in chronic, degenerative, neurodegenerative, metabolic, and infectious diseases [4,7].

In this context, studies have discussed the role of RONS and antioxidant defense mechanisms in malaria [9] Malaria is a potentially serious disease, causing more than 200 million malaria episodes and about 500,000 deaths annually **(Figure 1),** mostly in impoverished communities [10] Among others, *Plasmodium falciparum* and *Plasmodium vivax* pose the greatest threat to human health.

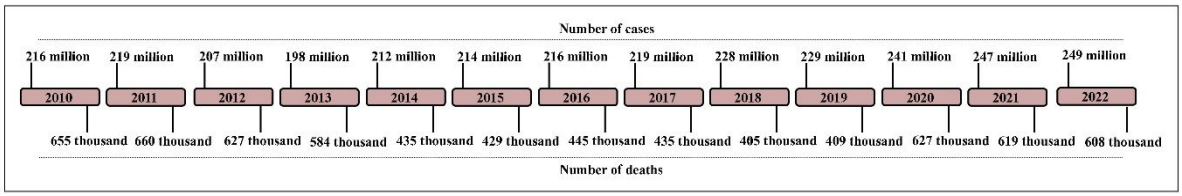

Figure 1. Timeline of the number of cases (above) and number of deaths (below) of malaria in the world.

Studies have associated the action of RONS with the various pathological manifestations in malaria, which can range from nonspecific symptoms, such as fever and mild anemia, to the complications of severe malaria, which include coma, prostration, respiratory difficulty, metabolic acidosis, renal failure, liver damage, and severe anemia [11–13].

According to SUZUKI et al. [14] the pathophysiological changes evident in malaria infection occur because mobilizable antioxidants are drastically reduced or consumed in oxidation-reduction reactions with RONS during infection.

Other factors evidenced in the infection are preponderant for the high production of RONS, including ischemia and reperfusion syndrome (IRS), parasite metabolism, as well as body's immune response against the parasite itself [5,6,12].

During infection, the parasites invade red blood cells and feed on hemoglobin, their main source of amino acids. As a consequence, red blood cells lose their function due to severe hemolysis. This eventually releases heme (Fe^{2+}) into the circulation. Heme can react with O_2 and generate $O_2^{\bullet-}$, subsequently increasing H_2O_2 levels through spontaneous dismutation, as well as increasing $HO^{\bullet}$ levels [15]. The increase in these RONS mediates the oxidation of lipids, proteins, and DNA, resulting in cellular and tissue damage, endothelial dysfunction, and loss of vascular homeostasis [16,17].

RONS can also activate toll-like receptor-4 (TLR4), triggering immune response. According to Ty et al. [18], RONS are a key factor into triggering the immune response during infection. According to the authors, immune cells are activated, including neutrophils and macrophages, which engulf and kill the parasites through the respiratory burst. One of the main responsible for this action is NO.

NO is a gaseous free radical, with an extremely short half-life in tissues, ranging from 3 to 10 seconds, synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). In macrophages NO synthesis is usually low, however, in response to infection, inducible nitric oxide synthase (iNOS) is activated, which can result in large amounts of NO produced over hours or days [19].

In addition to macrophages, endothelial, and neuronal cells synthesize NO, but at a constant rate, regardless of physiological demand. In endothelial cells, NO is synthesized by endothelial NOS (eNOS), acting as an important vasodilator, being able to increase the vascular permeability of smooth muscles [20]. Meanwhile, in neuronal cells, neuronal nitric oxide synthase (nNOS), releases NO which can act as a neurotransmitter [21].

However, in malaria the high production of NO favors its reaction with $O_2^{\bullet-}$, even at low concentrations, to produce highly oxidizing $ONOO^-$, which can act locally on the underlying vascular smooth muscle or on monocytes or platelets, promoting apoptosis in the microvascular endothelium and cytoadherence [22]. Localized action occurs because the parasites express cytoadhesion factors on the surface of infected red blood cells, allowing the binding and sequestration of red blood cells in the postcapillary venules, promoting vascular occlusion and tissue hypoxia [23]. Another important point to be considered is the fact that NO can interact reversibly with hemoglobin, which acts as an O_2 sensor and regulator of vascular tone in response to local O_2 partial pressure, binding to heme, generating nitrate and methemoglobin, which is unable to bind and carry O_2 for tissue perfusion, further potentiating ischemia, hypoxia, and anemia [24].

Thus, phagocytosis and the consequent action of RONS including $O_2^{\bullet-}$ and NO, as well as other toxic products, can aggravate the condition due to the rupture of parasitized erythrocytes, during which uninfected normal erythrocytes can also be destroyed, stimulating cytoadherence and consequently potentially blocking blood flow and leading to metabolic acidosis, renal failure, liver damage, and severe anemia [5,11,12,19].

Cellular damage produced by oxidative stress can be prevented or reduced by the action of consumable antioxidants present in food. Vitamins, including vitamins E and C, phenolic substances, such as flavonoids and resveratrol, carotenoids, including β-carotene and lycopene (LYC), and drugs such as N-acetylcysteine (NAC), among others, belong to this category [5,6].

Recent interest in carotenoids has focused on the role of LYC in human health. LYC is a natural, monounsaturated, lipophilic carotenoid synthesized by vegetables and fruits, such as tomatoes, watermelons, and carrots [25]. Tomatoes and tomato products are the main source of LYC and are considered an important source of carotenoids in the human diet [26]. It is widely used as a supplement in functional foods, nutraceuticals, and pharmaceuticals, as well as an additive in cosmetics [27,28].

LYC has potent activities, including antioxidant [29,30], anti-inflammatory [31], anticancer [32], neuroprotective [33], cardioprotector [34] and cholesterol reducers, and its concentration in multiple tissues is extremely important for these effects [35].

Consumable carotenoids react with a wide range of radicals, such as $O_2^{\bullet-}$, $OH^{\bullet}$, NO, and $ONOO^-$ by electron transfer, producing radicals with lower oxidizing capacity, or they can lead to hydrogen atom transfer generating neutral radicals [36,37]. This antioxidant action of LYC is favored by the conjugate double bond contained in its structure, which confers high antioxidant capacity, which is about 100 times greater than that of vitamin E [26].

Several studies have indicated an association between LYC use and a decrease in oxidative changes [38–40]. According to Pan et al. [41] the strong antioxidant activity was able to prevent cyclosporine-induced intestinal injury in mice by increasing antioxidant activity and decreasing the concentration of malondialdehyde (MDA), NO, interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α). These data reinforce the importance of LYC in the prevention of oxidative stress in malaria [6].

Thus, this study sought to clarify the possible antioxidant mechanisms of LYC, to determine whether LYC is an appropriate antimalarial candidate capable of reducing oxidative stress in male Balb/c mice infected with *P. berghei*, a strain of murine malaria responsible for inducing in the experimental animal model a syndrome similar to that caused by *P. falciparum* in humans and that is well characterized regarding the involvement of oxidative mechanisms in its pathophysiology. In this sense, this study provides evidence that, in mice with *P. berghei*-induced malaria, treatment with the carotenoid lycopene was able to improve several biochemical biomarkers related to oxidative stress. The levels of uric acid (UA), thiobarbituric acid reactive substances (TBARS), and NO in infected mice treated with LYC decreased below the levels found in the untreated infected animals, or those treated with NAC, reaching values similar to those of the non-infected animals. To the best of our knowledge, this study is the first to demonstrate important findings on the benefits of a therapy based on the natural compound lycopene for the management of biochemical changes in experimental malaria *in vivo*.

2. Results

Our data showed that *P. berghei* infection induced a significant increase in the level of TBARS compared to the Sham group in brain and lung tissues on days 4, 8, and 12 post-infection ($p < 0.0001$; **Figure 2**). Both the LYC+Pb and NAC+Pb groups have significantly lower TBARS levels than the Pb group in both tissues ($p < 0.0001$). In addition, the LYC treatment provided an apparent normalization in the level of TBARS in the treated animals, which was similar to the values presented by the animals of the Sham group in both brain and lung tissues.

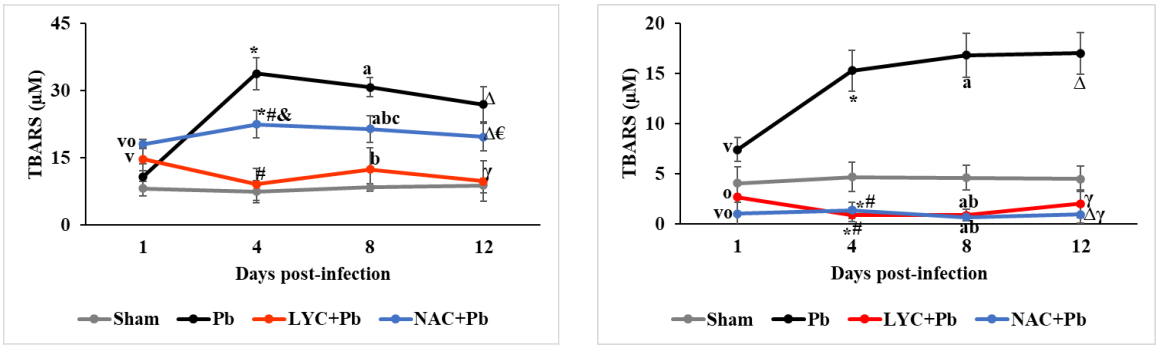

Figure 2. Concentration of thiobarbituric acid reactive substances (TBARS) in the brain and lungs of mice infected with *P. berghei*, treated or not with lycopene or N-acetylcysteine. Data are expressed as means ± standard deviation. **1 day**: $^{v}p \leq 0.02$ *versus* Sham; $^{o}p \leq 0.005$ *versus* Pb. **4 days**: $^{*}p \leq 0.001$ *versus* Sham; $^{\#}p < 0.0001$ *versus* Pb; $^{\&}p < 0.0001$ *versus* LYC+Pb. **8 days**: $^{a}p \leq 0.0002$ *versus* Sham; $^{b}p < 0.0001$ *versus* Pb; $^{c}p < 0.0001$ *versus* LYC+Pb. **12 days**: $^{\Delta}p < 0.0001$ *versus* Sham; $^{\gamma}p < 0.0001$ *versus* Pb; $^{\epsilon}p < 0.0001$ *versus* LYC+Pb.

In **Figure 3** we observed that *P. berghei* infection led to a significant increase in AC-ABTS and AC-DPPH in animals of the Pb group compared to the Sham group in brain and lung tissues ($p < 0.0001$). In the LYC supplemental group, a significant decrease in the level of AC-ABTS and AC-DPPH was observed in relation to the Pb group in both tissues ($p < 0.0001$), presenting behavior close to that exhibited by the Sham group in both tissues. However, after treatment with NAC, a significant increase in the level of AC-ABTS was observed in the brain tissue compared to the Sham and LYC groups ($p < 0.0001$).

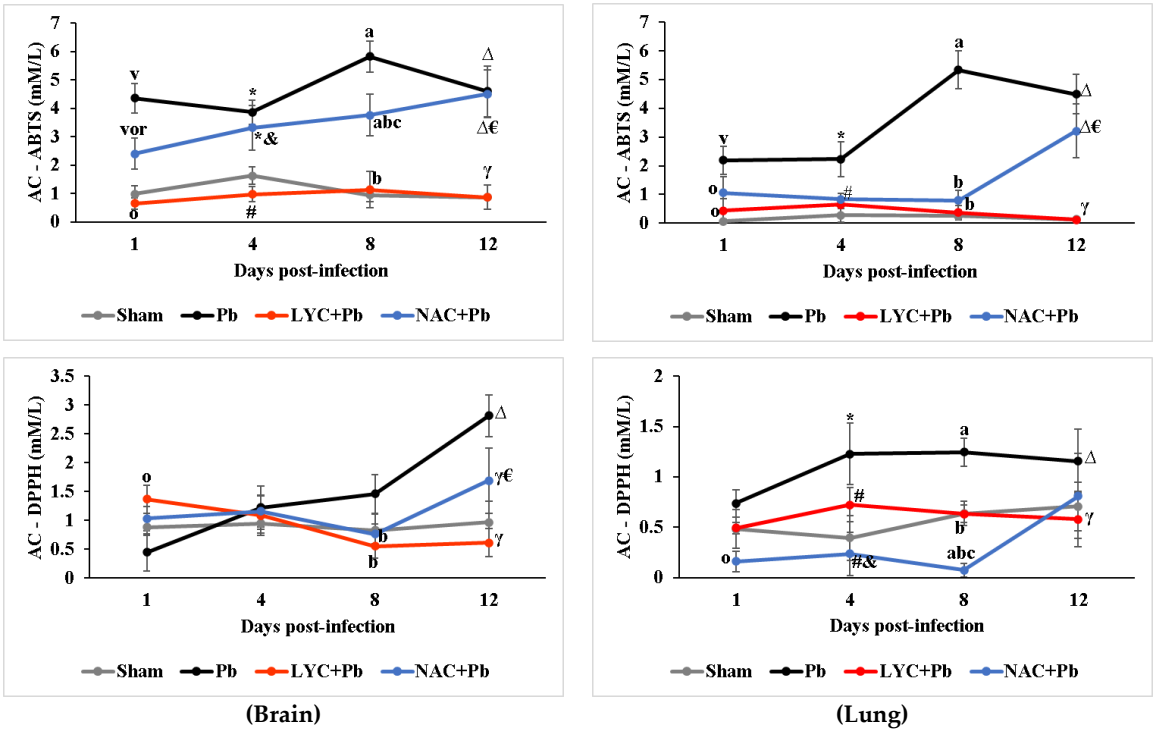

Figure 3. Antioxidant capacity (AC) by inhibition of ABTS and DPPH radicals in the brain and lungs of mice infected with *P. berghei,* treated or not with lycopene or N-acetylcysteine. Data are expressed as means ± standard deviation. **1 day**: $^{v}p < 0.0001$ *versus* Sham; $^{o}p \leq 0.005$ *versus* Pb; $^{r}p < 0.0001$ *versus* LYC+Pb. **4 days**: $^{*}p \leq 0.0001$ *versus* Sham; $^{\#}p \leq 0.01$ *versus* Pb; $^{\&}p \leq 0.02$ *versus* LYC+Pb. **8 days**: $^{a}p < 0.0001$ *versus* Sham; $^{b}p < 0.0001$ *versus* Pb; $^{c}p < 0.0001$ *versus* LYC+Pb. **12 days**: $^{\Delta}p \leq 0.005$ *versus* Sham; $^{\gamma}p \leq 0.001$ *versus* Pb; $^{\epsilon}p < 0.0001$ *versus* LYC+Pb.

Figure 4 shows that *P. berghei* induced a significant increase in NO levels in the Pb group compared to the Sham group in both tissues throughout the study period ($p < 0.0001$). After treatment with LYC, a significant reduction in the level of NO in brain tissue was demonstrated compared to the Pb and NAC+Pb groups ($p < 0.0001$). In addition, LYC led to normalization of the NO level in brain tissue. On the other hand, in the lung tissue, LYC induced a significant increase in the level of NO compared to the other groups ($p < 0.0001$).

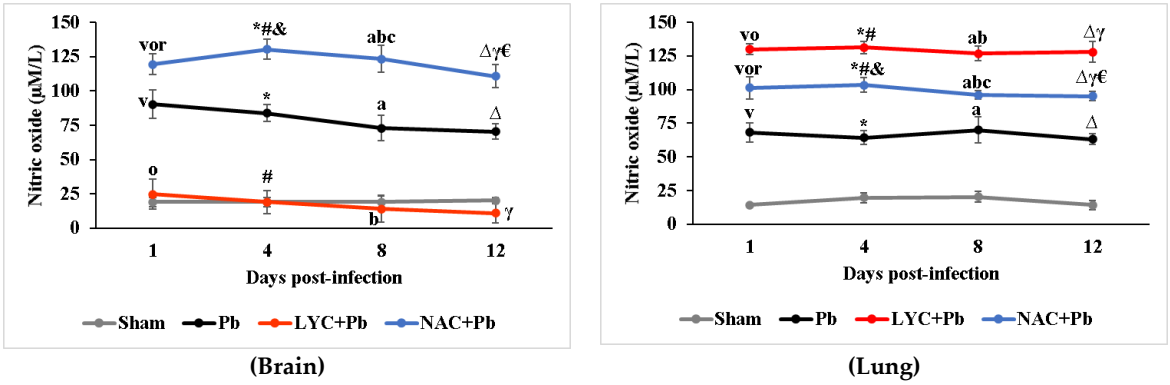

Figure 4. Nitric oxide concentration in the brain and lungs of mice infected with *P. berghei,* whether or not treated with lycopene or N-acetylcysteine. Data are expressed as means ± standard deviation. **1 day**: $^{v}p < 0.0001$ *versus* Sham; $^{o}p < 0.0001$ *versus* Pb; $^{r}p < 0.0001$ *versus* LYC+Pb. **4 days**: $^{*}p < 0.0001$ *versus* Sham; $^{\#}p < 0.0001$ *versus* Pb; $^{\&}p < 0.0001$ *versus* LYC+Pb. **8 days**: $^{a}p < 0.0001$ *versus* Sham; $^{b}p < 0.0001$ *versus* Pb; $^{c}p < 0.0001$ *versus* LYC+Pb. **12 days**: $^{\Delta}p < 0.0001$ *versus* Sham; $^{\gamma}p < 0.0001$ *versus* Pb; $^{\epsilon}p < 0.0001$ *versus* LYC+Pb.

Figure 5 shows that *P. berghei* induced a significant increase in UA levels in the Pb group compared to the Sham group in both tissues ($p < 0.0001$). On the other hand, LYC supplementation induced a significant reduction ($p < 0.0001$) of UA levels in relation to the Pb group in both tissues, and to the NAC+Pb group in the brain tissue. Treatment with lycopene provided a normalization of UA levels in both tissues, as well as NAC in lung tissue up to the eighth day of treatment.

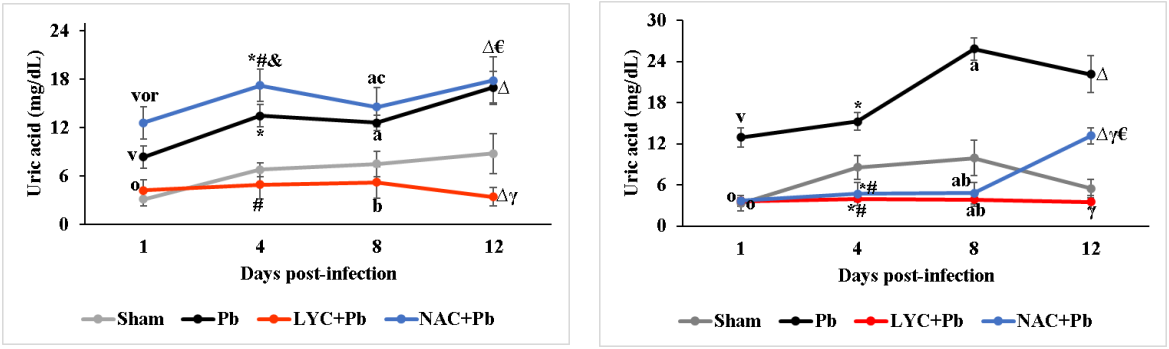

Figure 5. Uric acid concentration in brain and lungs of animals infected with *P. berghei* treated or not with lycopene or N-acetylcysteine. Data are expressed as means ± standard deviation. **1 day:** $^{v}p \leq 0{,}0001$ *versus* Sham; $^{o}p \leq 0.005$ *versus* Pb; $^{r}p < 0.0001$ *versus* LYC+Pb. **4 days**: $^{*}p \leq 0.0007$ *versus* Sham; $^{\#}p \leq 0.02$ *versus* Pb; $^{\&}p < 0.0001$ *versus* LYC+Pb. **8 days**: $^{a}p < 0.0001$ *versus* Sham; $^{b}p < 0.0001$ *versus* Pb; $^{c}p < 0.0001$ *versus* LYC+Pb. **12 days**: $^{\Delta}p < 0.0001$ *versus* Sham; $^{\gamma}p < 0.0001$ *versus* Pb; $^{\epsilon}p < 0.0001$ *versus* LYC+Pb.

3. Discussion

Malaria causes more than 500,000 deaths every year, most of them due to brain and/or lung complications induced by *P. falciparum* infection. In order to understand the pathophysiology involved in malaria, experimental infection in an mice model with *P. berghei* infection has been widely used for many studies due to its ability to induce oxidative biochemical changes, including lipid peroxidation and/or decreased antioxidant capacity in vital organs such as lung and brain, with several similarities to the human disease [42,43].

In order to investigate whether substances with antioxidant potential could reduce oxidative biochemical changes during malarial infection, Balb/c mice were induced to malaria by *P. berghei* inoculation, and treated with LYC or NAC.

Because NAC is a drug widely indicated for the treatment and/or prevention of several respiratory diseases and is involved in the reduction of oxidative stress in diseases including human immunodeficiency virus (HIV) infection, influenza A/H1N1 virus, and malaria, it was used in our study as a standard substance [44–47]. On the other hand, LYC, as an orally administered antioxidant agent, was tested for its antioxidant activity in mice infected with *P. berghei* and its action was compared to the effects of NAC. LYC is a carotenoid present in foods such as tomatoes, watermelon, guava, among other foods, which an antioxidant potential approximately twice the activity of β-carotene [48]. Its antioxidant property contributes to optimizing health status and reducing the risk of oxidative stress-based diseases such as cancer and malaria [38,49].

In this study, our data evidenced that LYC and NAC improved several biochemical biomarkers related to oxidative stress. However, LYC presented a more intense effect than NAC. LYC induced a reduction in the levels of UA, TBARS, and NO in mice with malaria, leading to concentrations significantly lower than those found in untreated infected animals, or in those treated with NAC, reaching values similar to those of uninfected animals. In this study, we confirmed important data on the in *vivo* benefits of a therapy based on this natural compound for the management of oxidative biochemical changes in experimental malaria.

Although there is still no consensus on the precise mechanism responsible for malaria severity, studies indicate that both the host and the parasite are under oxidative stress, due to the increase in

circulating RONS, produced during the inflammatory response by cells such as activated monocytes and neutrophils, and mainly due to the degradation of hemoglobin by the parasite [6,12,50–52].

During infection, the parasite is able to develop rapidly, because by consuming hemoglobin from the host erythrocyte, degrades it into amino acids, which are used for its nutrition. Fe^{2+}, also released in the cytosol, undergoes Fenton and Haber-Weiss reactions favored by the presence of O_2, generating RONS including $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$. These RONS can then induce lipid peroxidation, impair microvessel endothelial cells, and cause other important tissue damages [16,17].

In our study, we demonstrated that the experimental malaria induced by *P. berghei* infection caused a detrimental effect on the oxidative biochemical parameters studied in mice, resulting in an increase in the levels of TBARS, AC-DPPH, AC-ABTS, NO, and UA, which was expected, because, according to BAPTISTA et al. [53] about 60% of mice infected with *P. berghei* succumb to malaria between days 6 and 8 post-infection with moderate parasitemia between 6 and 11%.

To verify possible oxidative biochemical changes caused by RONS, we measured TBARS in lung and brain tissues of infected BALB/c mice. TBARS are mainly reactive α and β unsaturated aldehydes, such as MDA, 4-hydroxy-2-nonenal, 2-propenal (acrolein) and isoprostanes, and are products of the decomposition of polyunsaturated fatty acid hydroperoxides and laboratory markers of lipid peroxidation, which occurs by the action of RONS on lipids and, therefore, demonstrates the occurrence of oxidative stress [54].

Our data showed that the Pb group had a significantly higher level of TBARS than the Sham group in brain and lung tissues ($p < 0.0001$; **Figure 2**), reinforcing the action of RONS as an important mediator of oxidative biochemical changes caused by experimental malaria infection.

Previously, Reis et al. [46] reported increased production of MDA and conjugated dienes in the brains of C57BL/6 mice infected with Pb, indicating the occurrence of oxidative stress. Fernandes et al. [55] correlated increased plasma levels of reactive aldehydes in Pb-infected mice with malaria severity. Scaccabarozzi et al. [56] showed that infection with Pb in C57BL/6J mice induced at the same time biochemical changes in liver and lung tissue, as well as leading to acute respiratory distress syndrome, due to an exacerbated excessive oxidative response, demonstrated by the alteration of mobilizable antioxidant enzymes and the increase in MDA levels. Recently, Chuljerm et al. [57] reported elevated levels of TBARS in the plasma and livers of Pb-infected mice.

Our findings indicate that in *P. berghei* infected mice an excessive oxidative response seems to predominate, demonstrated by the increase in TBARS levels, which is an index of loss of structure and integrity of the cell membrane of brain and lung tissues, and these changes were related to high parasitemia.

However, treatments with LYC and NAC significantly reversed ($p < 0.0001$) the increase in TBARS concentration when compared to the Pb group (**Figure 2**). In addition, LYC treatment brought the TBARS level to similar values exhibited by the animals in the Sham group, which suggests that LYC may be successful in eliminating RONS, inhibiting lipid peroxidation, and protecting membrane lipids from oxidative damage in brain and lung of mice during malarial infection.

These data are in line with previous findings in the literature in which it has been reported that both LYC and NAC are able to reduce endogenous oxidant levels and protect cells against a wide range of pro-oxidative insults [58,59] by reacting directly with RONS [60]. According to Zhang [61], LYC has the ability to inhibit oxidative stress and TBARS, limiting the production of RONS. LYC activity has also been implicated in the elimination of *P. falciparum in vitro* [49]. However, the direct effect of LYC on the proliferation of the parasite *in vivo* is unclear. On the other hand, the antioxidant activity of NAC may have involved the direct action of its free thiol (SH) group, which can act as an electron donor, favoring the interaction of NAC with RONS.

By promoting cellular aggression processes, parasites can readily alter the concentration of mobilizable antioxidants in the body, triggering endogenous defense mechanisms against oxidative aggression. The action of these molecules helps protect biological membranes, which are susceptible to lipid peroxidation and oxidative damage in general. Mobilizable antioxidants include enzymes such as SOD, which dismutates $O_2^{\bullet-}$ into H_2O_2, CAT, and GSH-Px, which break down H_2O_2 and ROOH into poorly reactive molecules, such as H_2O, alcohol, and O_2, as well as other non-enzymatic molecules such as reduced glutathione (GSH) [5,6].

In this sense, we found a significant increase in AC-ABTS and AC-DPPH in animals in the Pb group compared to the Sham group in brain and lung tissues ($p < 0.0001$; **Figure 3**). The significant increase in AC-ABTS and AC-DPPH in the present study may reflect an adaptive response of the animal organism to an increased demand for mobilizable antioxidants, possibly to combat the RONS generated during *P. berghei* infection.

Reinforcing these results, we found a significant positive correlation between the concentrations of TBARS versus AC-ABTS ($r = 0.4$; $p < 0.0001$) and TBARS versus AC-DPPH ($r = 0.5$; $p < 0.0001$) in the Pb group. The increase in antioxidant capacity may have occurred due to the action of Pb on mobilizable enzymes or due to the high level of RONS, such as H_2O_2, $O_2^{\bullet -}$, $OH^{\bullet}$, $ONOO^-$, which form within cells in response to Pb infection.

On the other hand, LYC supplementation led to a significant decrease in the level of AC-ABTS and AC-DPPH in relation to the Pb group in both tissues (**Figure 3**). Additionally, the level of AC-ABTS and AC-DPPH was restored by treatment in the LYC+Pb group in brain and lung tissues. Similarly, to what occurred for infected and untreated animals, after treatment with NAC, a significant increase in the level of AC-ABTS in brain tissue was observed compared to the Sham and LYC+Pb groups. In the lung tissue, NAC restored the level of AC-ABTS to the levels presented by the animals of the Sham group.

In view of these findings, it can be suggested that, in mice infected with Pb, the administration of LYC may decrease the activity of mobilizable antioxidants, as a result of cellular redox regulation after the ingestion of exogenous antioxidants. On the other hand, NAC, being an analogue and precursor of GSH, may have induced the replacement of intracellular levels of GSH, also known as the main antioxidant produced by the body and which protects cells from oxidative stress.

In this context, some studies report that, in diseases in which oxidative stress is a pathogenic mediator, such as Alzheimer's, Parkinson's, Chagas, dengue, and malaria, the actions of mobilizable antioxidants are not sufficient to maintain an adequate cellular redox balance [62–65].

To counteract the deleterious effects of RONS, supplementation with consumable antioxidants, including vitamins E and C, phenolic substances such as flavonoids and resveratrol, carotenoids including β-carotene and LYC, and drugs such as NAC, are essential for maintaining optimal cellular function. LYC is a potent antioxidant that also has anti-inflammatory, anti-atherogenic, antidiabetic, neuroprotective, and anticancer effects [31,32,34,39,66–68].

Antioxidants can act by increasing the concentration of other antioxidants in the body, donating electrons to the RONS and neutralizing them, binding directly to RONS and inactivating them, or also by preventing the cascade of formation of highly reactive RONS, such as $OH^{\bullet}$ and $ONOO^-$ [69].

These RONS are reported to be a key factor in triggering the devastating inflammatory response that has been associated with disease progression and subsequent fatal outcome [70]. RONS also appear to act as second messengers in a signaling cascade and can activate mononuclear cells, as well as macrophages and dendritic cells that stimulate the release of High Mobility Group Box-1 (HMGB-1) into the intra- and extracellular space [18].

According to Techarang et al. [71] increased expression of HMGB-1 in endothelial cells may stimulate several receptors, including RAGE, TLR-4, and TLR-2, which activate nuclear factor kappa B (NF-κB) leading to the production of TNF-α, interferon-gamma (IFN-γ), IL-1β, and IL-6, involved in the pathogenesis of malaria.

These inflammatory mediators can also stimulate the activation of iNOS in macrophages. In macrophages, iNOS activity is normally low, but the expression of this enzyme is greatly stimulated by the release of inflammatory mediators such as IF N-γ, in response to infection, leading to an increased NO concentration [72].

NO is recognized as a mediator in a wide range of biological systems, plays an important role in the maintenance and regulation of bronchomotor tone by non-adrenergic and non-cholinergic neural mechanisms (NANC), and acts as an important vasodilator in the vascular endothelium [73]. In addition, it is essential for the phagocytic function of macrophages, generating oxidizing molecules such as $ONOO^-$ with the ability to destroy invading pathogens.

Despite participating in the body's defense when produced by macrophages, in our study the increase in NO concentration was not correlated with a decrease in parasitemia. According to our

results, there was a significant increase ($p < 0.0001$) in NO levels in the Pb group compared to the Sham group in both tissues throughout the study period (**Figure 4).** Therefore, it is possible that the exaggerated production of NO may have generated harmful effects to the animal organism, including generalized vasodilation resulting in hypotension and oxidative changes resulting from $ONOO^-$, highly reactive, capable of inducing peroxidation of membrane lipids.

However, after treatment with LYC, a significant reduction in the level of NO in brain tissue was observed in comparison with Pb and NAC+Pb groups (**Figure 4**). In addition, LYC led to normalization of the NO level in brain tissue. In lung tissue, LYC led to an increase in NO levels in the LYC+Pb group compared to the other groups.

LYC is an extremely hydrophobic molecule with a high capacity to permeate the double layer of phospholipids of the plasma membrane, and can accumulate in different tissues, such as the brain [74]. According to studies, LYC can be absorbed by passive diffusion and active process in which the transporter of the scavenger receptor protein class B type 1 (SR-B1) is involved [38,75]. Additionally, it has a long chain with 11 double conjugate links, which makes it an excellent scavenger for RONS, such as $^1O_2$, $O_2^{\bullet-}$, $OH^\bullet$, NO_2 and $ONOO^-$ [76,77].

In view of our results, it is possible that lycopene crosses the blood-brain barrier of the central nervous system and exerts neuroprotective effects against oxidative changes induced by malarial infection. Additionally, lycopene serves as an efficient antioxidant, acting on antioxidant defense in brain tissue by binding to RONS, inactivating them and preventing the reaction chains that lead to the formation of $^1O_2$, $O_2^{\bullet-}$, $OH^\bullet$, NO_2, and $ONOO^-$ highly deleterious to the body. In lung tissue, we believe that LYC can decompose $O_2^{\bullet-}$ and thus prevent the production of $ONOO^-$ and tissue oxidative nitration, releasing NO to promote vascular vasodilation, contributing to tissue perfusion and the arrival of more defense cells and lycopene molecules in the tissue. Therefore, lycopene may accumulate and exert protective effects on the pulmonary and cerebral vasculature.

Previous studies have shown the protective role of NO in the hemodynamic improvement of cerebral microcirculation and the reduction of vascular pathology in cerebral malaria [78,79]. On the other hand, in malarial infection, $ONOO^-$ can induce protein nitration, depletion of consumable antioxidants, peroxidation of the microvascular endothelium [22], resulting in the marked decrease in blood flow leading to ischemia and subsequent hypoxia, vasospasms, and tissue hypoperfusion along with cell-mediated congestion, resulting in pulmonary edema and stroke [80,81]. In addition, tissue ischemia may result from cytoadhesion caused by *Plasmodium* infection [5,12].

Prolonged hypoxia ceases oxidative phosphorylation in the mitochondria, promoting ATP degradation and accumulation of xanthine oxidase (XO) and hypoxanthine. In an attempt to restore tissue oxygenation, the body stimulates the expression of eNOS to increase the production of NO, aiming at tissue reperfusion. However, when the blood supply is resumed, XO acts on hypoxanthine resulting in the production of $O_2^{\bullet-}$, H_2O_2, and uric acid, the latter being used in the present study as a marker of the occurrence of IRS. On such IRS, $O_2^{\bullet-}$ can still react with NO generating $ONOO^-$ [82].

In this context, our data indicated that Pb infection was able to induce a significant increase in UA levels in the Pb group compared to the Sham group in both tissues ($p < 0.0001$; **Figure 5).** On the other hand, LYC supplementation induced a significant reduction ($p < 0.0001$) of UA levels in relation to the Pb group in both tissues, and to the NAC+Pb group in the brain tissue. In addition, treatment with LYC provided a normalization in the levels of UA in both tissues, as well as NAC in the lung tissue, suggesting its role in the prevention of damage caused by IRS.

These results are in agreement with previous studies that showed elevated UA levels in children infected with *P. falciparum* during acute episodes and with disease severity, suggesting that UA is an important mediator in the pathophysiology of malaria [83].

4. Materials and Methods

A total of 231 adult male mice of the species *Mus musculus* and Balb/c breed, 7-10 weeks of age, weighing between 25 and 40g (Vivarium of the Evandro Chagas Institute, Ananindeua, Pará-Brazil) were used. The animals were housed in the Experimentation Vivarium of the Oxidative Stress Research Laboratory (LAPEO) of the Institute of Biological Sciences (ICB) of the Federal University of

Pará (UFPA), with an ambient temperature of 24±2°C, a 12-hour light/dark cycle (lights from 7 a.m. to 7 p.m.), and free access to food and water. Before any experimental procedure, animals were acclimatized to laboratory conditions for 15 days.

All experimental procedures were performed at LAPEO. The animals were handled and cared for in accordance with the ethical standards of animal experimentation indicated by the Brazilian Society of Laboratory Animal Science. The project, filed under No. 3235130919, was approved by the Ethics Committee on the Use of Animals of UFPA (CEUA/UFPA).

2.1. Protocol for the preparation and administration of lycopene and N-acetylcysteine

The lycopene preparation and administration protocol was chosen based on a dose-response study on the effects of lycopene supplementation on biomarkers of oxidative stress [84], and the dose was calculated by allometric extrapolation [85]. Animals received 3.11mg/kg of body weight/day of lycopene via gavage.

The N-acetylcysteine preparation and administration protocol was chosen based on a randomized, double-blind, placebo-controlled study of chronic obstructive pulmonary disease [86], and the dose was calculated by allometric extrapolation [85]. Animals received 62mg/kg of body weight/day of NAC via gavage.

Mice in the LYC+Pb and NAC+Pb groups were pretreated with a dose of 3.11mg/kg bw/day of LYC or 62mg/kg bw/day of NAC via gavage, respectively, twenty-four hours before infection. Treatments then continued daily until the day before the animals were euthanized. Mice of the Sham group received only vehicle (water) via gavage.

2.2. *Plasmodium berghei* ANKA -infection protocol

The mice of the groups PB, LYC+Pb and NAC+Pb were performed by intraperitoneal injection (i.p.) of 10^6 *P. Berghei* ANKA-parasitized red blood cells (pRBC). On the other hand, the Sham group animals received 10^6 of non-parazitated red blood cells.

2.3. Protocol for subdivision of the experimental groups

In an experiment of 1, 4, 8 or 12 days of consecutive follow-up, 231 male mice (Balb/c) were randomly assigned to 4 groups (**Figure 6**), including **Sham** (n=28): mice received the vehicle (water; gavage) and non-parasitized red blood cells (i.p.); **Pb** (n=49): mice infected with P. *berghei* ANKA (i.p.); **LYC+Pb** (n=49): mice treated with **LY**C (gavage) and infected with *P. berghei* (i.p.); **NAC+Pb** (n=49): mice treated with **NAC** (gavage) and infected with *P. berghei* (i.p.).

The subgroups 1- and 4-days were formed by 7 animals each. The 8- and 12-days subgroups were formed by 15 and 20 animals, respectively, due to the higher mortality expected for these subgroups.

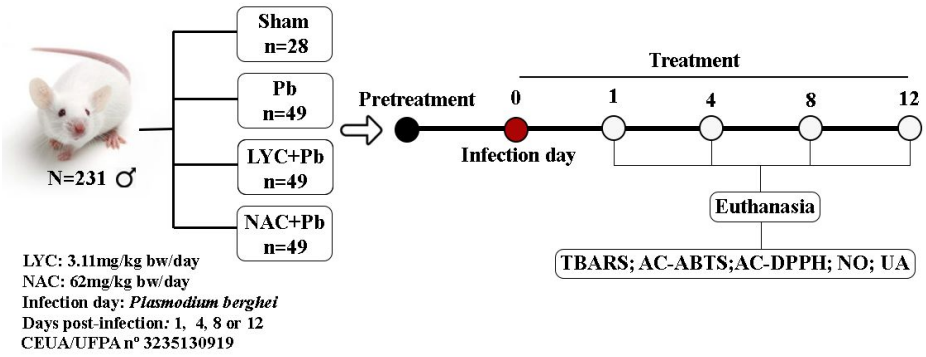

Figure 6. Schematic representation of the experimental protocol. Balb/c mice were pretreated with lycopene or N-acetylcysteine prior to inoculation with 10^6 erythrocytes parasitized with *Plasmodium Berghei* ANKA, and treatment continued daily until the day before euthanasia, for 1, 4, 8 or 12 consecutive days. After euthanasia, brain and lungs were collected for biochemical analyses: Antioxidant Capacity Equivalent to Trolox by inhibition

of ABTS (AC-ABTS) and DPPH (AC-DPPH) radicals. TBARS= Thiobarbituric Acid Reactive Substances; UA= Uric Acid; NO= Nitric Oxide.

2.4. Euthanasia Protocol and Sample Preparation

At the end of each study period, each animal was anesthetized intraperitoneally, using a combination of 0.5 mL of 10% ketamine hydrochloride (9 mg/kg) + 0.25 mL of 2% xylazine hydrochloride (10 mg/kg) + 4.25 mL of water for injection. After confirmation of unconsciousness and loss of corneal reflex, animals were euthanized by exsanguination through intracardiac puncture.

Subsequently, both lungs were extracted, as well as the brain of each animal. The organs were weighed and added phosphate saline-buffer solution (PBS) in a ratio of 1:10 (m:v). Subsequently, the ultrasonic disruption of tissues was performed to obtain a homogenate. After homogenization, the material was centrifuged at 2,500 rpm for 10 minutes and the supernatant was collected, stored in an *Eppendorf* microtube, and frozen at -20ºC until assayed.

2.5. Biochemical Measurements Protocol

2.5.1. Thiobarbituric acid reactive substances (TBARS)

The method was carried out according to the fundamentals proposed by Kohn and Liversedge [87], with chemical conditions of the reaction adjusted according to Percário et al. [88]. This method evaluates lipid peroxidation and has been used as an indicator of oxidative stress. The test is based on the reaction of thiobarbituric acid (4,6-Dihydroxypyrimidine-2-thiol, TBA; Sigma-Aldrich; T5500; São Paulo/SP) with by-products of lipid peroxidation (e.g. MDA), at acidic pH (2.5) and high temperature (94°C), forming chromogens with absorbance at 535 nm.

Initially, 0.5 mL of the sample or standard was mixed with 1 mL of the TBA solution (10 mM). Then, this solution was placed in a water bath at 94ºC for 60 minutes. Subsequently, 4 mL of n-butyl alcohol were added, the solution was stirred in a vortex-type agitator, then centrifuged at 3000 rpm for 10 minutes. After that, 3 mL of the supernatant was transferred to the cuvette and then spectrophotometry was performed at 535 nm (Spectrophotometer 800XI; Femto; São Paulo/SP).

A standard curve (1,1,3,3, tetrahydroxypropane; standard MDA; 20 μM; Sigma-Aldrich Chemical; 108383; São Paulo/SP) was performed in triplicate and, from the values found, the equation of the line ($y = 0.1419x - 0.0037$) was calculated, where y represents the absorbance value and x the concentration value, obtaining $R^2 = 0.9999$. From the equation of the line, the concentration of TBARS of the samples was determined.

2.5.2. Antioxidant capacity by radical $ABTS^{\bullet+}$ inhibition (AC-ABTS)

It was carried out according to the initial foundations proposed by Miller et al. [89], with reaction conditions modified by Re et al. [90]. The method is based on the ability of substances to eliminate the radical cation 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$), a blue-green chromophore with maximum absorption at 734 nm, resulting in the formation of the stable product ABTS, which is colorless.

Initially, the $ABTS^{\bullet+}$ solution (2.45 mM) was prepared from the reaction between ABTS (7 mM; Sigma-Aldrich; A1888; São Paulo/SP) and potassium persulfate (140 mM; $K_2O_8S_2$; Sigma-Aldrich; 216224; São Paulo/SP). Then, the initial reading (T0) of the $ABTS^{\bullet+}$ solution was performed in an 800XI spectrophotometer (Femto; São Paulo/SP) at 734 nm. Then, 30 μL of sample or standard was added to the solution and, after 5 minutes, the final reading (T5) was performed.

A standard curve (6-hydroxy-2,5,7,8-tetramethylcromono-2-carboxylic acid; Trolox®; 2.5mM; Sigma-Aldrich; 23881-3; São Paulo/SP) was performed in triplicate and, from the absorbance values found, the equation of the line ($y = 0.4324x + 0.0049$) was obtained, where y represents the absorbance value and x the concentration value, obtaining $R^2 = 0.9997$. From the equation of the line, the AC-ABTS of the samples was determined.

2.5.3. Antioxidant capacity by radical $DPPH^{\bullet}$ inhibition (AC-DPPH)

The test was performed according to the adapted method proposed by Blois [91] This assay evaluates the total antioxidant capacity of synthetic or natural substances to eliminate the $DPPH^{\bullet}$

radical (Sigma-Aldrich; D9132; São Paulo/SP), a violet chromophore with absorption at 517nm, resulting in the formation of the hydrogenated product DPPH, which is yellow or colorless.

First, the DPPH• solution (0.1 mM) was prepared from the reaction between DPPH (394.32 g/mol; Sigma-Aldrich; A1888; São Paulo/SP) and ethyl alcohol (P.A.; C_2H_6O; Sigma-Aldrich; 216224; São Paulo/SP). Subsequently, the DPPH• solution was read in an 800XI spectrophotometer (Femto; São Paulo/SP) at 517 nm. Then, 50 μL of the sample or standard was mixed in 950 μL of the DPPH• solution and placed in a water bath at 30°C for 30 minutes. After this period, the second reading was held.

A standard curve (Trolox; 2.5mM) was performed in triplicate and, from the absorbance values found, the equation of the line was obtained: $y = 0.2041x - 0.0031$, where y represents the absorbance value and x, the concentration value, obtaining $R^2 = 0.9973$. From the equation of the line, the AC-DPPH was determined in the samples.

2.5.4. Nitric oxide (NO)

The NO concentration was determined indirectly by the detection of nitrate (NO_3^-) or nitrite (NO_2^-) in the samples, using the NO colorimetric assay kit (Elabscience®, Catalog No: E-BC-K035-M). NO is readily oxidized to form nitrite *in vivo* or in aqueous solution, which can react with the chromogenic reagent nitrate, forming a pale red compound. The concentration of the compound is linearly related to the concentration of NO in the sample.

Initially, 100 μL of sample or standard was mixed with 200 μL of reagent 1 (sulfate solution), and 100 μL of reagent 2 (alkaline reagent). After resting for 15 minutes, the solution was centrifuged at 3000 rpm for 10 minutes. Then, 160 μL of the supernatant was transferred to the microplate, where 80 μL of the chromogenic reagent was added. After 15 minutes of incubation at room temperature, spectrophotometric readings were performed at 550 nm.

A standard curve (sodium nitrite; 100 μM) was performed in triplicate and, from the values found, the equation of the line was obtained ($y = 0.0022x - 0.0005$), where y represents the absorbance value and x, the concentration value, obtaining $R^2 = 0.9987$. From the equation of the line, the concentration of nitric oxide in the samples was determined.

2.5.5. Uric acid (UA)

The procedure was performed using the Liquiform Uric Acid Kit (Labtest). The technique consists of the oxidation of uric acid by uricase producing allantoin and H_2O_2. H_2O_2 in the presence of peroxidase reacts with 3,5-dichloro-2-hydroxybenzene sulfonate acid (DHBS) and 4-aminoantipyrine to form the antipyrylquinonymine chromogen. The intensity of the red color formed is directly proportional to the concentration of uric acid in the sample.

To perform the assay, 0.02 mL of the sample or standard was mixed in 1 mL of uric acid working reagent (4-aminoantipyrine, peroxidase, sodium azide, DHBS, and uricase). The solution was then incubated in a water bath at 37°C for 5 minutes. Then, absorbances were determined using an 800XI spectrophotometer (Femto; São Paulo/SP) at 505 nm.

A standard curve (Uric acid; 20mg/dL) was performed in triplicate and, from the values found, the equation of the line was obtained ($y = 0.0166x + 0.0012$), where y represents the absorbance value and x, the concentration value, obtaining $R^2 = 0.9986$. From the equation of the line, the concentration of UA in the samples was determined.

2.6. Statistical analysis

For each parameter analyzed, the analysis of possible outliers was performed by calculating the interquartile range, in which the difference between the third quartile (Q3) and the first quartile (Q1) was determined, called dj. Any value lower than Q1-3/2dj or greater than Q3+3/2dj was considered to be discrepancies, and was not considered in the statistical calculations. After the analysis of the discrepant points, normality was assessed using the Levene test. For homoscedastic distribution, the Analysis of Variance (ANOVA) test was applied, and for heteroscedastic dispersion, the Kruskal-Wallis test was applied. Significant differences were compared between the groups using Tukey's *post-hoc* test.

In the intragroup temporal progression analysis, the unpaired Student's t-test was performed. To verify the possible correlation between parameters, Pearson's correlation test was performed, considering the paired values of two parameters obtained for the same animal, and the calculations were performed with the data obtained from all animals simultaneously, according to the group to which they belong. For the pairs of values in which there was suspicion of a linear relationship, regression analysis was performed, using all animals in both groups simultaneously and each group individually. In all tests, a significance level of 5% ($p \leq 0.05$) was considered.

5. Conclusions

Lycopene prevented oxidative damage induced by *Plasmodium berghei* in brains and lungs of mice, restoring the levels of NO, TBARS, and antioxidant molecules, as well as preventing the occurrence of ischemia and reperfusion syndrome in infected animals. These results are striking evidence of the beneficial effect of lycopene supplementation on oxidative stress in experimental malaria *in vivo* and emphasize the importance of antioxidant supplementation in the treatment of the disease.

Thus, lycopene may become an important viable, safe, and innovative strategy for the development of therapeutic alternatives to mitigate the damage caused by malarial infection.

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Institutional Review Board Statement: The animal study protocol was approved by Comissão de Ética no Uso de Animais da UFPA (CEUA/UFPA; protocol code no. 3235130919, issued on September 10th, 2021).

Data Availability Statement: Data is freely available upon request.

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Conflicts of Interest: The authors declare no conflict of interest.

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5 CAPÍTULO IV: DISCUSSÃO INTEGRATIVA

A malária é uma doença que afeta milhares de pessoas em todo o mundo todos os anos. Sua patogênese está associada à produção de espécies reativas de oxigênio e nitrogênio (ERON) e níveis mais baixos de micronutrientes e antioxidantes humana (TORRE *et al.,* 2018; VANDERMOSTEN *et al.,* 2018). Pacientes com infecção mista por *Plasmodium falciparum* e *P. vivax* (ou *P. ovale*), sob tratamento medicamentoso como por exemplo artemeter + lumefantrina ou artesunato + mefloquina, que são drogas esquizonticidas sanguíneas eficazes para todas as espécies, associando-as à primaquina por sete dias, podem apresentar altos níveis de biomarcadores de estresse oxidativo nos tecidos do corpo (ABOLAJI *et al.*, 2013, 2016), o que limita o uso desses medicamentos.

Diante disso, estudos de Herbas *et al.* (2010) e Chuljerm *et al*. (2021) sugeriram que a inibição de ERON, aumentando a capacidade antioxidante do hospedeiro, pode representar uma estratégia terapêutica adjuvante no tratamento desses pacientes (HERBAS *et al.*, 2010; CHULJERM *et al.*, 2021). Nesse sentido, a suplementação com compostos antioxidantes como zinco, selênio ou vitaminas A, C ou E foram sugeridos como parte do tratamento (SHARMA *et al.*, 2012).

Entre os antioxidantes alimentares, o licopeno (LYC) tem se destacado entre os principais carotenoides, pois pode apresentar cerca do dobro da atividade antioxidante do β-caroteno (BÖHM *et al.*, 2002). Além disso, por estar presente em altas contrações em um dos alimentos mais consumidos pela população, que é o tomate.

Na busca de evidencias cientificas que confirmassem o potencial efeito antioxidante do LYC, o presente estudo realizou uma vasta revisão da literatura, na qual foi demonstrado o efeito antioxidante do LYC em várias doenças nas quais o estresse oxidativo está implicado como uma causa, incluindo câncer (CHENG *et al.*, 2020), doença crônicas associadas a idade como a doença de Alzheimer (QU *et al.*, 2016; ZHAO *et al.*, 2018; HUANG *et al.*, 2019) e hipertensão (FERREIRA-SANTOS *et al.*, 2018), uveite (GÖNCÜ *et al.*, 2016), Doença hepática gordurosa não alcoólica (MARTÍN-POZUELO *et al.*, 2015; NI *et al.*, 2020), aterosclerose (RENJU *et al.,* 2014; LIU *et al.,* 2021), diabetes (NEYESTANI *et al.*, 2007), entre outras. Além disso, foram fornecidas informações relevantes sobre possíveis mecanismo de ação descritos para o LYC e ainda, uma justificativa baseada em evidências experimentais

de que o LYC é eficaz contra a infecção malárica induzida por *P. falciparum in vitro* (AGARWAL *et al.*, 2014).

Na revisão da literatura, também, foram descritos alguns dos principais mecanismos que induzem o estresse oxidativo durante a malária, destacando a produção de ERON como um mecanismo de defesa contra a infecção, induzida pela síndrome da isquemia-reperfusão, o metabolismo do parasita e o metabolismo dos medicamentos antimaláricos.

Também, para confirmar experimentalmente os efeitos da infecção malárica e corroborar com a literatura acerca do papel importante da suplementação de micronutrientes na prevenção da malária, foram realizadas a infecção malárica de camundongos machos Balb/c com a cepa de *P. berghei* ANKA. Esta espécie induz a infecção malárica em roedores, sendo capaz de induzir uma síndrome em animais semelhante à causada pelo *P. falciparum* em humanos e que está bem caracterizada quanto ao envolvimento de mecanismos oxidativos em sua fisiopatologia. Além disso, foram realizados o pré-tratamento desses animais com uma dose de 3,11mg/kg de peso corporal/dia de LYC ou 62mg/kg pc/dia de N-acetilcisteina, e os tratamentos desses animais continuaram diariamente durante 1, 4, 8 ou 12 dias após a infecção.

Em nosso estudo, foi utilizada a NAC como uma substancia padrão. O fármaco antioxidante NAC tem sido proposto como um adjuvante ao tratamento da malária severa em ambos estudos *in vitro* e *in vivo* (WATT *et al.,* 2002; TREEPRASERTSUK *et al.,* 2003; ARRESRISOM *et al.,* 2007; GOMES *et al.,* 2015).

Após o período de infecção e tratamento, avaliamos o percentual da parasitemia e a taxa de mortalidade dos camundongos. Os resultados confirmaram que, a infecção malárica induzida pelo Pb, foi capaz de aumentar progressivamente a parasitemia de 0,6%, 5,6%, 15,8% e 40% nos dias 1, 4, 8 e 12 pós-infecção, respectivamente. Observamos também, que taxa de mortalidade dos camundongos infectados foi elevada, de 47% e 45% no 8° e 12° dia pós-infecção, respectivamente.

Apesar da elevação da parasitemia, a contagem de parasitos no sangue periférico pode ter subestimado o quadro real da parasitemia, uma vez que as populações de parasitos podem ter ficado aderidas dentro de microvasos do baço, rins, fígado, pulmões e cérebro (ZAID *et al,.* 2020), levando a menor disponibilidade de células infectadas na corrente sanguínea. Por outro lado, a presença de parasitos nesses órgãos pode ter sido o responsável pela elevada taxa de mortalidade apresentado pelos animais infectados pelo Pb.

Os camundongos do grupo Pb também apresentaram declínio progressivo do peso corporal associado à incidência de piloereção, olhos lacrimejantes e fechados, palidez, descoloração das orelhas, alteração da cor do sangue de vermelho escuro para vermelho claro, postura arqueada e letargia efetiva a partir do 6º dia. e 8 dias após a infecção, até o final do estudo. A presença de urina escura também foi observada nestes animais, embora sua incidência tenha sido inconsistente em todos os animais.

A partir desses dados, sugere-se que a palidez e a alteração da cor do sangue podem ser resultadas de anemia grave, e a piloereção pode estar relacionada ao estado hipotérmico durante a infecção, porque o mecanismo homeostático está se adaptando à perda de calor. O escurecimento da urina neste modelo pode ser devido à congestão no córtex e na medula renal durante a infecção (BASIR *et al.*, 2012). É possível, também, que essas alterações estejam relacionadas à interferência dos parasitas no influxo de nutrientes aos tecidos, resultando em perda de peso, postura arqueada e letargia.

Nesse sentido, estudos anteriores demonstraram que crianças e adultos com malária têm geralmente uma elevada prevalência de desnutrição e deficiências de micronutrientes, incluindo vitamina A, β-caroteno, LYC e zinco (THURNHAM E SINGKAMANI, 1991; ZEBA *et al.*, 2008), e esta situação cria uma complexidade de interações com graves consequências para a saúde do hospedeiro.

Outro fator importante é a produção exagerada de ERON durante a doença, que são responsáveis por induzir alterações bioquímicas oxidativas, responsáveis pela a redução da deformabilidade dos glóbulos vermelhos e consequente hemólise, acidose metabólica, anemia grave e malária cerebral (HALDAR *et al.*, 2007; SRIVASTAVA *et al.*, 2015; KUMAR *et al.*, 2018), em última análise, pode levar à morte do hospedeiro (GOMES *et al.*, 2015; BARBOSA *et al.*, 2021).

Estas alterações fisiopatológicas da malária têm início com o ciclo eritrocítico da infecção. Nesta fase, o parasito invade o eritrócito, consome e hidrolisa a hemoglobina intraeritrocitária, formando aminoácidos necessários para o seu desenvolvimento (TEKWANI e WALKER, 2005). Após a quebra da proteína, o ferro ferroso (Fe^{2+}) da ferroprotoporfirina liberada pode ser rapidamente oxidado a ferro férrico (Fe^{3+}), dando origem a ferriprotoporfirina IX, que sofre reações oxidação e redução, formando ERON, tais como os radicais livres superóxido ($O_2^{\bullet-}$), hidroxila ($OH^{\bullet}$), óxido nítrico (NO), peroxinitrito ($ONOO^-$), altamente reativos (MÜLLER, 2004; KLONIS *et al.*, 2013).

Para corroborar as evidencias cientificas da literatura e confirmar nossa hipótese sobre alterações bioquímicas oxidativas durante a infecção malárica, realizamos análises experimentais de marcadores laboratoriais bioquímicos, no cérebro e pulmões dos animais os animais sobrevivementes, para quantificação da concentração de concentração de Substâncias Reativas ao Ácido Tiobarbitúrico (TBARS), atividade antioxidante pela inibição dos radicais ABTS (AC-ABTS) e DPPH (AC-DPPHA), Óxido Nítrico (NO) e Ácido Úrico (UA).

De acordo com os resultados, a infecção malárica induzida pelo *P. berghei* foi capaz de induzir aumento significante no nível de TBARS, AC-DPPH, AC-ABTS, NO e UA comparado ao grupo Sham nos tecidos cerebral e pulmonar ($p < 0,0001$), o que eram achados esperados, pois, de acordo com estudos, cerca de 60% dos camundongos infectados com *P. berghei* sucumbem a malária entre os dias 6 e 8 pós-infecção com parasitemia moderada entre 6 a 11% (BAPTISTA *et al.*, 2010), devido ao estresse oxidativo (REIS *et al.*, 2010; FERNANDES *et al.*, 2014; SCACCABAROZZI *et al.*, 2018; CHULJERM *et al.*, 2021). Por outro lado, o tratamento com o LYC reduziu a porcentagem de parasitemia e a taxa de mortalidade dos camundongos infectados pelo Pb ($p < 0,0001$), para os quais o LYC exibiu maior potencial do que a NAC. Além disso, LYC e NAC melhoraram vários biomarcadores bioquímicos relacionados ao estresse oxidativo. No entanto, o LYC apresentou efeito mais intenso que a NAC. O LYC induziu a redução dos níveis de UA, TBARS e NO de camundongos com malária, levando a concentrações inferiores às encontradas nos animais infectados sem tratamento ou naqueles tratados com a NAC, atingindo valores semelhantes aos dos animais não infectados.

O efeito exercido pelo LYC demonstra que, o LYC pode ser bem-sucedido em eliminar as ERON, inibir a peroxidação lipídica e proteger os lipídios da membrana do dano oxidativo no cérebro e pulmão de camundongos durante a infecção malárica.

Estes dados estão alinhados com achados anteriores da literatura em que se relatou que ambos LYC ou NAC são capazes de reduzir os níveis de oxidantes endógenos e proteger as células contra uma ampla gama de insultos pró-oxidativos (EZERIŅA *et al.*, 2018; ELSAYED *et al.*, 2021), reagindo diretamente com as RONS (SAMUNI *et al.*, 2013). Segundo Zhang *et al.* (2020), o LYC tem a capacidade de inibir o estresse oxidativo e as TBARS, limitando a produção de RONS. A atividade do LYC, também foi implicada na eliminação de *P. falciparum in vitro* (AGARWAL *et al.*, 2014). Anteriormente, Metzger *et al.* (2001), apontavam que o aumento da concentração plasmática de LYC poderia estar associado a depuração da parasitemia em crianças com malária.

Por outro lado, a atividade antioxidante da NAC pode ter ocorrido através da ação direta, do grupo tiol (SH) livre da NAC, que pode atuar como doador de elétrons, favorecendo a interação da NAC com ERON. Além disso, a NAC por ser um análogo e precursor da GSH, pode ter induzido a reposição dos níveis intracelulares de GSH, também conhecida como o principal antioxidante produzido pelo organismo e que protege as células do estresse oxidativo.

Diante dos nossos resultados, é possível que o licopeno atravesse a barreira hematoencefálica para o sistema nervoso central e exerça efeitos neuroprotetores contra alterações oxidativas induzidas pela infecção malárica. Adicionalmente, devido aos hidrocarbonetos altamente poli-insaturados existentes em sua estrutura, o licopeno atua como um eficiente antioxidante na defesa antioxidante no tecido cerebral se ligando às ERON inativando-as e impedindo as cadeias reacionais que levam a formação de $^{1}O_2$, $O_2^{\bullet-}$, $OH^{\bullet}$, NO_2 e $ONOO^-$ altamente deletérias para o organismo. Já no tecido pulmonar, os dados sugerem que o LYC pode decompor o $O_2^{\bullet-}$ e então prevenir a produção de $ONOO^-$ e nitração oxidativa tecidual, liberando o NO para promover vasodilatação vascular, contribuindo para perfusão tecidual e chegada de mais células de defesa e moléculas do licopeno no tecido. Através dessas ações o licopeno pode acumular-se e exercer efeitos protetores na vasculatura pulmonar e cerebral.

Neste estudo confirmamos dados importantes sobre os benefícios *in vivo* de uma terapia baseada no fitonutriente licopeno para o manejo das alterações bioquímicas oxidativas na malária experimental.

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ANEXOS

1. Comprovante de aceitação de artigo de revisão.

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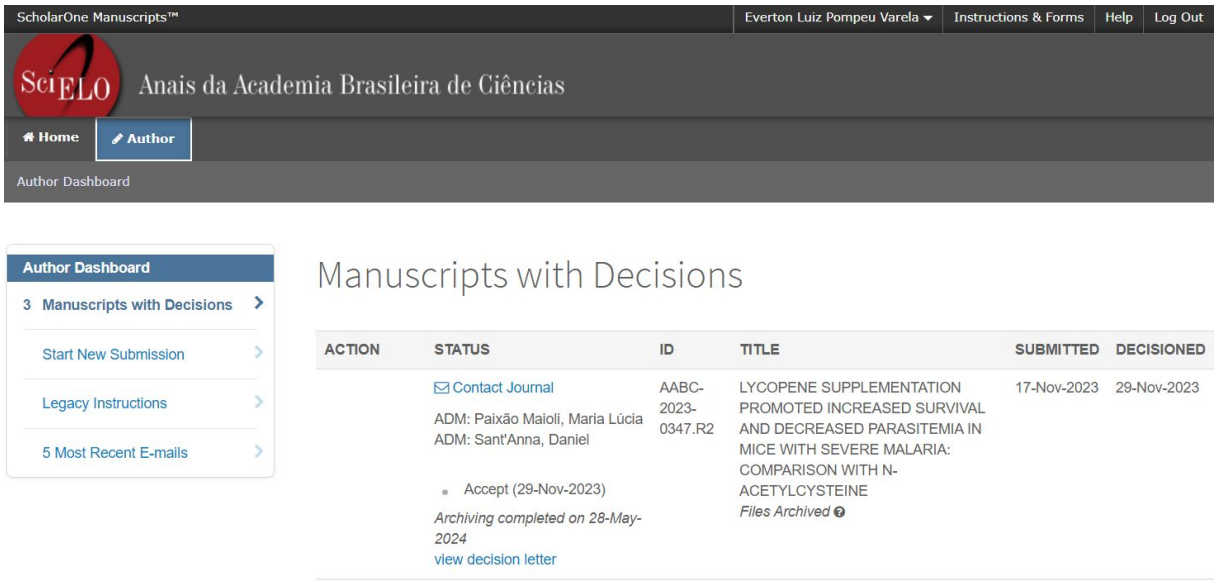

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