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INTERNATIONAL JOURNAL OF CLINICAL MICROBIOLOGY
ISSN NO: 2690-4721

Review Article

DOI: 10.14302/issn.2690-4721.ijcm-17-1676

Regulation of Expression of Reactive Oxygen Intermediates during *Plasmodium* Infection to Reduce Immunopathology Provides a Possible Antioxidant Adjuvant to Enhance Anti-Malarial Drug Therapy

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ABSTRACT

Malaria is a mosquito-transmitted infectious disease caused by intracellular protozoan parasites of the genus *Plasmodium*. In the absence of prompt and appropriate treatment contraction of primary infection by a human being often represents a medical emergency since it may progress rapidly to life-threatening complications. Exposure to parasites activates the immune system resulting in, among other effects, the release of reactive oxygen intermediates (ROI). This has the potential to induce oxidative damage, thereby causing cellular destruction, and hence to have a severe effect on vital organs of the body. Overexpression of ROI leads to immunosuppression and is a causal factor in the development of malaria-related disease symptoms. However, the body possesses various defence mechanisms, notably including the production of antioxidants, which are capable of reducing the cellular effects of ROI. Antioxidants are either sourced exogenously from the diet or synthesized through different intracellular mechanisms. Antioxidants that include glutathione peroxidase, catalase, EDTA and vitamin C suppress the initial production of ROI. Others such as uric acid, superoxide dismutase and vitamin E may also inhibit potentially damaging products of ROI metabolism. Current anti-malarial drugs often have damaging side-effects, as exemplified by memory impairment following treatment for cerebral malaria. Recent studies have explored the potential use of antioxidants alone or in combination with anti-malarials as a therapeutic means to negate *Plasmodium*-induced oxidative stress and its associated metabolic complications. It is indicated that when utilized in an adjuvant capacity antioxidants of natural and synthetic origin may improve anti-malarial therapy by causing less damage to the host during malaria infection.

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Keywords: Malaria; *Plasmodium*; infection; immunity; immunopathology; reactive oxygen intermediates (ROI); antioxidant; anti-malarial; drug; adjuvant; therapy

Received Date: June 21, 2017; **Accepted Date**: July 19, 2017; **Published Date**: Aug 14, 2017

Introduction

Malaria is an infectious disease of vertebrates caused by intracellular protozoan parasites belonging to the genus *Plasmodium*, of which five species are capable of infecting humans (1). Transmission between hosts occurs via the bite of an infected female *Anopheles* mosquito (1,2). Malaria is at present endemic in 110 countries and is found on every continent, bar Antarctica, but is confined to mainly tropical and sub-tropical regions (3,4). Of the estimated global population of 7.3 billion people, 3.5-3.7 billion are at risk of contracting malaria (3,5). In the absence of prompt diagnosis and appropriate treatment disease can escalate to life-threatening complications, such as cerebral and renal manifestations (6). It is routinely claimed that there are 300-500 million clinical cases worldwide annually (7). The 1-3 million deaths that result equate to a death every 10-30 seconds (8,9). 90% of deaths are due to *P. falciparum* and occur principally in young children in sub-Saharan Africa.

P. falciparum Infection Stimulates Host Immunity

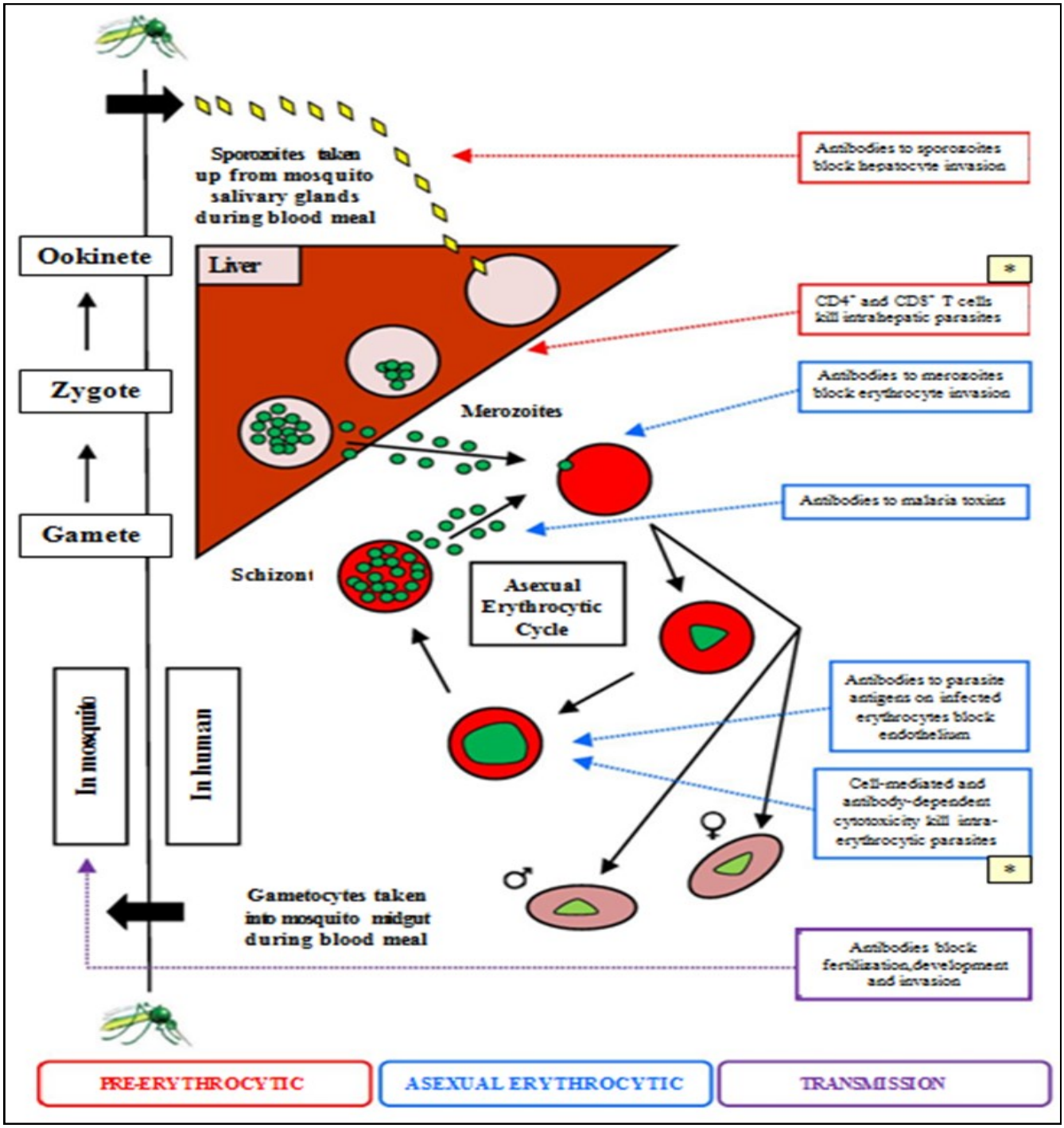
The life cycle of *P. falciparum* includes a symptomless, non-pathogenic liver stage of 7-10 days duration followed by the invasion of aged erythrocytes by infective forms, merozoites, which heralds the start of an intraerythrocytic vegetative growth cycle that is responsible for the pathogenic manifestations of disease (Fig. 1). Erythrocytes rupture once mature schizonts are produced, which occurs 46-48 hours after invasion. Every erythrocyte releases between 16-32 merozoite progeny, each of which may bind to and enter a new erythrocyte to initiate a further cycle of asexual replication (4).

The human immune response against malaria infection is complex and depends on different stages of the life cycle (10) (Fig. 1). Specific cytokines are released from human peripheral blood mononuclear cells which may activate the host's monocytes, $CD4^+$ and $CD8^+$ T lymphocytes, natural killer (NK) cells and neutrophils to react to liver stage and blood stage parasites (11). The immune mechanism to intraerythrocytic malaria parasites is not fully understood, yet a particular cytokine profile is known to be associated with protection that involves a pro-inflammatory response by $CD4^+$ T cells of the helper (Th) 1 subset, with a prevalence of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-12 (12-14). In contrast, the Th2 profile (notable for the production of IL-10, IL-5, IL-4 and transforming growth factor (TGF-β)) is noted to enhance severe conditions of the disease. However, since different immune responses act effectively at different stages of parasite elimination, there is no overall consensus on the respective pros and cons of these cytokine profiles (15,16).

P. falciparum Infection Activates ROI

Malaria infection activates the immune system resulting in the release of reactive oxygen intermediates (ROI) that induce oxidative damage which has the potential to kill parasite-infected hepatocytes and erythrocytes but also to cause cellular pathology (17). This triggers such severe effects on vital organs of the body as hepatomegaly, splenomegaly, endothelial and cognitive damage. ROI, together with reactive nitrogen intermediates (RNI) also linked with oxidative stress, are implicated in the development of systemic complications caused by malaria leading to the production of hydroxyl radicals in the liver that are thought to be a reason for the induction of oxidative stress and apoptosis (18). Additionally, erythrocytes infected with *P. falciparum* produce twice the concentration of hydroxyl radicals and hydrogen peroxide compared to uninfected erythrocytes (19). However, the body has various defence mechanisms to reduce the cellular effects of ROI including the production of antioxidants obtained either from the diet or synthesized via different intracellular mechanisms. Potential roles of an antioxidant are preventive or chain-breaking. Preventive antioxidants such as glutathione peroxidase, catalase, EDTA and vitamin C suppress the initial production of free radicals including ROI. So-called chain-breaking antioxidants like uric acid, superoxide dismutase and vitamin E inhibit damaging products of the ROI pathway (20). Thus, antioxidants of both natural and synthetic origin possess the potential to confer host-protective adjuvant effects on antimalarial therapy that may result in reduced immunopathology during malaria infection.

Figure 1. Schematic diagram showing the mechanisms of immunity against different life cycle stages of *Plasmodium falciparum*. * indicates those targeted immune responses in which reactive oxygen intermediates play an important role.



Oxidative Stress: A Host Defence Mechanism

The mechanisms of action of ROI and RNI involve reduction or elimination of parasite load (Fig. 1), which is the process exploited by most anti-malaria drugs for their activity. These reactive intermediates have a great influence on immune responses by activating or inhibiting the action of certain transcription factors or cytokines and also by modifying pathways of programmed cell death (21). An elevated level of nitric oxide (NO) results in immunosuppression and is causally linked to the development of cerebral malaria (22). However, as free radicals NO metabolites contribute to the destruction of *Plasmodium* (23,24). Malarial pigment is one of the few parasite-derived molecules that are known to elicit production of NO. Haemozoin is a potent inducer of NO generation in macrophages via the action of nuclear factor (NF)-κB and extracellular signal regulated by kinase (ERK). Haemozoin is associated with the mediator of IFN-γ mRNA synthesis through the enzyme inducible nitric oxide synthase (iNOS). During the hepatic stage of infection, defence mechanisms are linked to the release of IFN-γ by NK cells and NO synthesis (25). Haemozoin not only induces NO but is also responsible for macrophage activation by processes that are partially dependent on NO (26) and on other ROI/RNI such as the superoxide radical and hydrogen peroxide (27).

On a related theme, an elevated concentration of iNOS in human monocytes has not been linked with an exacerbation of malaria. Studies conducted on spleen cells from *P. berghei*-infected mice either resistant or susceptible to cerebral malaria suggested that the expression of cytokines and NO increases in cells of resistant animals compared to their susceptible counterparts (28). In addition, expression of a marker of TNF-α activity was also raised in resistant mice, suggesting that activation of macrophages is much greater in resistant animals (29). These findings corroborate radiometric assays used to quantify the anti -plasmodial effect of phagocytes. These prior studies showed that TNF-α increases the release of ROI by neutrophils infected by *P. falciparum*, which are toxic and contribute to the elimination of the parasite (30).

Derived from host erythrocytes, haemoglobin is a potential source of free radical synthesis in malaria. The parasite's utilization of this protein molecule as a source of amino acids to meet its nutritional requirements during the erythrocytic stage of its life cycle results in liberation of extensive amounts of haem into the circulation (31). However these haem groups induce intravascular oxidative stress due to the presence of Fe^{2+}-associated groups which cause metabolic flux in erythrocytes and endothelial cells and facilitate the internalization of the parasite in tissues such as the brain and liver (32). Moreover, free haem can activate neutrophil migration and ROI/RNI production with the help of the inhibitory Gα protein-coupled receptor that further activates protein kinase C, thus enhancing the inflammatory response (33) and delaying apoptosis, thereby contributing to the immunosuppression induced by malaria infection (34). Recently, it was shown that a transient oxidative insult to wild-type erythrocytes prior to infection with *P. falciparum* promotes phenotypic characters associated with the protective traits of haemoglobinopathic and fetal erythrocytes (35). This implies abnormal host actin remodelling and reduced cytoadherence are steps in a malaria host-protective pathway initiated by the redox imbalance that is inherent to sickle cell trait and other common hereditary blood disorders.

Of various cytokines examined, an increase in granulocyte-macrophage colony-stimulating factor (GM-CSF) has been correlated with a reduction of parasitaemia and with oxidative stress (13). GM-CSF is a cytokine that is known to have stimulatory action on granulocytes and macrophages and that helps to enhance the number and activity of both of these cells, working effectively to provide cellular immunity against blood stage malaria. Several studies have reported that administration of GM-CSF, individually or in combination with other factors, protects experimental models from infection. Similarly, mice lacking the ability to synthesize GM-CSF have an impaired anti-*Plasmodium* immune response. Such research suggests a possible relationship between GM-CSF and oxidative stress. IL-4 and GM-CSF receptors may be altered by lipid peroxidation products derived from haemozoin that are released upon rupture of parasitized erythrocytes. These include, for example, 4-hydroxynonenal (4-HNE). IL-4 and GM-CSF are activators of the differentiation of

haemozoin-loaded monocytes into dendritic cells which can be inhibited by 4-HNE and is an important immunosuppressive mechanism in malaria (36). In immature dendritic cells this appears to be linked to expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) by cells loaded with haemozoin after administration of (15) *S*-hydroxyeicosatetraenoic acid – which is a PPAR-γ ligand produced by haemozoin via peroxidation caused by haem – that prevents the differentiation of these cells (37). A relationship exists between GM-CSF and NO, illustrated by pre-treatment with GM-CSF and methionine encephalin (TGG) helping to protect mice from malaria. Conversely, for mice pre-treated with iNOS inhibitors the mortality rate is enhanced significantly, showing that the protection exerted by GM-CSF/TGG is at least partially due to NO.

Effects of Malaria on ROI

Both host and parasites are placed under oxidative stress during a malaria infection. ROI are multifunctional molecules associated with host defence, hormone biosynthesis, mitogenesis, necrosis, apoptosis and gene expression (38). Increased concentrations of ROI are produced during degradation of haemoglobin within the parasite and by activated neutrophils in the peripheral blood of the host. In cases of severe malaria, ROI are produced by parasite haemoglobin metabolism in erythrocytes, nicotinamide adenine dinucleotide phosphate oxidase in phagocytes, and iNOS in response to deficiency of arginine or cofactor tetrahydrobiopterin substrate. Plasma haemoglobin produced upon erythrocyte lysis can also catalyze the generation of ROI. Patients suffering from severe malaria have enhanced level of ROI products in their urine (39), reduced deformability of erythrocytes under shear stress and decreased α-tocopherol in erythrocyte membranes (40). ROI may bestow both both protective and pathological effects on the host during malaria. After administration of pro-oxidants, enhanced ROI production is directed against intra-erythrocytic parasites. The utility of ROI to the innate immune response was first investigated in phagocytic cells by examining ROI production by NADPH oxidases (NOX) followed by pathogen killing (41). During infection erythrocytes are exposed to continuous oxidative stress. Univalent reduction of oxygen occurs, resulting in generation of a series of highly reactive cytotoxic oxygen species such as superoxide, hydrogen peroxide and hydroxyl. This causes a wide spectrum of cell damage including inactivation of enzymes, lipid peroxidation, damage to DNA and modification of intracellular oxidation-reduction states (13).

Effects of Antioxidants on Malaria

Many protective mechanisms have evolved to reduce the harmful effects of free radicals on cells. These include antioxidants like glutathione (GSH) and also enzymes like glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase that catalyse the dismutation of hydrogen peroxide and superoxide anions to water at the expense of GSH (42). Reduced GSH is a tripeptide with an intracellular non-protein free sulfhydryl group, and is essential to overcome oxidative stress and thus to maintain the normal reduced state inside the cell. NADPH generated by glucose 6-phosphate dehydrogenase in the pentose phosphate pathway reduces oxidized glutathione. Cells with reduced levels of glucose 6-phosphate dehydrogenase are very prone to oxidative stress. Erythrocytes experience very acute stress because, without mitochondria, there is no other means of generating reducing power.

Human erythrocytes are rich in membrane sulfhydryl groups which play a major role in maintenance of oxidation-reduction status of the cell. A greater concentration of sulfhydryl groups in oxidatively stressed erythrocytes occurs because of their reduced GST activity (43). A longer association of toxic agents with sulfhydryl groups due to the decrease in GST activity may lead to their intraerythrocytic accumulation (44). Reduced levels of SOD and catalase in blood samples taken from malaria-infected patients resulted in heightened susceptibility of the erythrocytes to cell damage.

The current anti-malarial drugs of choice often have a harmful side-effect, such as reported in memory impairment after cerebral malaria. As a consequence, recent studies have aimed to use antioxidants, alone or in combination with anti-malarials, as a potential therapeutic method focused at reducing *Plasmodium*-induced oxidative stress and its associated

complications (45). However, some anti-malarials act by triggering oxidative stress; hence, the practical application of this strategy often yields conflicting outcomes. In general, researched antioxidants are. The effects on *Plasmodium* parasites *in vitro* of the antioxidants desferroxamine, vitamins C and E, folate and N-acetylcystein have been studied by direct administration and as adjunct therapy alongside standard anti-malarial regimens. The therapeutic relevance of antioxidants in malaria treatment depends on the targeted aspect of malaria pathology (46).

Conclusion

Oxidative stress plays an important role in the pathophysiology of malaria. This multifactorial phenomenon showcases a key aspect of the complex and intricate host-parasite relationship. Currently prescribed anti-malarials frequently have damaging side-effects. Thus, recent research has targeted the use of antioxidants, in combination with anti-malarials or alone, as a potential therapeutic method aimed at reducing *Plasmodium*-induced oxidative stress and its associated pathology. Release of ROI and RNI is integrally involved in the process by which parasitaemia is reduced and represents the main mechanism by which most anti-malaria drugs act. These metabolites regulate immune responses by activating or inhibiting release of transcription factors and cytokines and even by regulating programmed cell death. However, overex-pression of ROI results in immunosuppression, which, as a consequence, exacerbates disease. The use of antioxidants of both natural and synthetic origin provides the potential for improved adjuvantation of anti -malarial therapy that results in less damage to the host. Further research into this concept is required.

Funding Source

None to declare.

Conflict of Interest

The authors declare that they have no competing interests.

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