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Review Article

Oxidative stress: production in several processes and organelles during *Plasmodium* sp development

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Abstract

Worldwide, malaria parasites are increasingly resistant to available antimalarial drugs, which difficult treatment and control. Alternative drugs could exploit the differences between the hosts and parasite responses to reactive oxygen and nitrogen species. Malaria parasites (*Plasmodium* sp) are exposed to oxidative stress as a result of their metabolic processes and their hosts' responses to infection, both in vertebrate hosts and vector mosquitoes. Host erythrocyte hemoglobin digestion and heme production within food vacuoles and the synthesis and folding of proteins within the endoplasmic reticulum, as well as the production of the needed energy in the mitochondria are the main sources of oxidative stress. Parasites maintain the redox equilibrium with antioxidant systems (*i.e.* glutathione-thioredoxin). In this brief review, we discuss the most important processes involved in the production of high levels of free radicals during *Plasmodium* sp development, along with the parasite's protective responses and the strategic differences with the vertebrate hosts that are useful for specific antiparasitic drug design.

INTRODUCTION

Malaria is caused by parasites of the genus *Plasmodium.* The prevalence of this disease is currently at 200-300 million clinical cases worldwide, causing ~0.7 million deaths annually [1]. The increasing resistance of malaria parasites to available antimalarial drugs represents a major impediment for disease treatment and control [2]. An alternative drug design strategy could be based on the sensitivity of *Plasmodium* sp to oxidative stress.

Reactive oxygen (ROS) and nitrogen species (RNS) are produced in all cells [3] and play important roles in cell physiology, such as life cycle regulation, development, migration, induction of signaling pathways, activation of second messengers, and triggering of antioxidant responses [4]. Reactive species are common products of metabolic enzymatic reactions of cells. Their accumulation is prevented by the production of anti-oxidant molecules, which maintain the inner cellular environment in a homeostatic (redox) balance. The loss of the redox balance, either by an increase of oxidant molecules (ROS and RNS) or by decreased antioxidant system activities, causes a state of oxidative stress.

Reactive species can attack and degrade lipids, proteins and nucleic acids producing changes in their functions or increasing their susceptibility to proteolytic attack, which could eventually provoke changes in the structure and function of the cell [5]. One example of the deleterious effect of the accumulation of reactive species is the chain of events initiated by oxidation of cardiolipin, a phospholipid responsible for the insertion and retention of cytochrome C inside mitochondria [6]. The depletion of cardiolipin by mitochondrial oxidative stress produced by *Plasmodium berghei* infection in

mouse hepatocytes [7], leads to the opening of the mitochondrial permeability transition pore (MPTP) [8], the dissociation of cytochrome C [9, 10] and its release into the cytosol, where it induces apoptosis by the caspase pathway.

Processes and organelles involved in oxidative stress during the *Plasmodium* life cycle

Plasmodia are obligatory intracellular parasite members of the *Apicomplexa phylum*. Their life cycle includes an asexual phase in humans, with an initial schizogonic development in the liver, followed by an intra-erythrocytic cyclical multiplication. Some hematic merozoites differentiate into female and male gametocytes [11]. *Anopheles* mosquitoes, which during blood feeding ingest parasite sexual stages, are responsible for transmitting parasites between humans. Gametocytes taken in a blood meal by mosquitoes differentiate into gametes within the midgut of the insects, and after fertilization, the resulting zygotes transform into invasive ookinetes. After sporogonic multiplication, infective parasites reach the mosquito salivary glands and are transmitted during subsequent blood meals [12].

Plasmodia undergo several morphological and metabolic changes that lead to the production of reactive species during their complex life cycle (Fig.1). The parasite's mitochondrion and the apicoplast, two organelles located close to each other, are involved in the energy metabolism, lipid synthesis [13, 14] and consequently production of ROS. There are higher numbers of mitochondria in sexual compared to that in asexual stages, which is probably an adaptation to the additional energy requirement for parasite motility and a more active metabolism. Metabolism and motility represent the main source of free radicals and ROS production in these parasites [15].

Oxidative stress during *Plasmodium* sp development in the vertebrate host erythrocytes

Malaria infection causes a state of oxidative stress in vertebrates. This state is characterized by an increase of lipid and protein oxidation and a reduction of the antioxidant response, mainly mediated by glutathione [16]. The degree of oxidative stress correlates with the level of parasitaemia [17, 18]. Paradoxically, this metabolic mechanism activates signaling pathways that could result in host cell death, a situation unfavorable for the parasite [19]. Therefore, for a successful parasite life cycle, a perfect balance of the host and parasite redox states is required. For this purpose, as with other organisms, the parasite displays an effective and regulated antioxidant response, mediated by molecules capable of competing for oxidative substrates [20].

The intra-erythrocytic stages of *Plasmodium* sp are exposed to oxidative stress due to the great amount of oxygen present within the host cell [21]. Hemoglobin, taken within a food vacuole, is the main source of amino acids for the parasite [22], and the degradation of this protein results in the liberation of heme [23] (Fig.1A), a very toxic compound linked to high ROS and RNS generation [24, 25]. Residual heme is degraded by heme oxygenase-1 (HO-1), which has been found at increased levels during malaria infection [26]. Heme degradation causes the release of iron [22], which along with H_2O_2 induces the production of even more toxic radicals, such as the highly toxic hydroxyl radical (•OH) -by the Fenton reaction- that causes oxidative stress to the parasite (Fig.1B).

Vital to parasite functions are the processes of synthesis and folding, posttranslational modifications and the trafficking of secretory proteins [27] (Fig.1C). Anchored and secreted proteins require the formation of protein disulfide bonds between cysteine residues for molecule stabilization and function. *Plasmodium* sp also produces a large amount of secretory proteins for recognition of and adhesion to host cells, leading to differentiation and invasion. For example, the apical merozoite antigen-1 (AMA-1) is an essential protein for the invasion of the erythrocyte [28]. It has a complex scaffold structure and possesses eight disulfide bonds for stability [29]. Another example is the *P.falciparum* erythrocyte membrane protein 1 (PfEMP1), a member of a family of adhesive proteins. This protein requires a correct folding to display its cyto-adhesive and antigenic properties once inside erythrocytes [30, 31]. Protein folding takes place in the highly oxidized environment of the endoplasmic reticulum (ER), (Fig.1C) where protein disulfide isomerase (PDI) and ER oxidoreductase 1 (ERO1) participate in the formation of disulfide bonds [32]. PDI exerts two inter-related functions, depending of the redox state: as a chaperone assisting in protein folding and as a catalytic enzyme for disulfide bond formation (reduction and isomerization of disulfide bonds) [33]. PDI captures electrons from folded proteins, thereby oxidizing the thiol (SH) group in its cysteine residues, leading to the formation of disulphide bonds. ERO1 uses a FAD-dependent reaction to transfer electrons from PDI to oxygen (O_2), an oxidative process that generates great amounts of ROS in the form of hydrogen peroxide (H_2O_2) [34].

Blood stage parasite redox balance mechanisms

Plasmodium sp lacks catalase and a genuine glutathione peroxidase (GPx) [21], as well as copper/zinc-superoxide dismutase (Cu/Zn-SOD) [35], which are the main catalysts of peroxides in vertebrates. In the parasite GPx homologue, selenocysteine is replaced by cysteine and its reaction with reduced glutathione (GSH) is lower than typical

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human GPx [36]. In addition, the parasite's antioxidant defense response includes: iron (Fe)-SOD, manganese (Mn)-SOD), glutathione reductase (GR) and thioredoxin systems [37].

To counteract stress, the parasite sequesters most heme molecules into a crystalline structure, known as hemozoin (Fig.1). Some heme released into the cytoplasm produces superoxide anions ($O_2^{\bullet -}$), which are catalyzed by Fe-SOD to H_2O_2. Lacking a catalase (CAT) [21], this molecule is in turn transformed into H_2O by the thioredoxin peroxidase and glutathione redox cycles, which constitute a conservative dual antioxidant system (Fig.1D). This dual glutathione/thioredoxin system also function for maintaining the redox environment during the synthesis of parasite proteins and the formation of bridges between them, and this system is considered the major redox buffer for ROS exposure during *Plasmodium* sp intra-erythrocytic development [38].

In vertebrates, the glutathione system involves the activity of two enzymes, GPx and GR. GR is an ubiquitous flavoenzyme that catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of oxidized glutathione (GSSG) to its reduced form (GSH) [37]. Also, GR participates in the amino acid transport across membranes (γ-glutamil cycle), where it functions as a co-factor in enzymatic reactions and in bridge formation between proteins [39].

The redox plasmoredoxin protein links the antioxidant systems of glutathione and thioredoxin [40]. The thioredoxin system is composed of peptide thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH as reducing cofactors. TrxR is a flavo/oxidoreductase, which transfers electrons from NADPH to Trx, acting itself as a reductase for disulphide containing processes [41]. The thioredoxin system is also involved in a variety of cellular functions, including the reduction of ribonucleotides, DNA synthesis and the regulation of gene transcription by its interaction with transcription factors [42, 43]. In view of the fact that the two systems are linked and share functions, a functional failure of one member of this dual system must be compensated by the other. Nevertheless, Trx is essential for the survival of intra-erythrocytic parasite stages, and its functional failure provokes loss of parasite viability [44].

Although *Plasmodium* sp lacks a catalase and a genuine GPx to reduce peroxides [21], it has six peroxiredoxins (Prx) that are its principal enzymes for the reduction of cytotoxic peroxide and nitric oxide (NO) compounds. These enzymes include two thioredoxin-dependent 2-Cys Prx (TPx-1 and TPx-2) [45], one 1-Cys Prx [46], a 1-Cys antioxidant protein (AOP) [47], a glutathione peroxidase-like thioredoxin peroxidase (TPx_{G1}) [37], and a nuclear Prx (nPrx) [48, 49]. Whereas TPx-1, 1-Cys Prx and TPx_{G1} are localized in the cytosol, TPx-2 and TPx_{G1} are found in the mitochondria, and AOP and TPx_{G1} in the apicoplast. These peroxidases participate in the protection of macromolecules from H_2O_2, as second messengers in the transmission of redox signals, mediating cellular proliferation, differentiation and apoptosis [50, 51]. 1-Cys Prx is involved in scavenging oxidative compounds produced from hemoglobin digestion [52].

Malaria parasites also utilize host molecules for their own metabolic processes. For instance, they employ human peroxiredoxin 2 (hPrx-2) up-taken from the cytosolic compartment of human host cells as an enzymatic scavenger of peroxides [53].

Oxidative stress during *Plasmodium* sp development in the invertebrate host

Within the mosquito midgut malaria parasites are also exposed to oxidative stress. The blood meal is digested and metabolically processed for the insect's sustenance and oogenesis [54]. The great quantity of energy production, required during these processes, induces an increased production of reactive species along with a state of oxidative stress (Fig.1E). Concomitantly, during ookinete invasion of the mosquito midgut epithelium, several processes that produce reactive species occur. For example, recognizing and adhering to the epithelium (leading to invasion) requires the production of secretory proteins such as P25 and P28 [55], secreted ookinete adhesive protein (SOAP) [56], Von Willebrand factor A domain-related protein (WARP) [57], and circumsporozoite TRAP-related protein (CTRP) [58].

The ookinete invasion of the midgut elicits the local expression of nitric oxide synthase (NOS) [59]. NOS catalyzes the formation of NO, a toxic, reactive and unstable molecule. NO reacts with other molecules, producing RNS, such as peroxynitrite from the reaction of NO and superoxide anions [60]. An increased NOS toxicity to the parasite may originate from the three main enzymes induced during ookinete invasion: heme peroxidase (HPX2) and NADPH oxidase 5 (NOX5) [61]. The resulting great quantities of ROS and RNS, responsible for a state of oxidative stress, also prompt signal transcriptions for the mosquito immune genes, which are evolutionarily selected to limit vector infection [59, 62, 63].

Control of oxidative stress during *Plasmodium* sp development in the invertebrate host

Malaria parasites have evolved mechanisms to evade the immune response and to eliminate toxic metabolites

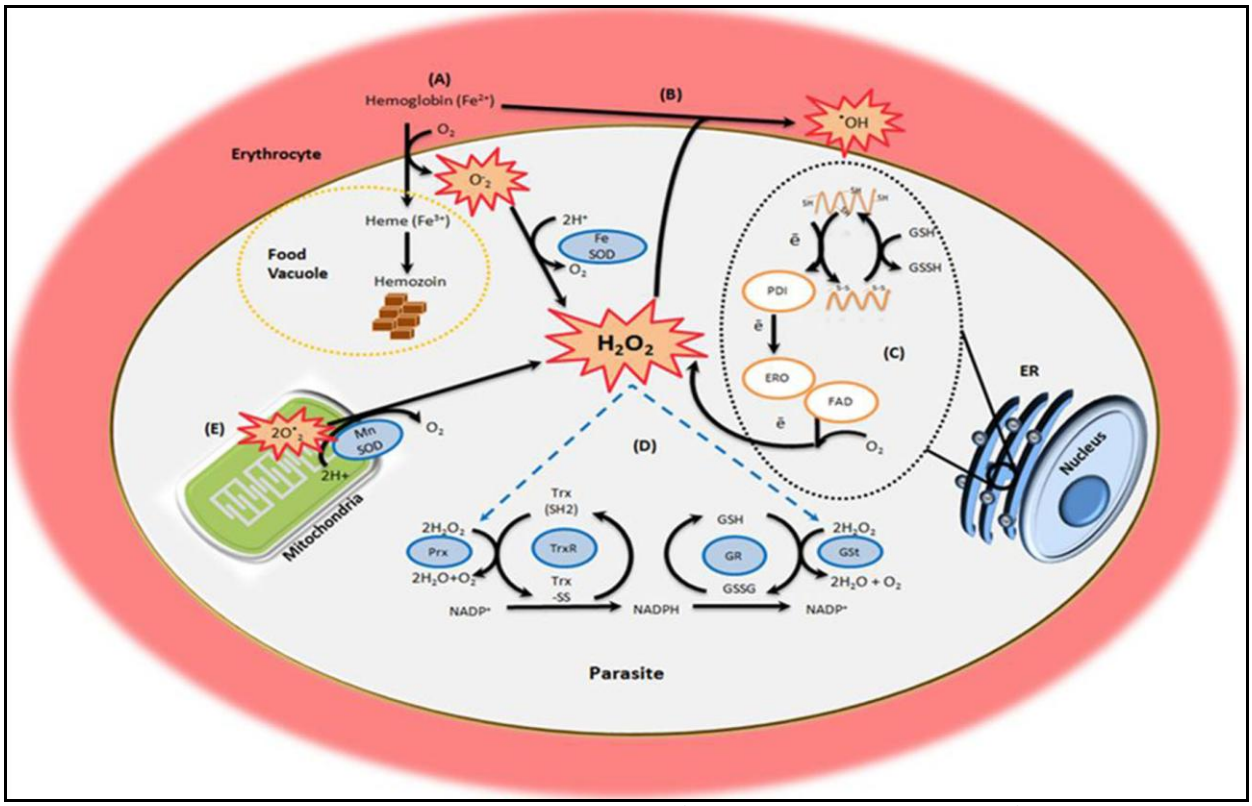

Figure 1. *Plasmodium* sp mechanisms to maintain the redox balance within infected erythrocytes: **(A)** Degradation of hemoglobin releases heme group that produces ROS in the food vacuole; **(B)** Heme group degradation causes the release of iron: which along with H_20_2 induces the production of •OH by the Fenton reaction; **(C)** Synthesis, folding and post-translational modifications of secretory proteins are oxidative processes that produce ROS in the endoplasmic reticulum (ER); **(D)** The effective and regulated antioxidant response which includes the Fe-SOD, Mn-SOD, peroxiredoxins, glutathione and thioredoxin systems maintain the redox balance.
[The nomenclature used in this figure is the same that in the text. This figure was originally prepared by Vianey Saldana-Navor.]

through an effective antioxidant response [61]. The dual glutathione/thioredoxin system maintains a reduced environment in the cytosolic milieu, which is crucial for its development inside the vector and during transmission. Studies in which the glutathione system was knocked-out showed reduced parasite development (reduced number of oocysts and a limited number of sporozoites in the mosquito salivary glands) [64, 65]. However, the fact that a decrease and not an absence of parasites was observed suggests that the participation of another antioxidant system (perhaps the thioredoxin and peroxidase system) may have countervailing functions. The disruption of TrxR [66], TPx-1 [67] and TPx-2 [66] resulted in parasites hypersensitive to ROS and RNS, and in some cases loss of viability. This suggests a degree of redundancy in the function of these antioxidant systems.

Antimalarial compounds and oxidative stress

Various antimalarial have been developed based on the disruption of the parasite redox balance to produce parasite death. A classification of various drugs according to the mechanisms involved in the modification of parasite oxidative balance is presented in Table 1. These are grouped in: **(1)** Compounds based on the accumulation of pro-oxidant molecules. In this group are those that interact with heme and inhibit hemozoin formation. These include the quinoles, 4-aminoquinolines such as chloroquine and amodiaquine and quinolinemethanols such as quinine and mefloquine. The efficacy of these compounds depends on their accumulation in the parasite food vacuole and affinity to heme. Terpene isonitrilies, diisocyanoadociane and axisonitrile-3 interact with heme through molecular bonds and inhibit its polymerization [68], and xanthones such as 2,3,4,5,6-pentahydroxyxanthone and 1,3,6,8-tetrahydroxyxanthone also bind to heme and their effect depends on the number of hydroxyl groups in their molecules [69]. Azole derivates also inhibit hemozoin formation, but are also selective inhibitors of the parasite lactate dehydrogenase (LDH), central to NAD^+ regeneration [70]. **(2)** Other compounds induce inhibition of antioxidant enzymes of the parasite and include those that inhibit TrxR such as Mannich bases, tertiary amides, nitro compounds, chalcone derivatives and those that inhibit the glutathione system like quinone derivatives, peroxynitrite and methylene blue [71]. **(3)** A third

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Table 1. Antimalarial compounds that induce oxidative stress

Inducing accumulation of pro-oxidant products: inhibiting hemozoin formation		Inhibiting antioxidant enzymes of parasite	Producing reactive oxygen species
Quinoline	**Nonquinoline**	Mannich bases*	
Chloroquine		Tertiary amides	
Mefloquine		Nitro compound*	
Pyronaridine		Chalcone derivative*	Pyrimethamine
Bisquinoline	Azoles derivatives	Eosin B*	Fluoromenadione
Amopyroquine	Xanthones derivatives	Naphthazarine derivative*	Curcumin
Mepacrine	Isonitriles derivatives	Isoxazole derivative (+)	Methylene blue
Quinine	(Aryl)aryl sulfanylmethylpyridines	Quinone derivatives (+)	
Amodiaquine		Peroxynitrite (+)	
Quinidine		Methylene blue (+)	

*Compounds that inhibit TrxR; (+) compounds that inhibit the glutathione system [72].

group comprises compounds that themselves produce ROS [72] such as pyrimethamine; this drug has multiple activities, interfering the synthesis of tetrahydrofolic acid inhibits DNA and RNA synthesis, but it also inhibits Fe-SOD, which in the parasite mitochondria catalyzes superoxide radicals [73]. On the other hand, the parasite depicts alternative means to compensate for oxidative stress and prevent its own damage, including the simultaneous participation of several parasite antioxidant mechanisms, such as glutathione, thioredoxin and peroxidases, and the use of antioxidant molecules recruited from the host. Furthermore, the pharmacological modification of the redox balance produces a selective pressure for the development of new parasite compensatory mechanisms and adaptation through antioxidant defenses.

Some anti-malarial compounds that inhibit the activity of the parasite antioxidant enzymes are effective in suppressing parasitaemia but they also inhibit human antioxidant enzymes [72] and cause undesirable damage to the host. Likewise, the high toxicity of ROS induced by other drugs may cause unselective damage to both parasites and hosts.

Whereas extensive efforts have been made to produce transmission-blocking compounds (directed at avoiding the infection of the vector) [74], no strategy has yet been explored aimed at modifying the redox balance within the mosquito vector. The latter strategy could lead to the development of novel drugs in the battle against malaria. In order to search for compounds that induce oxidative stress without affecting the host, it is necessary to have a better understanding of anti-oxidative responses mounted by the parasite within the apicoplast and mitochondrial metabolic pathways. The identification of specific active sites in enzymes that are involved in the synthesis and folding of parasite proteins, as well as those involved in the antioxidant defenses of the parasite (and that are distinct from those of the vertebrate host) could provide important insights for the pursuit of this strategy.

CONCLUSIONS

Plasmodium sp is exposed to different environmental stresses during its life cycle, making the maintenance of its redox balance of vital importance for survival. Overall, it seems that *Plasmodium sp.* is very sensitive to oxidative stress. Hence, it is of utmost importance to study the oxidative processes and the antioxidant enzymes of this parasite in order to understand the molecular mechanisms involved in the maintenance of its redox balance, which in turn could lead to the development of therapeutic targets and antiparasitic drugs, both in the vertebrate and mosquito hosts.

At present, most antimalarial compounds are based on oxidant attack, but they are increasingly inefficient due to the development of parasite resistance as well as their high toxicity and adverse effects for the vertebrate host. The search for new molecules targeting the antioxidant system and/or metabolism of the parasite, but that do not affect the host, offers opportunities for a renovated anti-malarial arsenal.

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COMPETING INTERESTS

The authors stated no conflicts of interest.

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