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Malaria and Reactive Oxygen Species: The Dance of Death

By

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Abbreviations

BSO, L-buthionine-S,R-sulfoximine
DCF-DA, 2'-7' dichlorodihydrofluorscein diacetate
DHE, dihydroethidium
ESR, electron spin resonance
FP IX, ferri/ferroprotoporphyrin IX
GCL, glutamate cysteine ligase
Grx, glutaredoxin
GSH, glutathione
GS, GSH synthetase GSSG, glutathione disulfide
HPLC, high performance liquid chromatography
NAC, N-acetylcysteine
RBC, red blood cells
SOD, superoxide dismutase
Trx, thioredoxin

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Abstract

Malaria is a serious, sometimes fatal, disease that mainly affects the warm regions of developing countries such as Africa, Southeast Asia, South and Central America. Malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted by the female *Anopheles* mosquitos when they feed on infected blood. *Plasmodium* parasites are highly sensitive to oxidative stress during their intraerythrocytic life stage as they consume haemoglobin inside their food vacuoles and generate toxic haem moieties and reactive oxygen species (ROS). Antimalarial drugs, such as chloroquine and artemisinin, treat malarial infection by increasing the oxidative stress inside the infected erythrocytes and thus killing the parasites. Furthermore, red blood cell (RBC) disorders like sickle cell anemia cause enhanced oxidative stress in *Plasmodium*-infected erythrocytes and confer a certain degree of resistance to malarial infection, suggesting that an increase in free radical production impairs the infection. Thus, a hypothesis is formulated that malaria parasites produce ROS as a necessary by-product of their metabolism and use the antioxidant defense systems to survive inside the host cell. Experiments designed to test this hypothesis are described and involve electron spin resonance (ESR) imaging and chemiluminescence assay for measurement of free radicals. Cell viability can be measured by Guava and Trypthan Blue dye exclusion assays. These experiments should help elucidate the potential role of ROS in malarial infection.

Introduction

I. Incidence of malaria: countries affected and death per year

Malaria is a serious disease caused by a parasite and is a leading cause of death worldwide. Symptoms of malaria include high fever, shaking chills, headaches, anemia and flu-like illness. The World Health Organization estimates that between 300 and 500 million people get infected with the malaria-carrying parasite, and between 1.5 and 2.5 million people die of malaria each year. Malaria is predominantly confined to developing countries, such as Africa, Southeast Asia, and South and Central America, where inadequate health care systems and poor socioeconomic conditions further exacerbate the spread of this disease [1].

II. Cause of malaria: four forms of malaria parasite

Protozoan parasites of the genus *Plasmodium* cause malaria. Four species of *Plasmodium* can infect humans, including *Plasmodium falciparum, plasmodium vivax, plasmodium ovale, and plasmodium malaria*. Of these four, *P. falciparum* is the most widespread and dangerous because it can cause fatal cerebral malaria if left untreated [1,2].

III. Transmission of malaria: from female anopheline mosquito to blood stage

Humans can contract malaria if bitten by a malaria-infected mosquito. The principal malaria mosquito is Anopheles gambiae [2]. Only female Anopheles mosquitoes transmit malaria parasite as they feed on blood while male mosquitoes feed on plant juices and do not transmit the disease. About 380 species of anopheline mosquito have been identified but only 60 species are able to transmit the disease [1].

When a mosquito bites a human, the malaria parasites (sporozoites) are injected into the blood and transferred to the liver, where they mature into schizonts (**Figure 1**). Schizonts rupture releasing merozoites, which then infect RBCs. The ring stage trophozoites can also mature into schizonts, which rupture and release merozoites. This blood stage of parasites is responsible for clinical manifestations of malaria. Some parasites enter a sexual erythrocytic stage and differentiate into gametocytes [1]. Female Anopheles mosquito ingests both male (microgametocytes) and female (macrogametocytes) gametocytes during its blood meal. Once inside a mosquito, the parasites multiply during their sporogonic cycle. Inside the stomach of the mosquito, the microgametes penetrate the macrogametes thus generating zygotes that mature into ookinetes. Ookinetes develop into oocysts, which grow, rupture, and release sporozoites. Sporozoites travel to the mosquito's salivary glands. When malaria mosquito inoculates the sporozoites into a new human host, the malaria life cycle is perpetuated [1,2].

IV. Treatment of malaria: antimalarial drugs

Previously, because malaria occurs mainly in tropical and subtropical regions of the world, it was thought that “miasma,” which literally means “bad air,” caused the disease [3]. The “miasma” theory was proved wrong in 1889 when Charles Louis Alphonse Laveran discovered the malaria parasite, and in 1897 when the mosquito was identified as the vector of malaria [3]. These findings inspired an eradication campaign against malaria. The campaign was started in 1950s but it failed due to numerous problems, including the development of resistance by mosquitoes to insecticides used to kill them and by malaria parasites to drugs used to treat them. Furthermore, the eradication campaign never extended to Africa, where malaria is the most common [1].

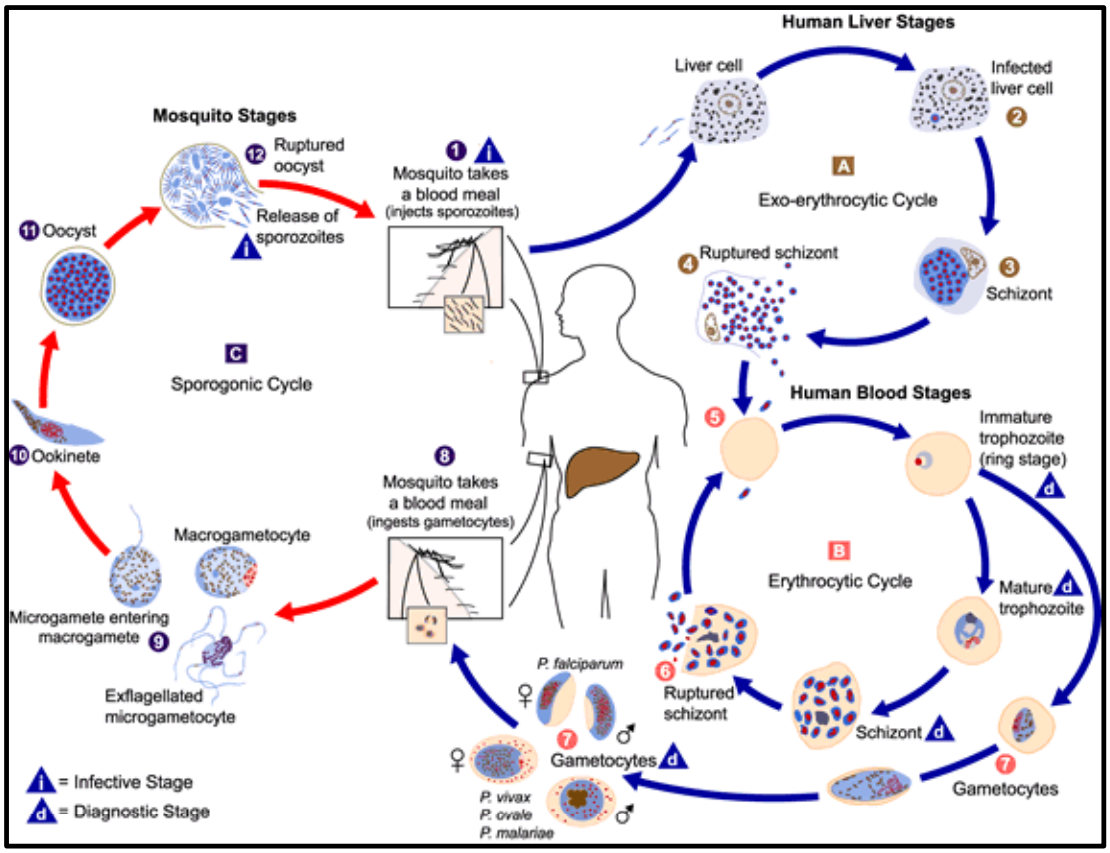

Figure 1. *Plasmodium* life cycle within a mosquito and a human [3].

Many drugs used to treat malaria exert their function primarily by increasing oxidative stress in parasite-infected erythrocytes. Antimalarial drugs include chloroquine, primaquine, artemisinin and xanthones [4]. The ability of antimalarial drugs to alter the oxidative status of parasitized host erythrocytes suggests that free radicals play an important role in malaria pathogenesis.

Malarial Infection and Oxidative Stress

I. Malaria parasite vulnerability to oxidative stress during its intraerythrocytic life

Like all aerobic organisms, malaria parasites generate reactive oxygen species (ROS) as by-products of their metabolism. Because of their high metabolic rate as the rapidly growing and

constantly multiplying parasites, *Plasmodium* parasites generate very large quantities of toxic redox-active metabolites, which can lead to oxidative stress [4]. Previous studies have shown that malaria parasites are most sensitive to oxidative stress during their erythrocytic life stages [5,6]. This is not surprising since the host cells also produce ROS, thus adding to the overall burden of the parasites [7]. Malaria parasites live in ROS-rich environment that contains the key prerequisites for formation of ROS via the Fenton reaction (Reaction 1 and 2) [4, 7]:

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \qquad \text{(Reaction 1)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \qquad \text{(Reaction 2)}$$

Haemoglobin is a major source of amino acids for malaria parasites. Ingestion of haemoglobin into the acidic food vacuole of the parasite leads to the spontaneous oxidation of the Fe^{2+} to Fe^{3+} and formation of superoxide ($O_2^{\bullet -}$) anion (**Figure 2**).

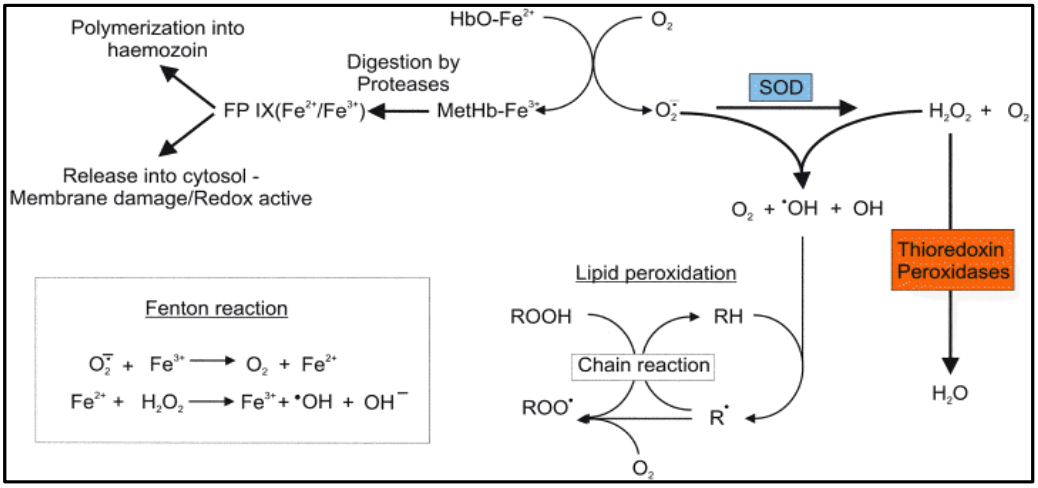

Figure 2. Sources of ROS in *Plasmodium* [7].

This reaction leads to the production of toxic oxygen intermediates, hydrogen peroxide and hydroxyl radicals [8]. In addition, haemoglobin is degraded resulting in the release of free haem ferri/ferroprotoporphyrin IX (FP IX). FP IX is highly toxic and needs to be detoxified. Up to 90% of FP IX is detoxified through biomineralization [9] but an appreciable amount of FP IX

can escape this transformation to inert haemozoin and has to be degraded by other means, such as binding to FP IX-binding proteins (histidine-rich proteins or HRP) or reacting with glutathione (GSH) [4,10,11]. If FP IX avoids neutralization it can cause membrane damage and parasite death [11]. It is these haem moieties and ROS that are targeted by antimalarial drugs (**Figure 3**). Chloroquine, for instance, functions by inhibiting FP IX detoxification. Primaquine and artemisinin are thought to react with haem moieties forming cytotoxic radicals. Xanthones interferre with haemozoin formation [4].

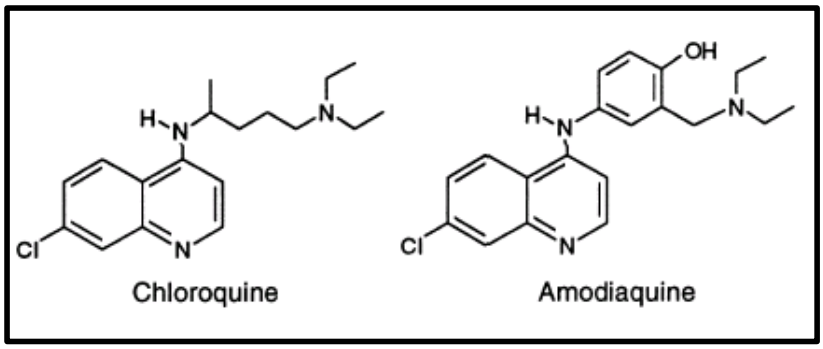

Figure 3. Structure of antimalarial drugs chloroquine and amodiaquine [10].

II. Antioxidant systems of the malaria parasite

In order to combat oxidative stress, malaria parasites are equipped with antioxidant defense systems, including GSH, superoxide dismutase (SOD), thioredoxin (Trx) and glutaredoxin (Grx) [4,7]. GSH is a tripeptide (γ-glutamyl-cysteinyl-glycine) and a major thiol antioxidant found in almost all aerobic cells. GSH cycles between its oxidized and its reduced state while it detoxifies hydroperoxides in concert with glutathione peroxidase using the reducing equivalents in NADPH and glutathione reductase. In the absence of oxidative stress, 90-95% of GSH is in its reduced state. In response to a stress, a cell undergoes enzymatic reactions that consume GSH by forming GSH conjugates with a great variety of electrophilic compounds or by forming

GSSG. GSH content can be maintained through *de novo* synthesis or through the enzymatic reduction of its disulfide form, GSSG, using the glutathione reductase/NADPH system [12]. *De novo* synthesis of GSH involves two enzymatically-controlled reactions. In the first, rate limiting reaction, glutamate and cysteine are combined by the enzyme glutamate cysteine ligase (GCL) to form γ-glutamylcysteine. In the second reaction, GSH synthetase (GS) catalyzes the formation of GSH by combining γ–glutamylcysteine and glycine (**Figure 4**). L-buthionine-S,R-sulfoximine (BSO) is a specific and potent inhibitor of GCL and thus GSH synthesis [4, 12].

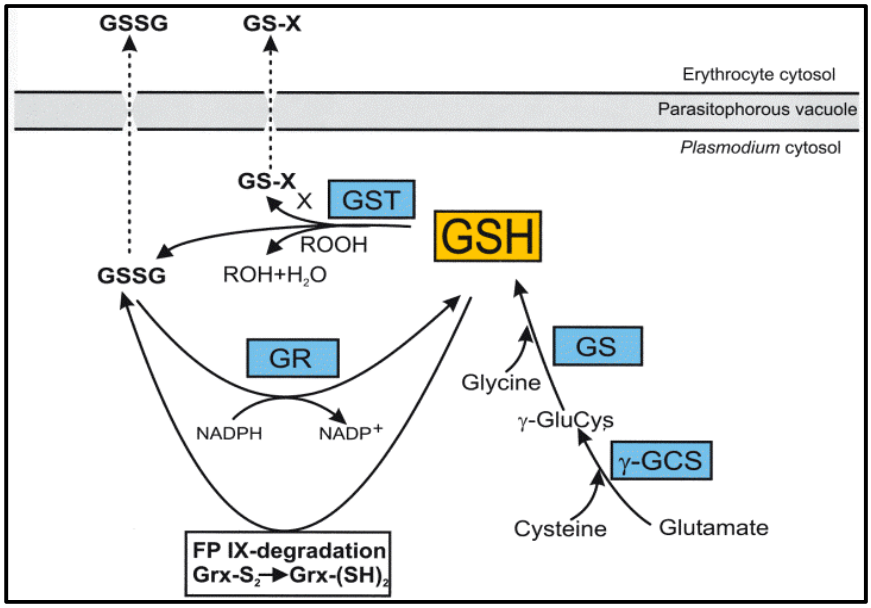

Figure 4. The GSH metabolism of *Plasmodium* [7].

The glutathione metabolism of erythrocytes infected with *Plasmodium* parasites has been studied extensively [4,7,11,12]. These studies have shown that, in addition to its role as an antioxidant and thiol redox buffer, GSH is involved in antimalarial drug resistance [4]. GSH functions as both a cofactor for enzymatic reactions and as a source of reductive detoxification of FP IX [1,

13]. *Plasmodium* parasites themselves have also been shown to possess the genes for GCL and GS, suggesting a functional glutathione synthesis pathway [14,15].

SODs are metalloproteins. Malaria parasites appear to contain SODs with only iron (Fe) as the metal cofactor [4]. All four species, *P. falciparum, P. ovale, P. malariae and P. vivax* possess a gene that encodes a Fe-dependent SOD termed SOD-1 [7]. SOD-1 is a cytosolic protein that is expressed throughout the erythrocytic life cycle of the parasite. Because SOD-1 is a cytosolic protein, it is not likely to act on superoxide anions generated during haemoglobin digestion in the parasite's food vacuole [7,12]. It seems more likely that the parasites take up Cu/Zn-SOD from host erythrocytes to remove superoxide anions or that superoxide dismutates spontaneously inside the food vacuole due to the acidic environment. There is also a mitochondrial SOD, SOD-2, but it is only found in *P. falciparum* [7]. Since malaria parasites clearly possess an active respiratory chain it is essential for SOD-2 to remove the superoxide anions that have leaked from the chain and, in doing so, prevent damage to the parasite [7].

Trx and Grx are the other major antioxidant systems present in *Plasmodium* parasites. Trx and Grx are characterized by a specific active site CXXC motif that allows them to act as redox messengers by interacting with various redox-active proteins and metabolites, such as transcription factors, ribonucleotide reductase and low molecular weight thiols [7]. *Plasmodium* parasites also contain a thioredoxin superfamily member unique only to them called plasmoredoxin [18]. Plasmoredoxin is thought to function by reducing ribonucleotide reductase [18]. Trx especially seems to be essential for parasite survival because knockout studies of thioredoxin reductase have proven to be lethal for *P. falciparum* [7].

III. The host erythrocytes and oxidative damage

Plasmodium parasites use their antioxidant proteins to defend against ROS generated as by-products of their own metabolism but also to detoxify ROS produced by the host immune system. RBCs infected with malaria parasites undergo various oxidative alterations as evidenced by changes in erythrocyte membrane fluidity [4,7]. Loss of membrane fluidity is thought to be caused by erythrocyte membrane lipid decomposition and protein cross-linking due to excessive ROS accumulation. Parker and colleagues [19] demonstrated that haemichrome accumulation and aggregation of erythrocyte band 3 occur on the inner surface of the infected RBCs, suggesting that the host erythrocytes are severely damaged by oxidative stress. These modifications resembled those observed in RBCs of humans with RBC disorders, such as sickle cell anemia and glucose-6-phosphate dehydrogenase deficiency [7].

It was proposed that enhanced oxidative stress was responsible for these transformations. Surprisingly, RBC disorders and glucose-6-phosphate dehydrogenase deficiency limited the severity of malarial infection by conferring a degree of resistance to *Plasmodium* [7]. Two theories were proposed to explain this apparent paradox. According to one theory, enhanced oxidative stress within the parasitized erythrocytes slows the growth rate of parasites and impairs the infection. Alternatively, malaria-infected erythrocytes are detected by the host immune system at an early stage of the infection so that oxidative stress is kept low and parasitemia under control [4]. Both theories implicate ROS as the two-edged sword for *Plasmodium* parasites. They inevitably produce ROS during normal aerobic respiration yet are highly sensitive to increased ROS production, including ROS generated by the infected host cells. It is likely that *Plasmodium* parasites are sensitive to increased oxidative stress because they are not fully

equipped to combat excessive free radical production since they lack the antioxidant redox systems used by higher organisms. Based on these observations it was possible to formulate the hypothesis on the relationship between ROS and malaria.

Hypothesis

Malarial infection does not involve ROS species. Malaria parasite produces ROS as a necessary by-product of its metabolism and it uses the antioxidant enzyme systems to survive inside the host erythrocytes.

Experimental Set Up: Part A

Question: Does malarial infection involve ROS?

To prove or disprove the hypothesis that malarial infection does not involve ROS, basic tests need to be performed. In the first experiment, a mouse model will be used to examine free radical production. A mouse is a valid model for the study of malarial infection because a mouse infected with the murine version of malaria, *P. yoleii*, reacts to the parasites the way humans react to *P. falciparum* parasites. To set up the first experiment, mice will be inoculated with *P. yoleii* and either treated with chloroquine or left untreated. Healthy mice will be used as the controls. In the second experiment, erythrocytes will be isolated from healthy human donors using immunomagnetic technique and infected with *P. falciparum* (clone 3D7) only or infected with *P. falciparum* (clone 3D7) and treated with chloroquine. Healthy human erythrocytes will be set up as the controls.

Free radical production will be examined in both murine and human model using electron spin resonance (ESR) imaging and chemiluminescence. ESR imaging has been used previously when measuring free radicals in murine heart [13] and rat RBCs (**Figure 5**) [14].

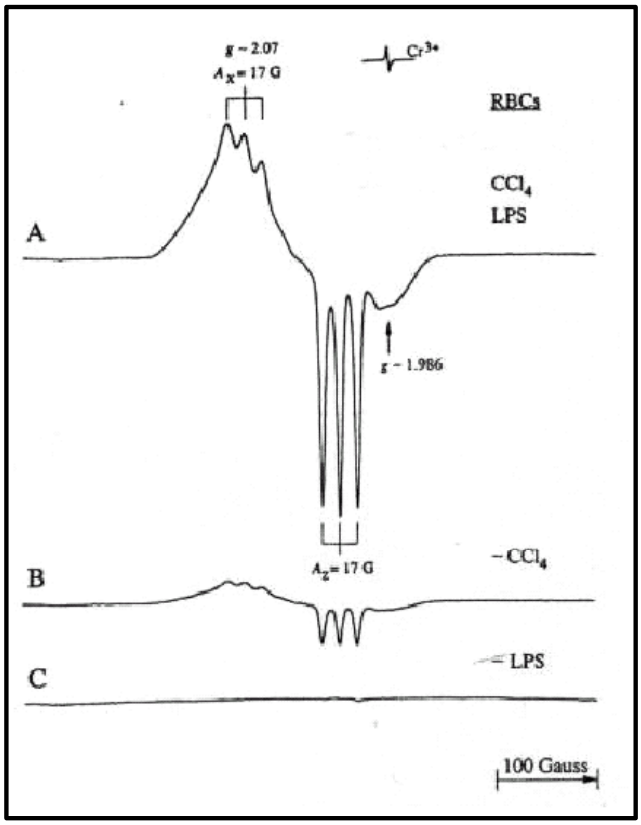

Figure 5. EPR spectra measured at 77K from rat RBCs. A) Rat injected with CCl_4 followed by an activating factor 20 hours later. The spectrum was taken 4 hours after the second injection. B) Activating factor replaced with saline. Spectrometer conditions were as follows: modulation amplitude, 4 G, Microwave power 10 mW, time constant 1.3 sec, scan rate 49 G/min. Adapted from [11].

When examining free radical content in rats after inducing endotoxic shock with carbon tetrachloride (CCl_4), Tilley and colleages observed signals at g = 2.5, 2.3, and 1.9 [11]. These signals were identified as different breakdown products of cytochrome P420 (**Figure 5**). In this study, ESR technique will be used to measure free radicals in murine spleen, liver, and blood, as well as in human erythrocytes. Furthermore, two fluorescent markers of cellular oxidant

production, 2'-7' dichlorodihydrofluorscein diacetate (DCF-DA) and dihydroethidium (DHE) [13] will be used to examine free radical production in the same samples. DCF-DA and DHE are freely diffusible and are oxidized to their fluorescent derivatives by various oxidant species. Cells generating ROS become fluorescent. These chemiluminescence assays are nonspecific but provide a general indicator of oxidation [13].

If the proposed hypothesis is correct, the control mice and human erythrocytes should have low levels of free radicals because they have intact cells with functioning antioxidant systems to maintain the redox equilibrium. In the presence of *P. yoleii*, murine hepatocytes, splenocytes and RBCs should have higher levels of free radicals due to malaria parasites invasion. In the presence of *P. falciparum*, human erythrocytes should also have an increased free radical content due to the infection. Finally, murine and human cells infected with *P. yoleii* and *P. falciparum*, respectively, should have several fold higher free radical levels as chloroquine prevents detoxification of FP IX and ROS. If the results obtained from this set of experiments differ from the results described in this paper, then the hypothesis is most likely incorrect.

Experimental Set Up: Part B

Question: Does inhibition of the antioxidants, such as GSH, impair the survival of the malaria-infected erythrocytes?

To determine if inhibition of antioxidant GSH affects the survival of parasitized erythrocytes, human erythrocytes will be treated with BSO to deplete GSH or N-acetylcysteine (NAC) to enhance cysteine levels for GSH synthesis. Next, human erythrocytes will be infected with *P. falciparum* (clone 3D7) and the infected cells treated with BSO or NAC or left untreated.

Healthy human erythrocytes will be used as the controls. After the indicated treatment, erythrocyte viability will be examined in all the samples using Guava dye exclusion assay or Trypthan Blue stain. These assays distinguish between viable and non-viable cells based on the differential permeability of the dyes. Non-viable cells take up the dyes while viable cells do not. Both assays provide rapid and reliable determinations of viability and total cell count.

If the proposed hypothesis is correct, vast majority (>90%) of the control cells should be viable. BSO-treated erythrocytes that have not been infected with *P. falciparum* should have a slightly lower percent of viable cells but not significantly lower. The infected erythrocytes treated with BSO should have a significantly lower percent of viable cells. It is estimated that greater than 70% of malaria-infected erythrocytes will be killed in the presence of BSO because GSH will not be available to detoxify any FP IX that escapes incorporation into haemozoin or to act as a redox buffer. Erythrocytes, whether infected with *P. falciparum* or not, would be as viable as the controls in the presence of NAC because NAC acts as a GSH precursor and replenishes GSH pools. Parasite viability can also be examined with Giemsa stain and analyzed by confocal microscopy. This technique should confirm that viable cells contain live malaria parasites while dead cells do not.

Once cell viability has been established, GSH pools will be measured in all these samples using high performance liquid chromatography (HPLC) because it is important to demonstrate that BSO effectively depletes and NAC restores GSH pools. Also, it is of interest to compare the levels of GSH in healthy and malaria-infected cells that have been cultured with BSO. Previously, *Plasmodium*-infected erythrocytes were shown to contain approximately half the

GSH levels of non-infected cells presumably due to the high efflux rate of GSSG from the infected cells [[14,15]. In the past, GSH levels have been measured successfully using the HPLC method [4,7]. Normally, standard thiols, such as GSH, NAC and L-cysteine, are used to generate a standard curve, which is then used to analyze the retention times of experimental samples and identify those samples. Samples are normalized to protein levels.

If this paper hypothesis is correct, BSO-treated infected and non-infected erythrocytes should have significantly lower levels of GSH compared to the controls. Non-infected erythrocytes will probably have a slightly higher GSH pools than infected erythrocytes due to the absence of *P. falciparum* and a decreased efflux rate of GSSG. In contrast, NAC should replenish GSH levels in both infected and non-infected BSO-treated cells. In fact, GSH in NAC-treated cells should be higher or equal to GSH inside the control cells depending on the amount of NAC used for this experiment. Alternative results could be obtained instead of those described above. NAC could act directly as a thiol antioxidant and replace GSH. In such a case, GSH levels would remain low in NAC-treated *Plasmodium*-infected cells majority of which should still be viable.

Conclusions

Malarial infection is caused by *Plasmodium* parasites with the *Anopheles* mosquito as the vector of the disease. The complex life cycle of malaria probably does not involve ROS species, which are produced by the parasites as a necessary by-product of metabolism. *Plasmodium* most likely uses its antioxidant defenses to survive inside the host erythrocytes. This hypothesis can be tested using ESR imaging and chemiluminescence to measure free radical content and HPLC to measure GSH levels. Also, cell viability can be analyzed using the Guava and Trypthan Blue

dye exclusion assays. These experiments would help one better understand if any relationship exists between ROS and the malaria pathogenesis.

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