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Review Article

Oxidative Stress in *Plasmodium:* Role of Glutathione Revisited

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ABSTRACT:
Malaria is still one of the three leading infectious disease in the world. *Plasmodium,* unicellular eukaryotic parasite responsible for the disease is under immense oxidative stress during the erythrocytic stages of its life cycle. The parasite overcomes the oxidative stress generated endogenously and by host immune system through its antioxidant and redox systems. *Plasmodium* possesses glutathione and thioredoxin redox systems with overlapping but distinct functions that help it to maintain redox state. Glutathione is the most abundant low molecular weight thiol redox buffer in all living cells that is detrimental for the maintenance of intracellular redox status. Glutathione functions as an antioxidant protecting cells against the deleterious effects of oxidant free radicals and also in detoxification process reactions in parasite. Interfering with glutathione redox system of parasite can be a novel way to combat the disease. The present review describes the recent findings in role and mechanism of glutathione in maintaining the redox status during oxidative stress in infection with *Plasmodium*

Keywords: Malaria, *Plasmodium*, Glutathione, Antioxidant, Oxidative stress.

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INTRODUCTION

Malaria has been a scourge of humanity since antiquityandremains soeventoday. It is one of the major public health problems recognized by World health Organisation (WHO) globally that is leading cause of morbidity and mortality in developing nations. According to WHOs world malaria report 2020 there were 241 million cases and 6,27,000 malaria deaths in world in year 2020 an increase from 227million cases in year 2019. Sub Saharan African countries carry the maximum malaria burden accounting for 95% of all reported cases and 96% of malaria deaths in 2020 (WHO 2020). The greatest burden of this disease caused by apicomplexean protozoan parasite *Plasmodium* is in the tropical and sub-tropical countries of the world. Malaria is a re-emerging life-threatening disease and due to emergence of resistance to antimalarials and non-availability of any potent vaccine and limited chemotherapeutic alternatives there is a dire need to elucidate the biochemical makeup of malaria parasite and identify and validate new potential drug targets in the parasites metabolism to develop new antimalarials and vaccine.

Oxidative stress is defined as a disturbance or imbalance that occurs in a cell or tissue when there is production and accumulation of reactive oxygen species (ROS). In a normal healthy cell balance is maintained between compounds (pro-oxidants) capable of producing harmful reeradicals and the compounds (antioxidants) that absorb or scavenge these free radicals. The oxidant-free radicals are independent existence species that possess one or more unpaired electrons, have a very short half-life, are highly reactive and exhibit damaging property towards macromolecules like conformational modifications in proteins that also functions as enzymes, lesions in deoxyribonucleic acid and in lipids causing their peroxidation which is a radical chain reaction that spreads rapidly affecting other lipids. These ROS that perform several physiological roles like cell signaling are generated as by-products of oxygen metabolism, environmental factors like UV rays, ionizing radiations, pollutants and xenobiotics (Pizzino et al., 2017). Theses ROS include O_2 (superoxide), OH^- (hydroxyl), HO_2 (hydroperoxyl), ROOC (peroxyl) as free radicals and H_2O_2, O_3 and O_2 (singlet oxygen) as non-radicals or nitrogen (N_2) derived species (RNS)mainly NO (nitric oxide), ONOO-(peroxynitrite), NO_2 (nitrogendioxide).

During oxidative stress when production of ROS increases or when the levels of antioxidants decreases there is serious cell damage if this stress is prolonged and massive. Oxidative stress and free radicals have been implicated with different degree of implication in onset and progression of many diseases like cancer, cardiovascular diseases and diabetes (Taniyama and Griendling 2003). Antioxidants play an important role in scavenging these reactive species by terminating the chain reaction of free radical formation by donating an electron to eliminate the unpaired condition of these species before they damage the cell and its structure like proteins, nucleic acid and lipids. Body maintain an antioxidant system by deploying certain enzymes called antioxidant enzymes that provide an important defense against free radicals. Glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR), thioredoxin reductase (TrxR), superoxide dismutase (SOD) are the most important ones. Cells also deploy a number of non-enzymatic antioxidants like vitamin E (tocopherol), vitamin C (ascorbate), and tripeptide glutathione (GSH) to protect themselves from ROS induced cellular damage. Glutathione besides acting as a cofactor for a number of enzymes also reduces ROS non-enzymatically and behave as a reductant for vitamin E and vitamin C.

OXIDATIVE STRESS IN MALARIA

Host parasite interactions are complex and still not completely understood. A delicate balance between pro-oxidants and antioxidant molecules exist between the parasite and its host as both is capable of producing reactive species. Oxidative stress and its role and mechanism during *Plasmodium* infection remains unclear even today. Oxidative stress generated by the production of free radicals have been proposed to play an important role in physiopathogenesis of malaria. *Plasmodium* is exposed to oxidative stress during its intraerythrocytic development and is highly sensitive to such stress being inimical for its growth and survival (Hunt and Stocker 1990, Becker et al., 2004). This involvement could be due to pathogenic mechanism triggered by the parasite, production of free radicals or hosts immune response to ward off the infection. *Plasmodium* infection induces activation of host immune defence mechanism causing respiratory burst involving macrophages and neutrophils that generate ROS and RNS. Over production of these radical species constitute the state of oxidative stress as a response from the host towards infection and in case of malaria can lead to destruction of *Plasmodium* parasite. In vitro studies showing killing of *Plasmodium yoeli* upon incubation with glucose and glucose oxidase by production of H_2O_2 and by superoxide O_{2^-} produced upon incubating *P. yeoli* with xanthin and xanthin oxidase demonstrate destruction of *Plasmodium* due to oxidative stress. Increased malondialdehyde (MDA) an important lipid peroxidation marker along-with other oxidative stress markers have been found in high levels in infected humans and rats as compared to normal controls suggesting increased production of free radical species (Sohail et al 2007, Guha et al 2006, Sobolewski et al 2005). Large amounts of toxic metabolites are generated by the parasite due to its high metabolic rate and rapid propagation leading to oxidative stress during erythrocytic

schizogony. *Plasmodium* living in ROS-rich environment require iron and oxygen for the formation of ROS via Fenton reaction (Liochev and Fridovich 1999).

Guha et al, (2006) showed that malaria infection induces hepatic apoptosis through augmentation of oxidative stress via mitochondrial pathway. Immune mechanism in malaria is not fully understood but it is generally accepted that free radical species kill the intraerythrocytic malaria parasite (Brunnet 2001, Clark and Cowden 2003). The parasite killing occurs by the production of ROS by phagocytes like monocytes and production of interferon gamma (IFNγ), TNF-α and IL-12 by Th1 cells that activate macrophages to secrete parasiticidal NO and ROS (Taylor et al 1993). Potter et al., (2005) has suggested that phagocyte derived ROS were not crucial for the clearance of malaria parasite, at-least in murine models but the sensitivity of parasite towards the oxidative stress can still be a critical factor for its survival. ROS generating systems are known to kill murine malaria parasites and *P. falciparum* (Berman et al, 1991, Marva et al, 1991). Moreover, the importance of ROS/RNS has been established in elimination of parasite as most of anti-malarial drugs act by the mechanism in which these species participate. These reactive species are also known to regulate immune responses either by stimulating or inhibiting the production of certain cytokines, transcription factors or even regulating cell death processes (Arruda et al 2004).

The role of antioxidants and oxidative stress in pathogenesis of malaria in humans remain unclear with some authors suggesting a protective role, whereas others suggesting a relation to physiopathology of the disease (Sohail et al 2007). Recent studies have suggested a crucial role of ROS/RNS produced as a result of oxidative stress in development of systemic complications caused by malaria. Atmana and Ginsberg (1993) have reported twice the amount of H_2O_2 and OH radical's production in *P. falciparum* infected RBCs as compared to normal erythrocytes. During oxidative stress functional and structural changes occur in plasma membrane of infected RBC due to lipid peroxidation that causes haemolysis, which has been linked to increased levels of thiobarbituric acid reactive substances (TBARS), which have been considered as index for lipid peroxidation. Chandra et al., (2006) reported an increase in TBRAS in P. falciparum infected individuals, while levels of antioxidant vitamins E and C were decreased.

Malaria parasite is enclosed inside parasitophorous vacuole and is surrounded by haemoglobin (Hb) inside host RBC. The parasite obtains its nourishment of amino acids by degrading Hb. Ingestion of Hb into acidic food vacuole of parasite leads to spontaneous oxidation of Fe2+ to Fe3+ with the formation of superoxide anions (O2-). This reaction leads to the formation of toxic O2 intermediates, H_2O_2 and hydroxyl radicals, thereby increasing the oxidative burden. Free heme is a powerful free radical generator, which is harmful to both host and parasite causing morphological and molecular damages. Fe^{2+} contained in heme group can catalyse Fenton and Haber Weiss reactions that generate free radicals (Fig. 1).

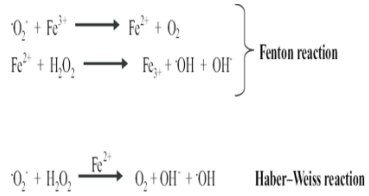

Figure 1:

Ferriprotoporphyrin IX (FP IX) which is released upon Hb degradation is toxic to *Plasmodium* (Banyal and Fitch 1982). Toxic FP IX can cause parasite death or damage to the membrane if it is not neutralized as *Plasmodium* does not possess haemoxygenase (Tilley et al, 2001). The parasite protects itself from the effects of toxic FP IX that latter is detoxified through biomineralization. Binding of FP IX to a substance similar to protein results in the formation of haemozoin. FP IX binding protein appears to be histidine rich protein or FP IX may also react with glutathione (GSH) (Atamna and Ginsburg 1993). Famin and Ginsburg (2003) identified a number of other FP IX binding enzymes in the parasite e.g., 6-phosphogluconate dehydrogenase, aldolase, lactate dehydrogenase and glyceraldehyde-3-

phosphate dehydrogenase. These heme moieties and ROS are targeted by chloroquine that prevents the polymerization of haeme to haemozoin, promoting accumulation of free haeme. Chloroquine thus increases the availability of intracellular haeme by disrupting plasma membrane structure and increasing oxidative stress in *Plasmodium*. Also, cellular response to haemozoin entails release of cytokine like TNF-α and IL-1 and generation of ROS like NO. Research on heme/haemozoin induced oxidative stress is exciting and opens new vista in development of new antimalarials. Sobolewski et al., (2005) reported that haemoglobin protects *Plasmodium* from ROS, but the parasite possibly possesses intrinsic defence mechanism against it. The parasite evades ROS as a consequence of ROS quenching by Hb, an antioxidant mechanism that has been overlooked (Alayash 2004). The oxidation process of haeme group is controlled within RBCs by methaemoglobin reductase system. Antioxidants or their associated enzymes have been implicated to play a role in preventing or reducing the formation of methaemoglobin in murine host and thereby in preventing the host from methaemoglobinemia during rodent malaria infection (Srivastava et al., 2001).

EFFECT UPON HOST

During its development inside host RBCs not only does the *Plasmodium* parasite exhibit structural changes but the host cells also undergo alterations that support the survival and propagation of the parasite. Development of parasite inside RBCs causes structural changes on erythrocyte surface that increases its viscosity and increases its adhesion to endothelial walls of capillaries thus avoiding its entry into spleen via circulation and preventing its destruction by immune cells present in blood and spleen, a defence mechanism adopted by parasite. There is change in erythrocyte membrane fluidity due to alteration in erythrocyte lipid composition and protein cross linking. Additionally, there is accumulation of erythrocyte band 3 protein, which forms skeletal multi-protein complex with lipid and proteins that confer mechanical integrity and viscoelasticity to RBCs to withstand sheer forces and squeeze through capillaries and also to involve in the attachment of *P. falciparum*-infected erythrocytes to endothelial cells in tissues (Winoqrad and Sherman 2004). This increased viscosity of RBCs is responsible for blocking of finer blood vessels in organs like kidney, lungs and brain causing cerebral malaria which causes maximum mortality in children infected with *P. falciparum* (Phiri et al, 2009). Other changes induced by *Plasmodium* on the surface of erythrocyte include lipid peroxidation of erythrocytes containing large amounts of polyenoic fatty acids (OH-PUFA) like 12 and 15-hydroxy-arachidonic acid (HETE) (Schwarzer et al 2003). This increased lipid peroxidation and oxidative stress affects membranes of parasitized erythrocytes making them stiff and rigid which are subsequently removed in spleen during circulation that further increases anaemia, a characteristic feature of malaria as *P. falciparum* infection accelerates aging of these cells and contribute to development of anaemia (Omodeo-Sale et al 2003). Histidine rich protein and 3-erythrocyte membrane protein of *P. falciparum* (PfEMP3) are the other important proteins that promote increased membrane stiffness in parasitized erythrocytes. Expression of *var* gene of *P. falciparum* produces protein *Plasmodium falciparum* membrane protein 1 (PfEMP1) that is expressed on surface of RBC that is responsible for cytoadherence of RBC by which it is able to connect to different host molecules located on vascular endothelium like intercellular adhesion molecule type 1 (ICAM1), platelet endothelial cell adhesion molecule (PECAM), hyaluronic acid and others which clog blood vessels and obstruct blood flow (Petterson et al 2005).

ANTIOXIDANTS IN *PLASMODIUM*

The nightmare for the parasite residing inside RBCs is ROS which are produced not only by the host in response to infection but also generated by parasite itself that interferes with physiology of RBCs and promote or felicitate its internalization in RBCs and hepatocytes. Aerobic respiration transport mechanism being the major source of free radical ROS/RNS generation in *Plasmodium* (Percario et al, 2012). The parasite is known to possess its own NADPH generating hexose monophosphate shunt pathway which wards off the damages caused by parasite's ROS or the host RBCs and immune cells (Atamana et

al, 1994). Also to avoid the oxidative stress the parasite has adapted to anaerobic mode of life style as it lacs catalases and glutathione peroxidase. *Plasmodium* protects itself against oxidative stress by a number of host or parasite encoded enzymes, vitamin C and E and proteins like glutathione and thioredoxin. Additionally, the parasite has adopted new mechanisms like apicoplast mechanism along with reduction in its own production of ROS to prevent oxidative damage arising from the host.

GLUTATHIONE

Glutathione is generally referred to tripeptide L-gamma-glutamyl-l-cystenylglyceine in both reduced and dimeric forms. Monomeric glutathione is known as reduced glutathione (GSH) and the dimeric form as oxidised glutathione, glutathione disulphide or di-glutathione (GSSG). Glutathione is the most abundant low molecular weight thiol redox buffer in all living cells and therefore, detrimental for maintenance of intracellular redox status. Various pathways involving biosynthesis of leukotrienes, proteins and nucleic acids depend on glutathione metabolism (Reed 1990). Liver is the net synthesiser of circulating GSH and organs like kidney salavage GSH through γ-glutamyl transpeptidase reaction (Denke and Fanburg 1989). The ratio between GSH and GSSG is maintained more towards reduced form of glutathione inside the cell mainly by the action of glutathione reductase (GR), which utilises NADPH as a cofactor supplying the necessary reducing equivalents. GSSG efflux pump exports excess GSSG to maintain the intracellular redox balance and the high levels of GSH are maintained by *de novo* synthesis (Griffith 1999). GSH functions as a general redox thiol buffer and a cofactor for a variety of proteins including glutathione-S-transferases (GSTs) and glutathione dependent peroxidases (Sies 1999). Glutathione protects the cell against the deleterious effects of oxidant-free radicals and pro-oxidants-drugs, whereas reduced GSH plays an important role in detoxification of FP IX. *Plasmodium* possesses a number of GSH dependent enzymes systems and is also important for detoxification and thiol disulphide exchange reactions.

Srivastava and Beutler (1969) have reported an increase in GSH turnover with accumulation of GSSG during oxidative stress and controlled through the ATP dependent transport. To replenish the lost glutathione, RBCs synthesise GSH from amino acids Glu, Cys, and Gly through the activity of gamma glutamyl cysteine synthetase(a rate limiting step that catalyses the ligation of L-glutamate and L-cysteine) and glutathione synthetase. Majority of GSH is consumed in the reaction catalysed by GST for the detoxification of xenobiotics and in the removal of toxic metabolic products. The loss of GSH is recovered by two step reaction mechanism from amino acids glutamate, cysteine, and glycine. The first rate limiting step is ligation of glutamate and cysteine by gamma glutamyl cysteine synthetase (γGCS) followed by the reaction of glutathione synthetase (GS) adding glycine and resulting in GSH (Meierjohann et al, 2002).

Enzyme glutathione reductase (E.C. 1.6.4.2) is responsible for keeping glutathione in its reduced state. It belongs to the pyridine-nucleotide disulphide oxidoreductase family of homodimeric flavoenzymes that also includes thioredoxin reductase and lipoamide dehydrogenase. Both human GR and *Plasmodium* GR are essential for the survival of malaria parasite inside the human erythrocytes (Gilberger et al, 2000). Plasmodial GR has been considered a potential therapeutic target for antimalarials and the difference in structure between human GR and *Plasmodium* GR may allow the design of inhibitors that specifically bind to parasite GR. The knowledge of three dimensional structure of human and Plasmodial GR will facilitate inhibitor formulation that specifically target the parasite protein. Methylene blue is one such compound known to be specific inhibitor of parasite GR (Becker et al, 2004). Marozine *et.al*., 2019 reported that nitroaromatic compounds non- or uncompetitively inhibited *Plasmodium falciparum*GR. During malaria infection the activity of GR increased in rodent RBCs parasitized with *P. yeolii*. Our studies also show increased GR activity in rodent RBCs parasitized with *P. berghei* infection (Kapoor and Banyal 2009). Activity of GR was higher *in P. falciparum* parasitized RBCs compared to non-infected RBCs in patients showing failure of therapeutic response to amodiaquine (Zuluaga et al, 2007).

Parasitized erythrocytes are under enhanced oxidative stress due to proteolytic oxidation of host haemoglobin inside the acidic food vacuole of the parasite and the levels of GSH are greatly reduced which indicate stress challenge. A significant amount of GSH is consumed in the reactions catalysed by the glutathione transferases (GSTs) for detoxification of xenobiotics and removal of toxic metabolic products. Balance is maintained in the parasite as it possesses capacity for *de novo* synthesis for GSH and reduction of GSSG. It is efficiently equipped with antioxidant measures that protect it from oxidative injury, whereas the host cell compartment is oxidatively distressed. The parasite possesses a functional glutathione synthesis pathway. The two enzymes involved in synthesis -γ-glutamylcysteinesyhthetase (γGCS) and glutathione synthetase (GS) have been reported in *P. falciparum* (Meierjohann et al,2002) and *P. berghei* (Sharma and Banyal 2007).

The parasitized host cells lose GSH via GSSG efflux pump, and the efflux of GSSG in infected cells is relatively more as compared to normal RBCs and the new permeability channels induced by the parasite in the RBC during invasion mediate it (Meierjohann et al 2002). With this efflux a redox ratio of GHS/GSSG cannot be maintained inside the cell. *Plasmodium*-infected cells contain about half the amount of GSH compared to non-infected RBCs despite having an active GSH de novo synthesis and functional glutathione redox system. This is attributed mainly due to high efflux of GSSG in infected RBCs. The increased oxidative stress due to malaria parasite causes oxidation of a large amount of GSH and its depletion in the cell. To maintain an adequate GSH/GSSG redox ratio there is a need for increased GSSG efflux from the cell. *Plasmodium* and its host erythrocytes contain functional GR but the enzyme is not able to reduce GSSG efficiently to prevent the efflux of GSSG from parasitized RBCs. Recent studies on *P. berghei* GR knockout have revealed it to be non-essential for the survival of parasite in infected erythrocytes and its functions being compensated by thioredoxin redox system (Pastrana Mena et al, 2010 and Buchholz et al, 2010).

Glutathione, besides serving as an antioxidant and acting as a redox buffer is involved in a variety of detoxification reactions in malaria parasite. FP IX produced Hb degradation and turning into haemozoin needs to be changed from its toxic form to the non-toxic state so that it does not lyse the parasite. This generally occurs due to the presence of different FP IX binding molecules in the parasite like histidine rich protein (HRP) and enzymes of glutathione metabolism. FP IX also binds to and inhibit the activity of parasite glyceraldehyde -3-phosphate dehydrogenase (PfGADPH), GR and protein disulphide isomerase in *P. falciparum* (Campanale et al 2003). GSH non-enzymatically degrades FP IX and if this process is weakened then more of toxic FP IX remains un-sequestered and damages the parasite.

Another important antioxidant enzyme found in *Plasmodium* is glutathione transferase (GST). GSTs are a group of multifunctional enzymes that directly depend on GSH and are involved in detoxification of xenobiotics by way of mercapturic acid pathway. Apart from their role in catalysing conjugation of electrophilic substrate to GSH they also have peroxidase and isomerase activity. *P. falciparum* is known to possess only single homodimeric GST (PfGST) that differs from its human counterpart. Compound CB-27 has exhibited antiplasmodial activity towards *P.berghei* GST without inhibiting the human ortholog (Colon-Lorenzo et al., 2020). GSTs protect cells from ROS-induced oxidative stress by detoxifying them. It conjugates to toxic reactive compounds like 4-hydroxynonenal and cholesterol oxide which are generated during oxidation of membranes. GST has also been proposed as a potential target for development of novel antimalarials.

Besides these antioxidant enzymes *P. falciparum* possesses a number of structurally related proteins like plasmaredoxin, glutaredoxin and thioredoxin which function as redox messanger which interact with variety of reactive proteins and metabolites (Holmgren 2000). Glutaredoxins are GSH utilising proteins that are characterized by the active site sequence CPYC and coded by the gene PFCO27IC in *P. falciparum* (Rahlfs et al, 2001). These proteins protect against the oxidative damages and also serve as hydrogen donor for ribonucleotide reductase and associated with transcriptional control.

Plasmaredoxin, a highly conserved and exclusively found in *Plasmodium* is a 22 kDa redox-active protein that provides electrons for ribonucleotide reductase, the enzyme catalysing the first step of DNA synthesis. Peroxiredoxins (Prx) are ubiquitous peroxidases that provide peroxide detoxifying capacity to malaria parasite in the absence of catalase and glutathione peroxidase. Prx help in reducing the oxidative stress by reducing the levels of iron released by interfering with GSH-mediated degradation of FP IX and help to keep FP-derived ROS at levels below the parasite antioxidant system can manage (Kawazu et al 2005).

CONCLUSION

Oxidative stress and free radicals are known to be detrimental to health and contribute to initiation and progression of several diseases. Antioxidants are able to counteract oxidative stress and mitigate its effects. Oxidative stress is a multifactorial phenomenon in malaria. Erythrocytic stages of malaria parasite responsible for the pathogenesis of disease are under enhanced oxidative stress. *Plasmodium* depends upon several antioxidant enzymes and proteins like glutathione for its protection from the deleterious effects of ROS. A delicate balance exists between the antioxidants and redox status in growing parasite and interference in this balance can be used as a mechanism to disrupt the growth of parasite and stop the progression of disease. Glutathione and its metabolites need to be assessed and validated as new antimalarial targets.

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